GENETIC STUDIES OF COLLAGEN TYPES XV AND XVIII

Type XV collagen deficiency in mice results in skeletal myopathy and cardiovascular defects, while the homologous endostatin precursor type XVIII collagen is needed for normal development of the eye.

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Abstract

Overlapping genomic clones coding for the α1 chain of mouse type XV collagen (Col15a1) were isolated. The gene was found to be 110 kb in length and to contain 40 exons. Analysis of the proximal 5'-flanking region showed properties characteristic of a housekeeping gene promoter, and functional analysis identified cis-acting elements for both positive and negative regulation of Col15a1 gene expression. The general exon-intron pattern of the mouse Col15a1 gene was found to be highly similar to that of its human homologue, and comparison of 5'-flanking sequences indicated four conserved domains. The genomic area encoding the end of the N-terminal non-collagenous domain nevertheless showed marked divergence from the human form. Due to the lack of two exons coding for the N-terminal collagenous domain and a codon divergence in one exon, the mouse α1(XV) chain contains seven collagenous domains whereas the human equivalent contains nine.

In order to understand the biological role of this protein, a null mutation in the Col15a1 gene was introduced into the germ line of mice. Despite the wide tissue distribution of type XV collagen, the null mice developed and reproduced normally and were indistinguishable from their wild-type littermates. After three months of age, however, microscopic analysis revealed progressive histological changes characteristic of myopathic disorder, and treadmill exercise resulted in greater skeletal muscle injury than in the wild-type mice. Irrespective of potential anti-angiogenic properties of type XV collagen-derived endostatin, the number of vessels appeared normal. Nevertheless, ultrastructural analyses revealed markedly abnormal capillaries and endothelial cell degeneration in the heart and skeletal muscle. Perfused hearts showed a diminished inotropic response, and exercise resulted in cardiac injury, changes that mimic early or mild heart disease. Thus type XV collagen appears to function as a necessary structural component for stabilizing cells with surrounding connective tissue in skeletal muscle cells and microvessels.

Mice lacking the type XV collagen homologue, type XVIII collagen, showed delayed regression of blood vessels in the vitreous body of the eye and abnormal outgrowth of the retinal vessels. This suggests that collagen XVIII plays a role in regulating vascular development in the eye. Moreover, type XVIII collagen was found to be important at the surface between the inner limiting membrane and the collagen fibrils of the vitreous body. Col18a1 deficient mice serve as an animal model for the recessively inherited Knobloch syndrome, characterized by various eye abnormalities and occipital encephalocele. The results presented in this thesis indicate diverse biological roles for the closely related collagen types XV and XVIII.

Keywords: transgenic mice, gene structure, angiogenesis, promoter analysis, skeletal muscle, heart, eye, endostatin, Knobloch syndrome
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Lauri Eklund
Abbreviations

ANP  atrial natriuretic peptide
β-AR  β-adrenergic receptor
BM  basement membrane
bp  base pairs
CAM  chorioallantoic membrane
ECM  extracellular matrix
ES  embryonic stem cell
FGF-2  basic fibroblast growth factor
HA  hyaloid artery
H&E  haematoxylin and eosin
HVS  hyaloid vascular system
kb  kilobase
kD  kiloDalton
MMP  matrix metalloproteinase
MQF  musculus quadriceps femoris
mRNA  messenger RNA
NC1  C-terminal non-collagenous domain
nNOS  neural nitric oxide synthase
PCR  Polymerase chain reaction
PDGF  platelet-derived growth factor
PHPV  persistent hyperplastic primary vitreous
PHTVL  persistent hyperplastic tunica vasculosa lentis
PM  pupillary membrane
TUNEL  terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labelling
TVL  tunica vasculosa lentis
VEGF  vascular endothelial growth factor
VHP  vasa hyaloidea propria
List of original articles

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


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1. Introduction

Collagens are defined as structurally related extracellular matrix (ECM) proteins providing mechanical strength for tissues. Large ECM proteins, including collagens, have been shown to contain globular domains that may have functions different from those of the parental proteins. This study was initiated in order to improve our understanding of the biological roles of the closely related type XV and XVIII collagens on the basis of observations in genetically modified mice. Transgenic techniques allows the analysis of various aspects of gene functions in the context of the whole animal and the generation of animal models of human diseases. Type XVIII collagen has been of particular interest recently due to its potential antiangiogenic functions, whereas the function of type XV collagen remained unknown. Because of certain evidence that type XVIII collagen-derived endostatin inhibits angiogenesis and reduces tumor growth in animal models, changes in the vessel formation were expected to occur in the mutant mice.

The results presented in this thesis indicate diverse biological roles for the closely related collagen types XV and XVIII and suggest a structural function for type XV in the skeletal muscle and capillaries, whereas type XVIII is needed in the developing vasculature of the eye. So far no human disease has been attributed to mutations in the type XV collagen gene, but while this work was in progress it was reported that mutations in the type XVIII collagen gene cause Knobloch syndrome, which is characterized by various eye abnormalities, confirming that collagen type is needed for the development and maintenance of the eye.
2. Review of the literature

2.1. Human diseases and animal models

2.1.1. Why are mouse models used?

Advances in modern DNA technology have led to a remarkable expansion in the genomic database information available, with complete characterization of the genomes of many species. Simultaneously, the development of techniques for manipulating the mammalian genome allows the introduction of modified genes into the germ lines of experimental animals. This has opened up a new avenue in the biological sciences. During the last two decades genetically modified animals have been central tools for studying the functions and regulation of genes and for understanding the pathophysiology of diseases in the context of the entire animal, in a manner which takes into account the interplay between cells and organs, which cannot be achieved in other ways. Thus animal models are valuable tools for studying human genetic diseases and for initiating testing of new therapeutic strategies, including gene therapy.

The mouse has become the most commonly used mammalian species for genetic manipulations. Genetically, the mouse and human are similar, having a comparable genome size and number of genes and related basic physiology and patterns of development. In addition, mice have a relatively rapid breeding time and large litter sizes, a large number of inbred and genetically defined strains have become available, and the complete mouse genome will soon have been sequenced. The most important consideration, however, is that at the present the embryonic stem cell (ES) technology used to modify precisely the gene of interest functions reliably only in the mouse.

2.1.2. Gene targeting, the introduction of site-specific modifications into the genome by homologous recombination

There are two basic techniques, which have been used to create genetically modified mouse lines: addition of an exogenous transgene, resulting in random chromosomal integration, and homologous recombination of introduced DNA, leading to targeted mutation of an endogenous gene (Roths et al. 1999).

The first method relies on transfection of foreign DNA into the fertilized oocyte. This results in random chromosomal insertion of the transgene (Gordon et al. 1980). The approach is frequently used to produce “gain-in-function” mutations in which the transgene is designed to overexpress the gene product or express a modified gene which may interfere with the function of the endogenous gene. The development of gene
targeting technology via homologous recombination in embryonic stem cells (ES) allows precise modification of the gene of interest. This gene targeting is frequently used to create "loss-of-function" mutations, which can provide important knowledge of the functions and biological significance of the encoded protein of the resulting null allele. Gene targeting also allows the production of subtle mutations such as amino acid substitutions without completely ablating the gene function (Müller 1999).

Two findings have made it possible to create exact modifications to the gene of interest: the isolation, growth and maintenance of mouse ES cells (Evans & Kaufman 1981, Martin 1981), and the observation that introduced DNA can recombine with its homologous chromosomal counterpart in the ES cells (Thomas & Capecchi 1987). Using standard gene technology, a targeting construct containing the desired alternation of the gene of interest and markers for selection flanked by sequences homologous to the endogenous target gene can easily be produced in vitro. The ES cells are then transfected with the targeting construct, which will homologously recombine with the resident gene and introduce the mutation at the right site in the genome (Joyner 1993).

ES cells are derived from the uncommitted inner cell mass of pre-implantation blastocyst-stage mouse embryos. After multiple passages under the appropriate in vitro cell culture conditions, they are still capable of contributing to the normal development of somatic tissues and to germ cell lineages when reintroduced into a host embryo’s blastocyst cavity (Bradley et al. 1984). The first description of gene targeting using homologous recombination in mouse ES cells was published in 1987, involving alteration of the selectable hypoxanthine phosphoribosyl transferase gene locus (Thomas & Capecchi 1987), which soon led to targeting of non-selectable genes and the creation of targeted mouse lines (Mansour et al. 1988).

In most cases targeted inactivation of genes is performed by introducing a positive selection marker, which will disrupt the gene structure. The development of Cre/loxP technology has added a new alternative for introducing mutations into the gene of interest (Nagy 2000). The Cre/loxP system has since been extended to produce conditional gene inactivation in mice that affects only selected cell types (Gu et al. 1994) or specific time points in an inducible manner (Kühn et al. 1995). It also allows genome engineering with respect to large deletions of up to 200 kb (Li et al. 1996) and translocations between non-homologous chromosomes (Smith et al. 1995).

### 2.1.3. Why is there “no phenotype”?

Several hundred mutant mouse strains have been generated by the ES cell technology to date [http://tbase.jax.org]. In many cases inactivation of an allele has the expected consequences, but phenotypes that could not be predicted from the supposed function of a given gene product or the pattern of its expression are also commonly observed. Certain null mutations in widely expressed genes have led to surprisingly restricted defects, as exemplified by the proto-oncogene c-src, which is expressed in every cell in the body, and particularly highly in the brain and platelets, but deficient mice unexpectedly showed osteopetrosis (Soriano et al. 1991). A large number of null mice manifest no phenotypes
or only very subtle ones, although the inactivated genes are well-conserved and are thought to have important functions, as in the case of myoglobin (Garry et al. 1998) or tenascin-C (Saga et al. 1992). It is unlikely that highly conserved and widely expressed genes will have no function. An unexpectedly minor phenotype may be caused by gene redundancy or developmental plasticity, and various adaptive mechanisms may exist to balance the null allele. This is highlighted by several physiological compensatory mechanisms observed in the surprisingly healthy myoglobin-deficient mice (Gödecke et al. 1999). In the case of tenasin-C knockout mice the development of apparent defects is dependent on the genetic background and pathological interventions (Mackie & Tucker 1999). The apparent lack of phenotype in vivo can be at least partly explained by compensation from other functionally related proteins, as between the myogenic regulatory factors Myod and Myf5 (Rudnicki et al. 1993) or between dystrophin and utrophin in skeletal muscle and heart (Grady et al. 1997), although such direct evidence for compensation is rare and the relatively small number of genes in the mammalian genome does not support vastly overlapping functions.

### 2.1.4. “An elephant is like an E. coli, only more so” (Jacques Monod)

The mouse is not always the perfect organism for modelling human diseases, as it is capable of showing a different and often a milder phenotype than the corresponding human patients, as observed in mdx mice, for instance, which lack dystrophin (Bulfield et al. 1984). Dissimilarities in the engineered mice used to mimic human diseases could be caused by redundant or altered biochemical pathways, patterns of transgene expression, absent or altered developmental pathways, different mechanical and environmental stresses due to small size and a sedate life-style, a short life-span, or an unknown mechanism of the mutation in question in humans. Importantly, some pathological changes may occur at the same absolute rate instead of being related to the shorter life-span of the mouse. In the case of some diseases which do not lead to symptoms in humans patients until an advanced age, the length of time needed for the development of degenerative changes could be much greater than the life-time of a mouse (Erickson 1989, Wynshaw-Boris 1996).

### 2.2. Collagens

The extracellular matrix (ECM) is a well-organized network of heterogeneous macromolecules that surrounds the cells and provides the necessary scaffold maintaining the structural integrity of tissues. In addition to serving as a structural support, it is involved in many other essential biological activities: it regulates the morphogenesis and development of tissues, influences tissue repair, provides a reservoir of ligands for cellular receptors, and stores and presents growth factors. The appearance and physical properties of the ECM, as in bone or skin, reflects variation in its components and assembly of the ECM.
The major components of ECM are collagens, non-collagenous glycoproteins, elastin and proteoglycans. Collagens, defined as structurally related ECM proteins providing mechanical strength for tissues, are the most abundant proteins of the ECM, and a characteristic of all collagen is the presence of at least one triple-helical domain built up of three polypeptides, called α-chains, containing a repeated (Gly-X-Y) amino acid sequence with glycine (Gly) as every third amino acid residue, and frequently hydroxyproline and hydroxylysine in the X and Y positions. This allows the three α-chains wrapped around each other to form stable triple-helical collagen molecules. The triple helix is relatively rigid and in many collagens it is interrupted by globular sequences that make the molecule more flexible and serviceable in a variety of macromolecular aggregates and biological activities, which can be different from those of the parental collagenous domains (Prockop & Kivirikko 1995, Bateman et al. 1996, Myllyharju & Kivirikko 2001).

Twenty distinct vertebrate proteins have been classified in the literature as belonging to the superfamily of collagens, as listed in Table 1, in addition to which there are at least three complete cDNA sequences encoding non-published collagen α-chains, raising the number of vertebrate collagens to 23 (Myllyharju & Kivirikko 2001). Some collagen types are formed by three identical α-chains, but some (types I, IV, V, VI, VIII, IX and XI) consist of more than one α-chain, encoded by separate genes, raising the number of collagen genes to over 30 (Prockop & Kivirikko 1995, Bateman et al. 1996, Myllyharju and Kivirikko 2001).

The collagens are divided into subgroups based on their structural features, as presented in Figure 1: fibril-forming collagens (types I-III, V and XI, Fig. 1A), fibril-associated collagens with interrupted triple helices, i.e. the FACIT collagens (types IX, XII, XIV, XVI and XIX, Fig. 1B), hexagonal network-forming collagens (types VIII and X, Fig. 1C), basement membrane collagen (type IV, Fig. 1D), beaded filament-forming collagen (type VI, Fig. 1E), collagen forming anchoring fibrils of basement membranes (type VII, Fig. 1F), collagens with transmembrane domains (types XIII and XVII, Fig. 1G), and the family of collagen types XV and XVIII, Fig. 1H (Prockop & Kivirikko 1995, Myllyharju & Kivirikko 2001).

### 2.2.1. Collagens in human diseases and mouse models

Collagen mutations are involved in a wide spectrum of human diseases. More than 1000 mutations have been found in 13 collagen types encoded by 23 distinct genes. Some of these cause severe malformations, leading to early lethal phenotypes, but others lead to certain common diseases. Naturally occurring and transgenic mice expressing mutant α-chains, and also targeted mice with null mutations, have led to advances in our understanding of the pathogenesis of collagen diseases. Human diseases caused by mutations in various ECM molecules, together with the corresponding mouse models, have recently been reviewed by Aszödi et al. (1998a), Gustafsson & Fässler (2000) and Myllyharju & Kivirikko (2001).

So far null mice for 10 collagen genes have been described in the literature (Table 1). The severity of the phenotypic consequences varies from embryonic lethality (e.g. the
lack of type II collagen) to relatively mild changes (e.g. in type X collagen null mice). In the case of human diseases (Table 2), most collagen mutations are caused by single base substitutions in the critical amino acid glycine, preventing the folding of the triple helix or producing an interruption in the helical domain, and the phenotypic consequences of these are often more severe than are seen due to a corresponding null allele (Gorski & Olsen 1999).

Table 2: Examples of Collagen Mutations and Their Consequences

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>Clinical Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine Substitution</td>
<td>Severe clinical phenotype</td>
</tr>
<tr>
<td>Nonsense Mutation</td>
<td>Null allele</td>
</tr>
</tbody>
</table>

Fig. 1. The members of the collagen family of proteins and their molecular assemblies. The rods indicate triple helical collagenous domains in each molecule, solid circles N and C-terminal non-collagenous domains and open circles interruptions in the collagen triple helix. GAG, glycosamino glycan; PM, plasma membrane. Modified with permission from Myllyharju & Kivirikko (2001).
### Table 1. Collagen types, their tissue distribution, and phenotype of the null allele

<table>
<thead>
<tr>
<th>Type</th>
<th>Occurrence</th>
<th>Target gene and mouse phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Most connective tissues</td>
<td>Col1a1 Vascular defects¹</td>
</tr>
<tr>
<td>II</td>
<td>Cartilage, vitreous body, cornea</td>
<td>Col2a1 Chondrodysplasia, intervertebral disc disease²,³</td>
</tr>
<tr>
<td>III</td>
<td>Most connective tissues</td>
<td>Col3a1 Vascular and skin defects⁴</td>
</tr>
<tr>
<td>IV</td>
<td>Basement membranes</td>
<td>Col4a3 Renal failure, progressive glomerulonephritis⁵,⁶</td>
</tr>
<tr>
<td>V</td>
<td>Tissues containing collagen I</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>Most connective tissues</td>
<td>Col6a1 Mild muscular dystrophy⁷</td>
</tr>
<tr>
<td>VII</td>
<td>Anchoring fibrils in many tissues</td>
<td>Col7a1 Skin blistering⁸</td>
</tr>
<tr>
<td>VIII</td>
<td>Descemet's membrane, many tissues</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>Tissues containing collagen II</td>
<td>Col9a1 Degenerative changes in articular cartilage⁹</td>
</tr>
<tr>
<td>X</td>
<td>Hypertrophic cartilage</td>
<td>Col10a1 Mild skeletal phenotype¹⁰,¹¹</td>
</tr>
<tr>
<td>XI</td>
<td>Tissues containing collagen II</td>
<td>Coll1a2 Hearing loss¹²</td>
</tr>
<tr>
<td>XII</td>
<td>Tissues containing collagen I</td>
<td></td>
</tr>
<tr>
<td>XIII</td>
<td>Many tissues</td>
<td></td>
</tr>
<tr>
<td>XIV</td>
<td>Tissues containing collagen I</td>
<td></td>
</tr>
<tr>
<td>XV</td>
<td>Many tissues in the BM zone</td>
<td>Col15a1 Skeletal myopathy and cardiovascular defects⁸</td>
</tr>
<tr>
<td>XVI</td>
<td>Many tissues</td>
<td></td>
</tr>
<tr>
<td>XVII</td>
<td>Hemidesmosomes in epithelium</td>
<td></td>
</tr>
<tr>
<td>XVIII</td>
<td>Many tissues in the BM zone</td>
<td>Col18a1 Vascular abnormalities in the eye⁶</td>
</tr>
<tr>
<td>XIX</td>
<td>Many tissues in the BM zone</td>
<td>Col19a1 Abnormal muscle layer in the oesophagus¹³</td>
</tr>
<tr>
<td>XXI</td>
<td>Many tissues</td>
<td></td>
</tr>
</tbody>
</table>


### 2.2.2. The subfamily of type XV and XVIII collagens

The collagen types XV and XVIII belong to the heterogeneous group of non-fibril-forming collagens and together form a distinct subgroup among the collagens (Pihlajaniemi & Rehn 1995, Rehn & Pihlajaniemi 1994). They are closely related in terms of their primary structures (Kivirikko et al. 1994, Muragaki et al. 1994, Oh et al. 1994a, Oh et al. 1994b, Rehn & Pihlajaniemi 1994, Rehn et al. 1994), as both have thrombospondin-I sequence homology in the N-terminus, seven homologous collagenous domains and highly similar C-terminal ends, termed the endostatin domain. Endostatin has been identified as a proteolytic fragment in type XVIII collagen having antiangiogenic properties (O’Reilly et al. 1997). In spite of their striking similarities in primary structure, similar occurrence in the basement membrane (BM) zones of most tissues (Muragaki et al. 1995, Myers et al. 1996, Hägg et al. 1997b, Saarela et al. 1998, Muona et al. 2001) and similar functions as potent angiogenic inhibitors (Ramchandran...
et al. 1999, Sasaki et al. 2000), there are some important differences between these collagens, which may suggest diverse roles for them. The only major difference at the primary structure level is the lack of N-terminal variants in type XV collagen and the presence of a frizzle domain in type XVIII (Muragaki et al. 1995, Rehn & Pihlajaniemi 1995). In terms of tissue distribution, type XV is absent from the sinusoidal endothelia (Muona et al. 2001), whereas type XVIII is very strongly expressed in the liver and is a prominent component of liver sinusoids (Saarela et al. 1998). Type XV collagen predominates in the skeletal muscle, and is also present to some extent in the fibrillar collagen matrix (Myers et al. 1996, Hägg et al. 1997b, Muona et al. 2001), while type XVIII collagen is restricted to the BM zones (Saarela et al. 1998). Moreover, although both of these collagens are post-translationally modified by intensive glycosylation, their carbohydrate chains are different, type XVIII being a heparan sulphate proteoglycan (Häßler et al. 1998) and type XV a chondroitin sulphate proteoglycan (Li et al. 2000).

Table 2. Human diseases caused by mutations in collagens

<table>
<thead>
<tr>
<th>Type</th>
<th>Gene</th>
<th>Human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>COL1A1, COL1A2</td>
<td>Osteogenesis imperfecta, osteoporosis</td>
</tr>
<tr>
<td>II</td>
<td>COL2A1</td>
<td>Several chondrodysplasias, osteoarthrosis</td>
</tr>
<tr>
<td>III</td>
<td>COL3A1</td>
<td>Ehlers-Danlos syndrome (type IV), arterial aneurysms</td>
</tr>
<tr>
<td>IV</td>
<td>COL4A3, COL4A4, COL4A5</td>
<td>Alport syndrome</td>
</tr>
<tr>
<td></td>
<td>COL4A5, COL4A6</td>
<td>Alport syndrome with diffuse oesophageal leiomyomatosis</td>
</tr>
<tr>
<td>V</td>
<td>COL5A1, COL5A2</td>
<td>Ehlers-Danlos syndrome (types I and II)</td>
</tr>
<tr>
<td>VI</td>
<td>COL6A1, COL6A2, COL6A3</td>
<td>Bethlem myopathy</td>
</tr>
<tr>
<td>VII</td>
<td>COL7A1</td>
<td>Epidermolysis bullosa</td>
</tr>
<tr>
<td>VIII</td>
<td>COL8A2</td>
<td>Corneal endothelial dystrophy</td>
</tr>
<tr>
<td>IX</td>
<td>COL9A1, COL9A2, COL9A3</td>
<td>Epiphyseal dysplasia, intervertebral disc disease, osteoarthrosis</td>
</tr>
<tr>
<td>X</td>
<td>COL10A1</td>
<td>Schmid metaphyseal chondrodysplasia</td>
</tr>
<tr>
<td>XI</td>
<td>COL11A1, COL11A2</td>
<td>Chondrodysplasias, non-systematic hearing loss, osteoarthrosis</td>
</tr>
<tr>
<td>XVII</td>
<td>COL17A1</td>
<td>Epidermolysis bullosa</td>
</tr>
<tr>
<td>XVIII</td>
<td>COL18A1</td>
<td>Knobloch syndrome</td>
</tr>
</tbody>
</table>


2.2.2.1. Genes encoding collagen types XV and XVIII

Exon-intron organizations have been reported for the human type XV collagen gene (Hägg et al. 1998) and the mouse gene for type XVIII collagen (Rehn et al. 1996). The human COL15A1 gene is about 145 kb in size and contains 42 exons (Hägg et al. 1998). It has been mapped to chromosome 9q21-q22 (Huebner et al. 1992), whereas its mouse counterpart has been localized to chromosome 4, B1-3 (Hägg et al. 1997a). Type XVIII collagen is expressed as three N-terminal variants in a tissue-specific manner (Muragaki et al. 1995, Rehn & Pihlajaniemi 1995), the variants being encoded by two promoters and further modified by alternative splicing (Rehn et al. 1996). The human COL18A1 gene has been localized to chromosome 21q22.3 and mouse Col18a1 to chromosome 10 (Oh et
al. 1994b). Comparison at the genomic level has indicated high structural homology between the genes, suggesting that the two collagens have a common ancestor (Hägg et al. 1998). This is further supported by the discovery of a single gene in C. elegans homologous with both vertebrate XV and XVIII collagens (Ackley et al. 2001). Genes for collagens XV and XVIII are conserved between vertebrates, the nematoda C. elegans and the fruit fly D. melanogaster (Hynes & Zhao 2000), suggesting a unique importance within the animal kingdom.

2.2.2.2. Type XV collagen

Type XV collagen is thought to be a homotrimer consisting of three α1(XV) chains (Rehn & Pihlajaniemi 1994, Sasaki et al. 2000). It is characterized by a central highly interrupted triple helical domain and large N and C-terminal NC domains. The human α1(XV) chain contains nine collagenous domains, whereas the mouse equivalent contains seven (Muragaki et al. 1994, Myers et al. 1992, Hägg et al. 1997a). The mRNAs are expressed in many tissues, but the highest levels in the mouse can be detected in the heart and skeletal muscle (Hägg et al. 1997a). The main producers of type XV collagen in the human fetus are mesenchymally derived cells, particularly fibroblasts, muscle cells and endothelial cells (Kivirikko et al. 1995). The protein has been shown by immunostaining to have a widespread tissue distribution and has been localized mainly to the BM zones, although it can also be found in the fibrillar collagen matrix near the BMs of certain human tissues (Myers et al. 1996, Hägg et al. 1997b). Detailed immunofluorescence studies of developing and mature mouse tissues have revealed early prominent expression in the skeletal muscle, peripheral nerves and capillaries of most tissues. Expression in the heart, kidney and lung appeared to be developmentally regulated. Immunoelectron microscopy of skeletal muscle and peripheral nerve has localized type XV collagen to the outermost layer of the lamina densa and to the interstitial collagen fibres near the BMs (Sormunen R, Muona A, Pihlajaniemi T, unpublished). This suggests that it may serve to link BMs to the underlying fibrillar collagen matrix. Some interactions between type XV collagen and other extracellular matrix proteins have been identified, e.g. the fact that the NC1 domain binds strongly to fibulin-2 and nidogen-2 (Sasaki et al. 2000).

2.2.2.3. Type XVIII collagen

The α1(XVIII) chain contains ten triple-helical domains and large N and C-terminal NC sequences (Oh et al. 1994b, Rehn et al. 1994). The longest variant is characterized by a cysteine-rich sequence, which is homologous with the presumed ligand-binding domain of the frizzled proteins (Muragaki et al. 1995, Rehn & Pihlajaniemi 1995). The frizzle proteins are involved as receptors in the wingless signalling pathway, possibly by being able to store or sequester them (Bhanot et al. 1996). Type XVIII collagen mRNAs are expressed in many tissues, particularly large amounts of the longest variant is being found in the liver (Rehn & Pihlajaniemi 1995). α1(XVIII) mRNAs are produced by several cell types, including epithelial and endothelial cells, cardiac muscle cells,
keratinocytes and hepatocytes (Saarela et al. 1998). Type XVIII collagen has been located in the BM zones throughout the body by antibody staining, particularly in vascular and epithelial structures (Muragaki et al. 1995, Saarela et al. 1998). The multidomain structure of the molecule is suggestive of multiple roles. In agreement with this, it may have a role in organ morphogenesis and epithelial development (Karumanchi et al. 2001, Lin et al. 2001).

2.2.2.4. Mutations in type XVIII collagen cause Knobloch syndrome

Patients suffering from the recessively inherited Knobloch syndrome have recently been found to have a splice site mutation leading to premature termination of the short form of collagen XVIII (Sertie et al. 2000). The same phenotype is also observed in patients affected by mutations inactivating all three tissue forms (Marneros & Olsen 2001). The syndrome is characterized by various eye changes, including high myopia, vitreoretinal degeneration with retinal detachment, and macular abnormalities. Another diagnostic feature is a posterior skull defect, an occipital encephalocele (Knobloch & Layer 1971).

2.3. Angiogenesis and endogenous inhibitors

Proper vascular development is essential for growth during embryogenesis and for subsequent tissue functions. The blood vessels in the embryo are formed by two distinct mechanisms, vasculogenesis and angiogenesis, processes in which the maturation, proliferation and migration of endothelial cells, together with tube formation, are elementary events (Carmeliet 2000, Yancopoulous 2000). Vasculogenesis is defined as the differentiation of mesoderm-derived precursor cells, called angioblasts, into endothelial cells and the formation of an immature vascular plexus within previously avascular tissue, while in angiogenesis, new capillaries form from the pre-existing primary vascular network by sprouting or splitting (Risau 1997). After formation the primary vasculature it is progressively remodelled to meet the local physiological requirements. Maturation into a fully functional circulatory system requires the recruitment of supporting cells, pericytes and smooth muscle cells and the production of an ECM around the endothelial cell tubes (Carmeliet 2000). In a healthy adult the formation of new blood vessels is observed only in certain situations, such as tissue repair or in the placenta during pregnancy. On the other hand, angiogenesis is centrally involved in pathological conditions, including retinopathies and cancer (Folkman 1995b).

The mechanisms underlying the physiological and pathological control of vascular development are not fully understood, but the fate of the endothelial cells and the angiogenic phenotype are thought to be the result of interactions between inducible and antiangiogenic factors. Studies with genetically engineered mice, deficient for or misexpressing potent growth factors and their receptors have revealed some important molecular bases for underlying events taking place during the blood vessel development. Vascular endothelial growth factor (VEGF) and its cellular receptors have been found to be major regulators of the development of the primary vasculature (Carmeliet et al. 1996,
Ferrara et al. 1996, Fong et al. 1995, Shalaby et al. 1995). Secreted growth factors are also involved in vessel maturation, maintenance and regression, exerting their effects by means of cellular receptors acting on endothelial cells. Angiopoietin1 and its receptor tie2 are important for vessel stabilization and the maintenance of supporting cells and ECM associations (Suri et al. 1996, Thurston et al. 1999), while angiopoietin2 antagonizes these effects and results in destabilization of the vascular structures and ECM contacts, as is required for subsequent angiogenic remodelling in the presence of VEGF or regression without angiogenic stimuli (Maisonpierre et al. 1997). The ephrins are a family of growth factors and some of them are essential for vascular development. The lack of ephrin-B2 and its receptor ephB4 is known to lead to defects in early angiogenic remodelling and to be also important for establishing arterial and venous identity (Wang et al. 1998, Gerety et al. 1999). In addition to the growth factors discussed above, researchers have described additional members of the VEGF and angiopoietin families, number of other growth factors, such as fibroblast growth factors and platelet-derived growth factors involved in angiogenesis and lymphangiogenesis. (Carmeliet 2000, Ferrara 2001).

Temporal, spatial and quantitative regulation and cooperative functioning of inducers and repressors is essential for the development of a normal vasculature. Numerous endogenous inhibitors of angiogenesis have been described (Cao 2001), including thrombospondin (Good et al. 1990) and proteolytic fragments derived from type IV collagens (Colorado et al. 2000, Kamphaus et al. 2000, Maeshima et al. 2000), fibronectin (Homandberg et al. 1985), serbin antithrombin (O’Reilly et al. 1999), and MMP-2 (Brooks et al. 1998). Moreover, naturally occurring angiogenic factor antagonists such as soluble receptors for FGF-2 and VEGF may interfere with the actions of growth factors (Hanekken et al. 1994, Kendall & Thomas 1993).

### 2.3.1. Endostatin

A highly interesting feature of type XVIII collagen is the 20-kDa C-terminal peptide, endostatin, which is capable of inhibiting endothelial cell activities, angiogenesis and tumor growth (O’Reilly et al. 1997). Tumor growth and metastasis are dependent on the formation of blood vessels, so that inhibition of tumor angiogenesis could be used as a strategy for treating cancer (Folkman 1971). Numerous endogenous inducers and inhibitors of angiogenesis have been described (see section above), some of the latter having been isolated from normally avascular tissues, according to the hypothesis that the expression of inhibitor(s) retains the quiescent state of the vasculature, as in the cornea and vitreous body (Dawson et al. 1999). On the other hand, tumor tissues produce circulating antiangiogenic factors (Folkman 1995a), which led to the discovery of angiostatin in tumor-bearing animals (O’Reilly et al. 1994). Using a similar strategy, endostatin was isolated from conditioned murine haemangioendothelioma cell culture media as a tumor-derived specific inhibitor of endothelial cell proliferation (O’Reilly et al. 1997).

One of the common features of endogenous antiangiogenic factors is that they appear as cryptic fragments of the large parental proteins associated with vascular structures (Cao 2001). This suggests that proteolytic processing of parental molecules may have an
important role in the regulation of their activities. This is also the case with regard to endostatin. The NC1 domain of type XVIII collagen comprises three functional elements: the endostatin region (~180 residues), which is separated from an upstream trimerization region (~50 residues) by a protease-sensitive hinge region (~70 residues) (Sasaki et al. 1998). Several proteases, including various matrix metalloproteinases, cathepsins and elastase, can separate endostatin-containing protein fragments from the parental molecule in vitro (Wen et al. 1999, Felbor et al. 2000, Ferreras et al. 2000, Lin et al. 2001). Cleavage within the hinge results in the release of monomeric endostatins and larger endostatin-like protein fragments that can be detected in tissue extracts and as circulating forms in the blood, at concentrations of 120-300 ng/ml, indicating that cleaved endostatin fragments exist in the body in vivo under physiological conditions and may be involved in the homeostatic control of angiogenesis (Ständker et al. 1997, Sasaki et al. 1998, John et al. 1999, Miosge et al. 1999).

2.3.1.1. Endostatin suppresses endothelial cell activities in vitro

Angiogenesis is a complex, multi-stage process, and potential inhibitors may act in different steps. They may interfere with endothelial cell activation by growth factors, by preventing degeneration of the BM of the endothelium, by antagonizing endothelial cell survival, proliferation and migration, or by affecting blood vessel tube formation (Risau 1997, Carmeliet & Collen 2000, Cao 2001). Endostatin suppresses the endothelial cell angiogenic phenotype, blood vessel formation and tumor growth by affecting some of the endothelial cell functions.

The exact molecular mechanisms by which endostatin acts have remained unclear. Various pathways have been suggested, based mainly on in vitro cell culture experiments, but with conflicting results. Endostatin has been reported to inhibit endothelial cell proliferation (O’Reilly et al. 1997, Dhanabal et al. 1999b) and migration (Yamaguchi et al. 1999, Dhanabal et al. 1999b, Shichiri & Hirata 2001). It reduces endothelial cell survival to some extent by inducing endothelial cell apoptosis (Dhanabal et al. 1999a, Dixelius et al. 2000). A prerequisite for angiogenesis is proteolytic activity leading to degradation of the ECM and dissolving of the cell attachments prior to their migration. Endostatin may suppress certain proteolytic activities (Kim et al. 2000, Wickstöm et al. 2001), and endostatin treatment is accompanied by changes in the structures required for cell mobility and matrix invasion, including the disassembly of focal adhesions and reorganization of the cytoskeleton (MacDonald et al. 2001, Wickstöm et al. 2001).

2.3.1.2. Endostatin inhibits angiogenesis and tumor growth in vivo

The ability of endostatin to inhibit angiogenesis and its anti-tumor activity has been observed in numerous in vivo studies. Its antiangiogenic activity has been documented in chicken chorioallantoic membrane (CAM) assays (Sasaki et al. 1999, Dixelius et al. 2000), and endostatin treatment has been found to inhibit angiogenesis and tumor growth in mouse models, and to result in persistent tumor dormancy without any apparent side

2.3.1.3. Endostatin receptors

It is likely that endostatin exerts its biological functions via cellular receptors. Two types of potential receptor have been identified, namely certain integrins (Rehn et al. 2001) and the proteoglycan glypican (Karumanchi et al. 2001). Rehn et al. (2001) suggest that endostatin interacts with integrins α5β1, αvβ3 and αvβ5. Interestingly, its soluble and immobilized forms may have different functions. Immobilized endostatin was found in in vitro cell culture assay to support endothelial cell survival and migration in an integrin-dependent manner, whereas soluble endostatin inhibited endothelial cell functions (Rehn et al. 2001). Glypicans are glycosyl-phosphatidylinositol anchored heparan sulphate proteoglycans which are able to bind various ligands and enhance the formation of their receptor-signalling complexes. It has been proposed that endostatin may mediate it’s antiangiogenic signals through co-receptor complexes of this kind, in which glypicans serves as low-affinity receptors presenting endostatin to other, as yet unidentified, high-affinity receptors, which are then involved in intracellular signalling (Karumanchi et al. 2001).

Endostatin binds to heparin in affinity chromatography, and its crystal structure indicates a prominent heparin/heparan sulphate-binding site (Hohenester et al. 1998, Sasaki et al. 1998). This suggests that endostatin may competitively inhibit the binding of angiogenic growth factors to heparan sulphate proteoglycans, which are known to act as co-receptors for a number of cytokines (Hohenester et al. 1998). The importance of the heparin binding domain of endostatin has been evaluated in several experiments, resulting in evidence both for (Sasaki et al. 1999, Dixielius et al. 2000) and against (Chang et al. 1999, Yamaguchi et al. 1999, Sasaki et al. 2000) the essential role of heparin binding.

2.3.1.4. Trimeric and monomeric endostatins have different effects

Endostatin is a trimer when present in the full-length parental molecule, type XVIII collagen, or in the proteolytically released NC1 domain (Sasaki et al. 1998), and different functions have recently been proposed for trimeric and monomeric endostatins (Ackley et al. 2001, Kuo et al. 2001). Studies with a C.elegans mutant, Cle-1, which lacks the endostatin portion indicate that in this avascular nematode endostatin is involved in neurogenesis and is needed to induce cell migration and axon guidance (Ackley et al. 2001). Furthermore, the authors showed that the trimeric NC1 domain and the monomeric endostatin have opposite functions. Whereas the trimeric NC1 is needed for
cell migration, the monomeric endostatin inhibits the resulting migratory activity. This may suggest that the trimeric form binds and clusters cellular receptors or matrix molecules in order to activate cell migration (Ackley et al. 2001). Interestingly, a similar anti-migration function of monomeric endostatin and motogenic and morphogenic activity of the trimeric NC1 domain or dimeric endostatin emerged in mammalian Matrigel cell culture experiments (Kuo et al. 2001), where it appeared that the oligomeric NC1 domain has motogenic activity which is not restricted to endothelial cells. These studies also suggested a negative autoregulatory feedback mechanism for proteolytically cleaved monomeric endostatin which opposes the activity of its parental NC1 molecule (Kuo et al. 2001). Moreover, different binding properties have been observed for the trimeric NC1 domain and the endostatin fragment with respect to ECM components in solid phase assays in vitro. The trimeric NC1 domain has been found to bind with perlecan and the laminin-1-nidogen complex, whereas monomeric endostatin has very much weaker interactions with the same molecules (Sasaki et al. 1998). It has been proposed that the pro-migrational effect might be due to the ability of the trimeric NC1 to interfere with cell attachment to the underlying BM by binding ECM molecules, which are important for cell adhesion and migration (Marneros & Olsen 2001).

2.3.1.5. Type XV-derived endostatin

The endostatin domain has been found only in collagen types XV and XVIII, and the high amino acid homology of these fragments (60% sequence identity) allows assuming that their functional properties may be similar. Type XV-derived endostatin and trimeric NC1 have been shown to have antiangiogenic functions, as expected, but they differ somewhat from those of type XVIII-derived endostatin. Endostatin-XV inhibits FGF-2-induced endothelial cell migration in vitro, but not proliferation (Ramchandran et al. 1999), while systemic administrations in vivo suppressed tumor growth in a mouse model (Ramchandran et al. 1999). Both XV-derived NC1 and endostatin fragments inhibit VEGF-induced angiogenesis in the chick CAM assay, but only the NC1 domain is effective after FGF-2 stimulation, the endostatin-XV fragment having no activity (Sasaki et al. 2000). Proteolytically released endostatin- XV fragments are found in mouse tissues (Sasaki et al. 2000) and in human haemofiltrate (John et al. 1999). The NC1 fragment associates into trimers (Sasaki et al. 2000), but oligomerized endostatin-XV does not have any motogenic activity (Kuo et al. 2001). Endostatin-XV does not contain zinc (Sasaki et al. 2000), nor does it bind heparin (Ramchandran et al. 1999, Sasaki et al. 2000), properties that may be specific to endostatin-XVIII (Boehm et al. 1998, Sasaki et al. 1999, Yamaguchi et al. 1999, Dixelius et al. 2000).

2.4. Muscle diseases

This section focuses on changes in ECM proteins and cellular receptors related to the pathogenesis of muscle diseases, indicating the importance of cell and matrix interactions for the normal functioning and maintenance of skeletal muscle. The molecular basis for

The proteins associated with muscle diseases can be subdivided into three groups according their localization: extracellular, transmembrane and subsarcolemmal proteins (Table 3, Figure 2). Mutations in several components of dystrophin and associated proteins, and in other ECM and transmembrane proteins, have been found to be the primary causes of muscular dystrophies. The generation of genetically engineered mouse models for muscle diseases has resulted in major advances in our understanding of their pathogenesis (Table 3). A common feature of skeletal muscle diseases is that the cardiac muscle is also affected, as indicated in Table 3.

2.4.1. Molecular basis of muscular diseases

The first important step for understanding the molecular basis of muscular diseases was the characterization of dystrophin, a large subsarcolemmal protein which is absent in cases of Duchenne muscular dystrophy (Monaco et al. 1986). Its isolation and initial characterization showed that it co-purified as part of a protein complex that includes a number of transmembrane glycoproteins (Campbell & Kahl 1989, Ervasti & Campbell 1991, Suzuki et al. 1992). Characteristic of dystrophin-deficiency is a secondary reduction in the levels of other molecules in the complex found at the sarcolemma (Rafael & Brown 2000, Hack et al. 2000). In addition, the levels of cytoplasmic and membrane-associated α-dystrobrevin, α1-syntrophin and nNOS are dependent on the presence of dystrophin and are dramatically reduced in dystrophin-deficient mdx mice (Peters et al. 1998, Brenman et al. 1995, Chang et al. 1996).

2.4.2. Extracellular matrix and basement membrane

The muscle ECM has an important bridging role between muscle fibres and the surrounding BM and interstitial connective tissue in order to resist frictional and tensional forces. This is emphasized by primary defects in the ECM proteins responsible for muscular disorders. The BM of muscle fibres plays a crucial role in the development and function of skeletal muscle. Laminin-2 is assembled from α2, β2 and γ1 chains and represents the BM component that binds the dystrophin complex to the ECM (Miyagoe-Susuki et al. 2000). In dy/dy mice, loss of laminin α2 causes severe abnormalities in muscle BM organization and almost complete absence of the BM around muscle fibres (Xu et al. 1994).

Fukuyama congenital muscular dystrophy (FCMD) is caused by a mutation in a novel protein named fukutin. Preliminary data suggest that this is a secreted protein localized to the BM (Ishii et al. 1997, Kobayashi et al. 1998), and that it may interact with α-dystroglycan (Hayashi et al. 2001) and the laminin α2 chain (Hyashi et al. 1993). Fukutin is thought to function as an enzyme for modifying cell surface glycoproteins or
glycolipids (Aravind & Koonin 1999) and is needed to maintain BM integrity, since its absence leads to thin, disrupted BMs in FCMD (Ishii et al. 1997, Matsubara et al. 1999).

Type VI collagen is a heterotrimeric, widely expressed beaded filament-forming collagen, which interacts with the BM, and its absence, presumably caused by defective linkage between the muscle cell BM and the surrounding connective tissue, leads to myopathy in humans and mice (Kuo et al. 1997).

**Fig. 2.** Muscle diseases and their molecular mechanisms show a wide diversity. Dystrophin is a key molecule linking the actin cytoskeleton to the ECM component laminin-α2 chain via dystroglycans. β-Dystroglycan in the sarcolemma is surrounded by other transmembrane glycoproteins, the sarcoglycans and sarcospan (SPN). On the intracellular side, dystrophin is associated with dystrobrevin (DB), a syntrophin complex, neural nitric oxidase synthase (nNOS) and caveolin (C-3). In addition, other transmembrane components (integrin α7, type XIII collagen) or matrix components (fukutin and type VI collagen) cause muscular diseases. Type XV collagen may link the BM to the underlying fibrillar collagen matrix. For references, see text.
Table 3. Human disorders and mouse models for muscular diseases with the involvement of ECM components and their cellular receptors

<table>
<thead>
<tr>
<th>Protein and location</th>
<th>Proposed function</th>
<th>Human disorder</th>
<th>Cardio-myopathy</th>
<th>Mouse model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular matrix</td>
<td>Connects cells and their BMs to the interstitial connective tissue layer</td>
<td>Bethlem myopathy&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td>Col6a1&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Type VI collagen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminin α2 chain</td>
<td>BM component, binds α-dystroglycan</td>
<td>Merosin-deficient CMD&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Mild&lt;sup&gt;4&lt;/sup&gt;</td>
<td>dy/dy&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fukutin</td>
<td>BM component</td>
<td>FCMD&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Modifying enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Dystroglycan</td>
<td>Links β-dystroglycan to laminin-2</td>
<td>No (embryonic lethal in mice&lt;sup&gt;3&lt;/sup&gt;)</td>
<td></td>
<td>Dag1&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Transmembrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α7 integrin</td>
<td>Laminin receptor, links muscle fibre to ECM Signalling module</td>
<td>CMD&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Mild&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Itga7&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;10&lt;/sup&gt;</td>
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<tr>
<td>β-Dystroglycan</td>
<td>Links α- and β-dystroglycan to dystrophin</td>
<td>No (embryonic lethal in mice&lt;sup&gt;3&lt;/sup&gt;)</td>
<td></td>
<td>Dag1&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Sarcoglycan</td>
<td>Stabilizes DGC Signalling module</td>
<td>LGMD&lt;sup&gt;11&lt;/sup&gt; type 2D</td>
<td>Rare&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Sgca&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-Sarcoglycan</td>
<td>Stabilizes DGC</td>
<td>LGMD&lt;sup&gt;14, 15&lt;/sup&gt; type 2E</td>
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<td>Gsg&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;18&lt;/sup&gt;</td>
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<td>δ-Sarcoglycan</td>
<td>Stabilizes DGC Signalling module</td>
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<td>Sgcd&lt;sup&gt;-/-&lt;/sup&gt;&lt;sup&gt;20&lt;/sup&gt;</td>
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<tr>
<td>ε-Sarcoglycan</td>
<td>Stabilizes DGC Signalling module</td>
<td></td>
<td></td>
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<tr>
<td>Sarcospan</td>
<td>Stabilizes DGC s at the sarcolemma</td>
<td></td>
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<td>Caveolin-3</td>
<td>Intracellular signaling</td>
<td>LGMD&lt;sup&gt;21&lt;/sup&gt; type 1C</td>
<td></td>
<td>Col13a1&lt;sup&gt;N/N&lt;/sup&gt;&lt;sup&gt;22&lt;/sup&gt;</td>
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<tr>
<td>Type XIII collagen</td>
<td>Links muscle fibre to the BM</td>
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Table 3. Continued.

<table>
<thead>
<tr>
<th>Protein and location</th>
<th>Proposed function</th>
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<th>Cardiomyopathy</th>
<th>Mouse model</th>
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<td>Links transmembrane glycoproteins to actin</td>
<td>DMD, BMD23</td>
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<td>Syntrophins (α, β1, β2)</td>
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<td>-</td>
<td>Snta-/-26</td>
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<tr>
<td>Dystrobrevins (α, β)</td>
<td>Signalling mediator links nNOS to DGC</td>
<td>Yes27</td>
<td>-</td>
<td>Adbn-/-27</td>
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<tr>
<td>Nitric oxide synthase (nNOS)</td>
<td>Increases in muscle blood flow</td>
<td>-</td>
<td>-</td>
<td>NOS-/-28</td>
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</table>


2.4.3. The cytoskeleton-extracellular matrix axis

Dystroglycan functions as an adhesion molecule, anchoring cells to the ECM and thereby providing transmembrane linkage between the matrix and the cytoskeleton (Henry & Campbell 1999). α-Dystroglycan is an extracellular peripheral membrane glycoprotein attached to the transmembrane β-dystroglycan (Ervasti & Campbell 1991), which in turn connects the intracellular cytoskeleton to the ECM by binding dystrophin (Jung et al. 1995), whereas α-dystroglycan itself links the muscle cell membrane to the ECM by binding the BM laminin-2 (Ervasti & Campbell 1993, Gee et al. 1993).

The sarcoglycan subcomplex contains five subunits that are laterally associated with β-dystroglycan: α-, β-, δ-, γ- and ε-sarcoglycans. Sarcoglycans may function to couple mechanical and chemical signals in the muscle (Hack et al. 2000) and are required for proper targeting of sarcospan to the muscle plasma membrane (Crosbie et al. 1999). Sarcospan is a member of the tetraspan family, a group of proteins thought to mediate transmembrane protein interactions, including the integrins (Maeker et al. 1997, Crosbie et al. 1999).

The integrins are a large family of heterodimeric transmembrane cell surface receptors involved in a wide variety of cell interactions (Burkin & Kaufman 1999). Studies of integrin α7 deficient mice suggest that α7β1 integrin may function as a link between the
muscle fibre and the ECM that is independent of the dystrophin-dystroglycan complex (Mayer et al. 1997, Cohn et al. 1999).

Changes observed in mice lacking the cytosolic and transmembrane domains of type XIII collagen, a transmembrane collagen found at sites of cell adhesion, suggest a function for this collagen in the linkage between the muscle fibres and the BM (Kvist et al. 2001).

### 2.4.4. Cytoplasmic components

The syntrophins (α, β1, β2) and dystrobrevins (α and β) are intracellular proteins associated with dystrophin (Suzuki et al. 1994, Ahn et al. 1995, Sadoulet-Puccio et al. 1997). Syntrophin binds to α and β-dystrobrevins, two closely related proteins that share homology with dystrophin (Peters et al. 1997). The functional role of these proteins is not well established, but they may form a modular adapter protein complex linking signalling enzymes to the sarcolemma (Brenman et al. 1995, Bredt 1996, Chang et al. 1996, Kameya et al. 1999).

The interaction of neuronal nitric oxidase synthase (nNOS) with the dystrophin complex is mediated by syntrophins (Brenmann et al. 1996, Hashida-Okumura et al. 1999). It has important vasodilator functions in actively contracting muscle, increasing local blood flow to match the metabolic demands by attenuating sympathetic vasoconstriction (Thomas et al. 1998).

Caveolins are proteins thought to have a role in the formation of caveole membranes, specialized components of plasma membranes involved in a subset of transmembrane signalling events. Caveolin 3 is a muscle-specific caveolin believed to function in intracellular signalling (Engelman et al. 1998).

### 2.4.5. Theories of pathogenesis

#### 2.4.5.1. Mechanical weakness and contraction-induced damage

Despite the existence of extensive biochemical and genetic information on dystrophin and its associated proteins, the exact pathogenesis of Duchenne and other muscular dystrophies remains unknown. The presence of distinct subcomplexes suggests that these have different roles.

Mutations in some proteins in the complex almost certainly affect the structure of the sarcolemma while others do not (Bredt 1999). It is believed that the linkage between the actin cytoskeleton and the ECM protects the sarcolemma from mechanical stress during contraction, and it has been proposed that muscular dystrophy entails enhanced susceptibility to mechanically-induced plasma membrane disruption (Schotland et al. 1977, Ervasti & Campbell 1991, Menke & Jockusch 1991, Ohlendieck et al. 1993, Campbell 1995). In this model, the lack of one or more proteins renders the muscle
structures more vulnerable to mechanical injury, leading to sarcolemmal disruption, followed by an influx of calcium, activation of numerous proteases and subsequent segmental necrosis (Petroff 1998). Despite the great genetic heterogeneity of muscular diseases, a common pathogenic feature could be disruption of the link between the ECM and the cytoskeleton, which may occur in the subsarcolemmal part (e.g. dystrophin), at the sarcolemma level (e.g. sarcoglycans and integrin α7) or in the ECM (e.g. laminin α2 chain and type VI collagen). The structural hypothesis behind such a pathogenesis is further supported by exercise and immobilization studies with mdx mice designed to analyze the role of mechanical stress in the degeneration of dystrophin-deficient muscle (Brusee et al. 1997, Brusee et al. 1998, Vilquin et al. 1998, Mokhtarian et al. 1999).

2.4.5.2. Integrated structural and signalling system

Dystrophin deficiency is associated with specific defects in muscle cell signalling, and it may offer a scaffold for locating signalling molecules at appropriate positions in the sarcolemma or for regulating signal-transduction pathways. Nitric oxide (NO) is the major endothelial-derived relaxation factor in the body, and skeletal muscle-derived NO may also have important vasodilator functions in actively contracting muscle (Thomas et al. 1998). Interestingly, neural oxide synthase in the skeletal muscle sarcolemma is absent in Duchenne muscular dystrophy (Breman et al. 1995). α-Dystrobrevin deficiency leads to mild skeletal dystrophy and cardiomyopathy in mice (Grady et al. 1999). The distribution of dystrophin-associated proteins, including β-dystroglycan, α-sarcoglycan, dystrophin and α1-syntrophin, remains normal, but the mice show impaired nitric oxide-mediated signalling, suggesting that the muscle dystrophy in adbn−/− mice reflects non-structural roles for the complex (Grady et al. 1999). That is also the case with certain sarcoglycans (Hack et al. 2000). γ-Sarcoglycan is necessary for the proper assembly and localization of β and δ sarcoglycans at the sarcolemma, but its absence leads to dystrophic changes with an intact dystrophin-dystroglycan-laminin axis, which favours a function as a cell signalling mediator (Hack et al. 1998). Moreover, Corel-Vazquez et al. (1999) provided evidence for a novel mechanism in the pathogenesis of muscular dystrophy and cardiomyopathy by knocking out δ-sarcoglycan, which perpetuated vascular smooth muscle function and caused ischaemic damage to the cardiac and skeletal muscles. The absence of α-sarcoglycan produces a secondary absence of other sarcoglycans and sarcospan, whereas dystrophin and dystroglycans are still present at the cell membrane, suggesting that the mechanical link between the cytoskeleton and the extracellular matrix is still preserved (Duclos et al. 1998). Finally, the identification of a mutation in the caveolin-3 gene in muscle dystrophy patients further suggests that certain muscle diseases may be due to defects in signalling mediators (Minetti et al. 1998).

2.4.6. Exercise-induced muscle damage

A single episode of excessive physical exercise cause acute damage to the skeletal muscle fibres of humans and animals (Ebbeling & Clarkson 1989, Komulainen & Vihko 1998). A
sudden physical loading is thought to induce focal lesions in muscle fibres, leading to cell necrosis (Armstrong et al. 1983, McNail & Khakee 1992, Komulainen et al. 1998). This transient damage is repaired by the proliferation of satellite cells during the next two weeks, after which regenerated muscle fibres, indicated by central nuclei, are detectable in the previously damaged areas (Vihko et al. 1978). Muscle swelling (Peeze Binkhorst et al. 1990), associated with an inflammation reaction and an increase in the activity of acid hydrolases such as β-glucuronidase, are also characteristic features of muscle injuries after acute exercise. Changes in the level of β-glucuronidase are the result of enhanced lysosomal activity in the affected muscle fibres and of infiltrating macrophages (Vihko et al. 1978, Salminen 1985). An increased level of β-glucuronidase has been shown to be a reliable quantitative marker of muscle damage (Salminen & Kihlström 1985). β-Glucuronidase is measured directly from the muscle of interest, and it reflects the amount of exercise-induced damage in the muscle more specifically than serum creatine kinase levels, for example, which are a commonly used marker of muscle injury (Komulainen & Vihko 1994).

Interestingly, mice manifesting muscle phenotypes respond differently to exercise, suggesting variations in the aetiology of muscle cell damage. Dystrophin deficiency in mdx mice leads to impaired performance in running, so that adult mice of this strain ran less than half of the distance achieved by wild-type mice in voluntary treadmill experiments (Carter et al. 1995, Wineinger et al. 1998). Mdx mice have been shown to become exhausted more easily during treadmill exercise (Vilquin et al. 1998) and their muscles are more readily damaged than those of normal mice during sudden exercise (Brussee et al. 1997, Vilquin et al. 1998). A targeted mutation affecting the cytosolic and transmembrane of type XIII collagen causes histological changes in the muscle fibres and impairs running performance as well as leading to an increased incidence of skeletal muscle injuries in treadmill exercise (Kvist et al. 2001). Type VI collagen deficiency results in myopathy in both the mouse (Bonaldo et al. 1998) and man (Jöbsis et al. 1996), but despite histological signs of muscular disease, Col6a1−/− mice did not show significantly reduced running activity during two months spent in wheel cages (Bonaldo et al. 1998). Similarly, γ-sarcoglycan null mice, characterized by pronounced dystrophic muscle changes (Hack et al. 1998), tolerated a rigorous swimming protocol well and did not experience increased exercise-induced muscle injuries, suggesting non-mechanical causes of cell degeneration (Hack et al. 1999).

2.5. Vascularization of the mammalian eye

The development of the ocular circulation is related to the requirement of nourishing the eye without interfering with visual functions. The avascular structures, the cornea, lens and vitreous body, receive oxygen and nutrients via the internal circulation of the aqueous humor. The uveal blood vessels supply nutrients to the iris and the ciliary body. The retina is supported by two separate circulation systems, the superficial retinal circulation supplying two-thirds of it, whereas the outer retina is avascular and receives its nutrients and oxygen from the chorioideal vascular bed (Fig. 3).
The development of vascularization in the mammalian eye is characterized by both regression of primary vessels (Goldberg 1997) and the simultaneous formation of a vasculature in the retina (Wechsler-Reya & Barres 1997). The change from a highly vascularized primary vitreous body and avascular retina to an avascular transparent vitreous body and well-developed retinal blood vessels takes place during the first postnatal weeks in rodents. After regression of the primary vessels, the vitreous body and cornea remain avascular. In certain situations the hyaloid vasculature shows abnormalities in the normal regression pattern, possibly due to an unbalanced ratio of inducible and antiangiogenic factors, leading to the severe complications (Golderg 1997). On the other hand, conditions such as diabetic retinopathy, age-related macular degeneration or retinopathy of prematurity are characterized by inappropriate vascularization followed by the pathological growth of new blood vessels, leading to severe loss of vision or blindness (Neely & Gardner 1998, Campochiaro 2000).

2.5.1. The structure of the hyaloid vasculature

The primary function of the hyaloid vascular system (HVS) is to support the internal structures of the developing eye. It is present during the early phase of development and then regresses in a genetically programmed manner, reflecting the reduced metabolic activity of the lens during its maturation and the completion of the retinal vessels (Goldberg 1997).
The structure and regression of the HVS have been described in a number of mammalian species, including humans (Strek et al. 1993, Zhu et al. 2000), primates (Hamming et al. 1977), the dog (Boeve et al. 1988), the rat (Braekevelt & Hollenberg 1970, Latker & Kuwabara 1981), the rabbit (Jack 1972a, 1972b, 1972c) and the mouse (Ito & Yoshioka 1999). The findings have indicated some species-specific differences among mammals, in that the intraocular vessels regress and the retinal vasculature develops before birth in humans but postnatally in rodents. Although the general morphological pattern of the vasculature of the vertebrate eye is conserved, the source of the blood supply to the retina shows a clear difference in frogs, where the hyaloid vasculature in the vitreous body is a permanent component of the mature eye, the retina itself remaining avascular and receiving nutrients and oxygen from the chorioidal circulation and the permanent superficial hyaloid system adjacent to the inner limiting membrane on the anterior surface of the retina (Miodoński & Bär 1987).

The intraocular hyaloid vessels are classified based on their subanatomical location (HA, VHP, TVL, PM, Fig. 3). The hyaloid artery (HA) arises from the ophthalmic artery and enters the developing eye through the embryonic fissure. It contains bundles of arterioles and proceeds to the eye through the optic nerve and runs to the posterior pole of the lens through Cloquet’s canal. At the posterior pole of the lens, the HA subdivides into vessels covering the posterior part of the lens, termed tunica vasculosa lentis (TVL). In the vitreous body, the HA branches to form several vessels radiating over the internal surface of the retina and forming the vasa hyaloidea propria (VHP), the capillaries of which grow through the primary vitreous body and join with the TVL at the equator of the lens (Braekevelt & Hollenberg 1970, Jack 1972b, Strek et al. 1993, Ito & Yoshioka 1999). Some branches of the VHP pass through the lens laterally and drain into the vessels of the ciliary process, or bend over the edge of the iris and drain into the outer choriocapillaries (Strek et al. 1993, Los et al. 2000). The peripheral vessels of TVL in the anterior portion of the lens communicate with the network of the pupillary membrane (PM) in the anterior chamber of the eye (Strek et al. 1993). The PM is also supplied by the long posterior ciliary arteries (PCA) and attached to vessels of the ciliary processes and the initial segments of the choriocapillaries (Strek et al. 1993).

### 2.5.2. Normal hyaloid vasculature development and regression

The HVS in the mouse is detected at day 10.5 of gestation and is essentially complete by day 13.5 (Bremer et al. 1988, Mitchell et al. 1998). Regression is clearly detectable postnatally, although the first signs can be observed as early as embryonic day 17.5 (Mitchell et al. 1998). This regression occurs simultaneously with the development of the retinal vasculature and the formation of the avascular secondary vitreous body (Bischoff et al. 1983).

The regressing vessels show certain morphological changes in polarization, location and structure. They lose their polarization and their contact with the lens capsule and retina. Endothelial cells and pericytes show typical apoptotic changes, the vessels being collapsed, flattened and narrowed in diameter, decreasing in tortuosity and losing their small interconnecting vessels. The capillary wall is hyalinized and acellular in appearance.
and the vessel lumina are occluded by cell debris and hyalocytes (tissue macrophages). Hyalocytes are also visible in the vicinity of the regressing vessels. The BMs of the regressing capillaries become discontinuous. At the final stage of regression, the pericytes and endothelial cells disappear, leaving BM-like remnants behind (Latker & Kuwabara 1981, Meeson et al. 1996, Mitchell et al. 1998, Ito & Yoshioka 1999, Zhu et al. 2000)

There are no dramatic changes in the number of VHP in the mouse until the eighth postnatal day, when the vessels decrease rapidly in number within two days (Ito & Yoshioka 1999). During the first postnatal days the VHP changes its position and is apparently pushed towards the lens as a consequence of a secondary vitreous body formation in the space between it and the retina. Eventually the VHP overlies the posterior part of the lens (Bischoff et al. 1983, Ito & Yoshioka 1999). Five days after birth, macroscopic changes such as breakdown in the capillary structure and loss of the interconnecting capillaries become evident in the TVL (Mitchell et al. 1998), and the number of longer branches of capillaries around the lens decreases moderately up to postnatal day 21 (Mitchell et al. 1998, Balazs et al. 1980, Ito & Yoshioka 1999). The HA is the only vessel structure which continues its development after birth, persisting at least up to day 16, but disappearing by the end of the third postnatal week. Unlike the rapid involution of the VHP, the PM shows a progressive slow decrease in its number of vessels after birth and is removed from the mouse eye between the fourth and ninth days postnatally (Lang & Bishop 1993, Ito & Yoshioka 1999).

Regression normally takes place before birth in man, while in the other primates studied it occurs during postnatal development (Hamming et al. 1977). The HVS in humans is well established in the ten-week-old fetus and fully developed by 12-13 weeks of gestation (Zhu et al. 1999). In contrast to the mouse, development of the avascular secondary vitreous body and complete retinal vascularization takes place in the fetus, between the fourth month of gestation and birth (Goldberg 1997).

2.5.2.1. Mechanism of the vessels regression

Although the intraocular hyaloid vessels are derived from the same mesodermal tissue and are similar in appearance to a continuous endothelium, BM and pericyte covering (Braekevelt & Hollenberg 1970, Jack 1972a, Zhu et al. 1999), they show differences in the time course of regression and pattern of initial vessel closure (Jack 1972c). This suggests the presence of various triggering factors and pathways in the process of regression.

The vessels in the primary vitreous body degenerate in a developmentally programmed manner. The factors(s) triggering the cascade of events, whether derived from endothelial cells or from exogenous sources, are not well known. Several mechanisms have been proposed to explain the background to vascular regression (Figure 4). These include increasing ocular size and retinal angiogenesis (Latket & Kuwabara 1981, Bischoff et al. 1983), obstruction of the vessels (Jack 1972b, Jack1972c), physical vascular stretching and loss of close contact between the endothelial cells and the posterior lens capsule (Latket & Kuwabara 1981, Mitchell et al. 1998), changes in haemodynamic forces and the cessation of blood flow (Meeson et al. 1996, Ito &
Several studies have shown that obstruction of the hyaloid capillaries by macrophages occurs in the early phase of degeneration, and this has been proposed as one of the triggering factors (Jack 1972b, Jack 1972c, Hamming et al. 1977, Ito & Yoshioka 1999).

HVS atrophy during postnatal development in the mouse is accompanied by development of the retinal vasculature. The hyaloid and retinal vasculatures share a common arterial supply from the optic nerve head, suggesting that these developmental events could be linked (Fig. 4A). Interestingly, in a high-oxygen atmosphere the retinal vasculature regresses and the HVS persists and dilates, suggesting that normal regression of the HVS is coupled with development of the retinal vasculature, and an experimental elevation of pressure in the hyaloid vessels inhibits the normal process of regression (Bishoff et al. 1983).

It has been suggested that survival factors such as vascular endothelial growth factor (VEGF) are present in the neonatal plasma, and that the diminished blood flow affects the endothelial cells within the obstructed vessels (Lang et al. 1994, Alon et al. 1995). During the early development of the lens the posterior epithelial cells express VEGF, which is available for the TVL vessels located at the posterior surface of the lens (Fig. 4B). In the later stages, VEGF expression is restricted to the anterior and equatorial areas of the lens, due to epithelial cell differentiation and thickening of the lens capsule. The regression process may be initiated by the physical separation of the source of survival factors (e.g. VEGF) from the endothelial cells (Mitchell et al. 1998).

It has been demonstrated on a number of occasions that the hyaloid vessels regress segmentally by apoptosis (Lang & Bishop 1993, Lang et al. 1994, Diez-Roux & Lang 1997, Mitchell et al. 1998, Taniguchi et al. 1999). More specifically, Meeson et al. (1996) observed a direct relationship between the extent of cell death and the amount of blood flow: as the flow decreased, apoptosis of the endothelial cells increased. In addition to reduced availability of survival growth factors, reduced flow and declining haemodynamic forces may lead to changes related to a low oxygen level and the expression of certain genes regulated by the shear force (Meeson et al. 1996).

2.5.2.2. The role of tissue macrophages, hyalocytes

A number of investigators have suggested that hyalocytes may play an active role in HVS regression (Jack 1972c, Hamming 1977, Latker & Kuwabara 1981, Lang & Bishop 1993, Meeson et al. 1996, Diez-Roux & Lang 1997). The first evidence for the involvement of hyalocytes came from histological studies, where they were observed to be in close contact with regressing vessels, leading to the idea of the presence of direct signalling processes and a scavenger role for macrophages (Ito & Yoshioka 1999, Zhu et al. 2000). Genetic studies revealed that macrophages could be directly involved in the apoptosis of capillary endothelial cells in the PM. Lang and Bishop (1993) showed that macrophage ablation in genetically modified mouse eyes results in a lack of normal cell death and tissue remodelling, causing persistence of the HVS. Subsequently, Diez-Roux et al.
(1997) demonstrated that hyalocytes are essential for regression of the HVS by injecting toxic liposomes into the eye in order to eliminate them. This resulted in the persistence of functional PM vessels and survival of the endothelial cells. Furthermore, the phenotype was rescued by replacement of the eliminated hyalocytes with bone-marrow-derived macrophages.

After the initial observations of an interrelationship between apoptosis, macrophages, serum survival factors and regression of vessels, Meeson et al. (1996) proposed the following hypothesis regarding a two-stage process of eye vessel involution (Fig. 4C). The first stage is the macrophage-dependent induction of endothelial cell apoptosis, which reduces the blood flow (Lang & Bishop 1993, Lang et al. 1994, Diex-Roux & Lang 1997). The second is the coordinated apoptosis of capillary endothelial cells, which is dependent on the cessation of plasma flow (Meeson et al. 1996). The macrophages drive most aspects of capillary regression in this model (Meeson et al. 1996). Zhu et al. (2000) have proposed three mechanisms that may be involved in involution of the HVS: firstly, cytolysis of vascular cells, apparently mediated by juxtavascular hyalocytes, resulting in apoptotic or necrotic cell death, secondly, loss of vascular cells, leading to the thinning and partial blockage of vessels, which is associated with blood flow stasis in segments of the vasculature, and thirdly, elimination of vascular debris by neighbouring cells, including hyalocytes.

2.5.2.3. Inhibition of vascular cell growth by the vitreous humor

After regression of the HVS, the cornea, lens and vitreous body have to remain avascular in order to ensure normal vision. The vitreous body is normally able to resist endothelial cell invasion and the disturbing consequences of neovascularization which may occur under pathological conditions (Neely & Gardner 1998, Campocchiaro 2000). The regression and prevention of vessel invasion may partly be due to the influence of angiogenic inhibitors (Lutty et al. 1985), as several studies have assigned antiangiogenic properties to the vitreous humor. Tumor vascularization and growth within the intact vitreous body is prevented (Brem et al. 1976), extracts from it inhibit endothelial cell proliferation and viability in vitro (Lutty et al. 1985, Zhu et al. 1997), and suppress tumor-induced neovascularization (Felton et al. 1979) and angiogenesis in the chick CAM assay in vivo (Lutty et al. 1983). Putative antiangiogenic molecules present in the vitreous humor have been described in some studies (Fig. 4D), and these inhibitory factors are thought to derive from hyalocytes (Raymond & Jacobson 1982, Zhu et al. 1997). Such molecules include chondromodulin-1 (Funaki et al. 2001), thrombospondin-1 (Sheibani et al. 2000), a pigment epithelial-derived factor (Dawson et al. 1999), transforming growth factor β (Eisenstein & Grant-Bertacchini 1991), and unidentified proteins of certain defined molecular weights (Lutty et al. 1985, Taylor & Weiss 1985).
Fig. 4. Proposed mechanisms for the regression of vessels in the primary vitreous body. A, The hyaloid and retinal vasculatures share a common arterial supply from the optic nerve head, and it has been suggested that regression of HVS is coupled to and triggered off by the development of the retinal vasculature. B, In the case of TVL, regression may be induced by physical separation of the cells producing ‘survival factor’ (VEGF) from the vascular endothelial cells due to the differentiation of the lens epithelial cells and thickening of the lens capsule. Removal of the cellular debris is completed by phagocytosis (on the right). C, A macrophage-dependent apoptosis model suggests that hyalocytes associate with endothelial cells and actively induce their death in a two-step process. It is proposed that the hyalocytes induce ‘initiating apoptosis’ in single endothelial cells in normal capillary segments, which reduces the blood flow and consequently leads to ‘secondary apoptosis’ of the remaining endothelial cells. In the last phase of regression, the hyalocytes phagocytose the cell debris (on the right). D, The regression of the embryonic vasculature and maintenance of the avascularity of the mature vitreous body could be due to the ability of the latter to inhibit blood vessel growth, possibly involving chondromodulin-1 (ChM-I), thrombospondin-1 (gb140), pigment epithelial-derived factor (PEDF), transforming growth factor β (TGFβ) and unidentified proteins with molecular weights of 5700, 6200 and 12000. Hyaloid vascular system (HVS), retina (R), optic nerve (ON), tunica vasculosa lentis (TVL), pupillary membrane (PM). For references, see text.
2.5.3. Abnormalities in regression of the hyaloid vasculature

2.5.3.1. Animal models

Persistence of the HVS is a rare abnormality in mammals, and many of the reported cases are sporadic, only one eye being affected. Certain engineered and naturally occurring mutations nevertheless indicate a heredity basis for this (Table 4). Typically, the presence of the HVS is associated with abnormal proliferation of fibrovascular tissues posterior to the lens and the development of cataract.

Although detailed morphological descriptions of conditions with persistent HVS in different species have been provided, the exact causes of the disease have not been determined. Various reasons have been proposed for this developmental abnormality. Spitznas et al. (1990) described it as a result of imperfect development of the lens, Boeve et al. (1988) and Lutty et al. (1985) suggested that persistence of the fetal vasculature could have been caused by an imbalance in regulating growth factors, an increased growth signal or decreased inhibition of intravitreal angiogenesis. It has also been hypothesized that the condition may be due to failure in hyalocyte function (Jack 1972c; Balazs et al. 1980, Latker & Kuwabara 1981).

2.5.3.2. Human diseases

Persistence of the fetal intraocular vasculature in humans causes a disorder known as persistent hyperplastic tunica vasculosa lentis/persistent hyperplastic primary vitreous (PHTVL/PHPV), or more accurately persistent fetal vasculature (PFV) (Goldberg 1997). This is a rare abnormality and usually occurs unilaterally, affecting one eye without apparent hereditary influence in an otherwise normal child (Goldberg 1997, Silbert & Gurwood 2000). About 10% of cases are bilateral, however, and familial inheritance with autosomal dominant, autosomal recessive and X-linked gene mutations have been reported (Haddad et al. 1978, Goldgerg 1997, Silbert & Gurwood 2000). Persistent vasculature in both eyes is often associated with systemic diseases, including trisomy 13, trisomy 15 and trisomy 18, Walker-Warburg syndrome and Norrie disease. Without treatment, it may cause progressive complications, leading to severe loss of vision or blindness (Goldberg 1997). Anatomically defined components of the fetal intraocular vasculature (HA, TVL, VHP, PM) may persist in combination with others, but any of the vascular remnants can dominate in various combinations or may occur alone, and in rare cases they are all found. In a typical case, HA is connected with a fibrous tissue membrane covering the posterior surface of the lens, which usually results in cataract (Goldberg 1997). The most typical clinical presentations in humans are leukocoria, microphthalmia, cataract, retinal detachment, glaucoma and phthisis bulbi (Haddad et al. 1978, Goldgerg 1997, Silbert & Gurwood 2000).
Table 4. Abnormalities in regression of the HVS due to spontaneous mutations in domestic animals and genetically modified mice

<table>
<thead>
<tr>
<th>Genetic manipulation or inbred strain</th>
<th>Possible gene function and consequences</th>
<th>Eye phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic mouse expressing diphtheria toxin under a macrophage-specific promoter</td>
<td>Ablation of hyalocytes in the eye</td>
<td>Lack of normal cell death and tissue remodeling causing persistence of the hyaloid vasculature(^1)</td>
</tr>
<tr>
<td>p53-null mice</td>
<td>Tumor suppressor, essential for cell cycle arrest and for activation of the apoptotic death pathway</td>
<td>Reduced apoptosis in the HVS, persistence of TVL and HA, cataract and development of fibrovascular plaque posterior to the lens(^2)</td>
</tr>
<tr>
<td>“Norrie disease gene”-null mice</td>
<td>Secreted growth factor, homologous with TGF(\beta), involved in regulation of angiogenesis in the eye</td>
<td>Persistence of fetal hyaloid vessels in the vitreous body and abnormalities in retinal vascularisation(^3)</td>
</tr>
<tr>
<td>Angiopoietin-2 null mice</td>
<td>Vascular growth factor, involved in angiogenic remodelling</td>
<td>Persistence of fetal hyaloid vessels in the vitreous body and abnormalities in retinal vascularisation(^4)</td>
</tr>
<tr>
<td>Inbred SAM-P/9 mouse line</td>
<td>Accelerated senescence, at least two genes contribute to the eye defects, genetic loci not identified</td>
<td>Persistence of VHP and posterior TVL, cataract, and development of a hyperplastic fibrovascular plaque posterior to the lens(^5)</td>
</tr>
<tr>
<td>Spontaneous mutation in deer mice(^6)</td>
<td>Genetic loci not identified</td>
<td>Persistence of the primary vitreous vascular system, cataract, rudimentary iris and ciliary body(^6)</td>
</tr>
<tr>
<td>TgN3261Rpw transgenic mouse line</td>
<td>Produced by insertional mutagenesis, genetic locus not identified</td>
<td>Persistent hyperplastic primary vitreous body and TVL, failure of cleavage of the anterior chamber, retrolental fibrovascular membrane, posterior polar cataract, and detached retina(^2)</td>
</tr>
<tr>
<td>Staffordshire bull terrier</td>
<td>Spontaneous mutation, genetic locus not identified</td>
<td>Persistent fetal intraocular vessels, leukocoria, cataract, lens coloboma, haemorrhage and development of hyperplastic fibrovascular plaque posterior to the lens(^6)</td>
</tr>
<tr>
<td>European ferret</td>
<td>Spontaneous mutation, genetic locus not identified</td>
<td>Persistent fetal intraocular vessels, proliferation of fibrovascular tissue in the posterior lens capsule, cataract, and retinal detachment(^7)</td>
</tr>
</tbody>
</table>

References: \(^1\)Lang & Bishop (1993), \(^2\)Reichel et al. (1998), \(^3\)Richter et al. (1998), \(^4\)Yancopoulos et al. (2000), \(^5\)Hosokawa et al. (1993), \(^6\)Burns & Feeney (1975), \(^7\)Colitz et al. (2000), \(^8\)Curtis et al. (1994), \(^9\)Lipsitz et al. (2001).

2.5.4. Development of the retinal vasculature

In rodents, development of the retinal vascularization begins at a very late stage in gestation and proceeds during the first two weeks postnatally (Calay et al. 1972, Conolly et al. 1988, Jiang et al. 1995, Stone et al. 1995, Benjamin et al. 1998), whereas in
humans it occurs during the third trimester (Hughes et al. 2000). Retinal vascularization may occur through a combination of vasculogenesis by ‘spindle-shaped’ endothelial precursor cells and angiogenesis by sprouting from pre-existing vessels, as suggested by several authors, including McLeod et al. (1987), Chan-Ling et al. (1990), Jiang et al. (1995), Schaepdrijver et al. (1995) and Hughes et al. (2000). The extent of vasculogenesis involved in retinal development may have been overestimated in previous studies, however (Provis 2001), and the significance of the vasculogenic process for the development of the retinal vasculature remains to be elucidated. After initial vascularization of the superficial retina, new vessels sprout from the superficial retinal vascular plexus and invade the deep layers. Once the primary vascular bed is formed, it is remodelled in accordance with the oxygen tension in the tissue. The model for retinal vascularization presented in Figure 5 is based on Stone et al. (1995), Wechsler-Reya & Barres (1997) and Benjamin et al. (1998).

2.5.4.1. Development of the retinal vasculature is regulated by oxygen and mediated by VEGF

It was first demonstrated in the early 1960’s that the development of the retinal vasculature is regulated by oxygen levels, when it was noted that exposure of young animals to high levels of oxygen (hyperoxia) in an artificial atmosphere led to obliteration of the newly formed vessels, whereas a reduction in the level of oxygen (hypoxia) had the opposite effect (Ashton 1966). It was later demonstrated that vascularization is mediated by the vasoformative function of the retina itself, and vascular endothelial growth factor (VEGF) was identified as a major stimulatory factor (Miller et al. 1994, Pierce et al. 1995). The essential role of VEGF was confirmed by the observation that VEGF was expressed in neuroglial cells (astrocytes and Müller cells) in the avascular peripheral retina at the same time and in the region where the vessels formed, but always spreading ahead of the developing vessels (Stone et al. 1995). Moreover, the neuroglial cells showed direct oxygen-dependent expression of VEGF, in that endogenous VEGF production was suppressed in oxygen-enriched atmospheres and vessel formation was inhibited (Alon et al. 1995), whereas hypoxia upregulated VEGF expression and stimulated angiogenesis (Alon et al. 1995, Pierce et al. 1995, Stone et al. 1995). VEGF is not only needed for the induction of vessels formation, but also acts as a survival factor. Premature down-regulation of VEGF expression in the retina, induced by hyperoxia, results in endothelial cell apoptosis and obliteration of the newly formed vessels (Alon et al. 1995, Yamada et al. 1999), which can be prevented by VEGF treatment (Alon et al. 1995).

2.5.4.2. Astrocyte migration precedes retinal vascular development, which is induced by retinal ganglion cells and mediated by PDGF-A

Cell migration, secreted growth factors, endothelial cells, astrocytes, Müller cells and retinal ganglion cells play a central role in the early phase of retinal vascularization
Astrocytes migrate from the optic disc to the retinal periphery in advance of retinal vascular development and form a physical template for the vessels (Ling & Stone 1989, Watenabe & Raff 1988, Ling et al. 1989, Jiang et al. 1994). Their growth and spreading is dependent on signals from the retinal ganglion cells, mediated by secreted platelet-derived growth factor A (PDGF-A). Blocking of the PDGF-A signalling pathway will prevent normal astrocyte migration and spreading, indicating that the migration of astrocytes is highly dependent on PDGF-A and is guided by the retinal ganglion cells (Fruttiger et al. 1996). The close relationship between retinal ganglion cells, secreted PDGF-A, astrocytes and the developing vasculature has been further demonstrated by generating transgenic mice that over-express PDGF-A in their retinal ganglion cells. Such mice showed a hyperproliferation of astrocytes, which consequently led to overproduction of retinal capillaries (Fruttiger et al. 1996). Vascularization of the superficial retinal layer may be preceded by an invasion of endothelial precursor cells into the nerve fiber layer from the optic disc (McLeod et al. 1987, Chan-Ling et al. 1990, Jiang et al. 1995, Schaepdrijver et al. 1995, Hughes et al. 2000).

2.5.4.3. Angiogenesis of deeper retinal layers

In the second stage, beginning in approximately four-day-old mice and continuing over the second and third postnatal weeks, the deeper retinal layers are vascularized (Fig. 5). During this process the vessels emerge as buds from the superficial layer and start to grow towards the sclera. This happens without being preceded by populations of either vascular precursor cells (Chan-Ling et al. 1990) or astrocytes (Chan-Ling & Stone 1991). During the process, the Müller cells, which are found through the entire thickness of the retina, transiently express VEGF, driving vessel formation in the deeper layers (Stone et al. 1995).

2.5.4.4. Remodelling and maturation of the capillary plexus

The last stage is characterized by maturation of the preformed capillary plexus, leading to the final vascular pattern with appropriate branching of arteries, veins and capillaries (Conolly et al. 1988). The remodelling occurs within a defined time, the 'plasticity window', this being during the first two postnatal weeks in mice. The most pronounced remodelling event is a massive regression of unnecessary capillaries (vascular pruning), resulting in the formation of capillary-free zones around the arterial vessels and a decrease in vascular density (Benjamin et al. 1998). The remodelling occurs in transiently pericyte-free endothelial plexuses and is regulated by PDGF-B and VEGF (Benjamin et al. 1998) (Fig. 5).
Fig. 5. Cell migration and secreted growth factors are essential for normal vascularization of the retina. 1, Astrocytes (A) migrate across the surface of the retina (R) from the optic nerve (ON) in a manner that is dependent on signals from retinal ganglion cells (G) expressing PDGF-A (circles). 2, Vascularization of the superficial retinal layer may be preceded by the migration of endothelial precursor cells (EPC). 3, Increased metabolic activity in the growing retina causes “physiological hypoxia”, which is detected by the astrocytes and Müller cells (M). 4 and 5, In response to this, neuroglial cells secrete VEGF (asterisks) and activate the formation of retinal vessels. 6, The newly formed vessels become perfused and the physiological oxygen level down-regulates VEGF expression. 7, The preformed vascular plexus is further remodelled by ‘vascular pruning’. The maturation of vessels witnessed by pericyte (P) coating lags behind the formation of the blood vessels to a significant degree. Vitreous body (V). For references, see text.
3. Outlines of the present research

The advances made in modern molecular biology during the last two decades have led to a remarkable expansion in genomic information and to the discovery of a large number of new genes, as witnessed in the case of the collagen family, for instance. At the time when this work was started, two new collagens, types XV and XVIII, had recently been found and their primary structures and genomic organizations were partly known. In order to understand their biological functions, to evaluate their possible involvement in human diseases and to study the related pathogenic mechanisms, the following aims were set for this work:

1. To isolate mouse *Col15a1* genomic clones and characterize the gene structure and promoter elements in order to study genetic conservation by comparing the mouse gene with its human counterpart and to facilitate the generation of transgenic and targeted mouse lines.

2. To produce type XV collagen-deficient mice and analyze the consequences of the lack of the gene product.

3. To analyze the consequences of type XVIII collagen deficiency in mice, especially bearing in mind possible changes related to angiogenic mechanisms, which may be associated with the antiangiogenic properties of the type XVIII collagen-derived endostatin fragment.
4. Materials and methods

4.1. DNA and RNA analyses

4.1.1. Isolation of genomic clones (I)

A mouse genomic lambda library (Stratagene) and a cosmid library (Stratagene) were screened using cDNA and genomic fragments coding for human or mouse α1(XV) chains. DNA fragments were radioactively labelled with [α-32P]dCTP (3000 mCi/mmol, Amersham) using random priming (Pharmacia Biotech) or nick-translation methods (GIBCO). When cDNA clones for the human α1(XV) chain were used as probes, the screenings were performed under cross-species conditions: at 37°C in 35% (v/v) formamide in 6xSSC (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 6.8), 1% (w/v) bovine serum albumin, 1% Ficoll (w/v), 1% polyvinylpyrrolidone (w/v), 0.25 mg denatured salmon sperm DNA/ml and 0.1% SDS. The final washes for the filters were carried out in 2xSSC and 0.1% SDS at 55°C. To isolate clones from the 3’ end of the gene, a mouse α1(XV) cDNA clone (HM-29.2, Hägg et al. 1997a) was used as a probe under stringent hybridization conditions (Sambrook et al. 1989). To obtain clones from the 5’ end of the Col15a1 gene, a 260 bp PCR-fragment LE-MXV5/6 was generated using the oligonucleotides LE-MXV5 (5’-ATGGAAATTCGACTCAGCGCGGGCA GC-3’) and LE-MXV6 (5’-ATGGAAATTCGCGGTCGCTGGACAGCGG-3’) containing non-cDNA sequence-specific EcoRI recognition sites (underlined) in the 5’ end of the primers for cloning. To cover gaps in previously found genomic clones, a cosmid library was screened with three probes under stringent conditions. Screening with a 470-bp PCR fragment PHMXV26/47, generated using the oligonucleotides PHMXV26 (5’-GACCTTCAGCAGATCC-3’) and PHMXV47 (5’-GTTAACCCAGCTGTGT-3’), Sac I-fragments HP3’ (3’ end of clone MG5’8) and 3.18S2 (5’ end of clone MG-3.1), then yielded clones covering the gap in the previously isolated genomic clones.
4.1.2. Characterization of genomic clones and DNA sequence analysis (I, II, III)

DNA from genomic cosmid and λ clones was isolated by standard methods (Sambrook et al. 1989) and analyzed by restriction enzyme mapping and Southern blotting. Appropriate restriction fragments were subcloned into the plasmid pBluescript SK (Stratagene) or pSP72 (Promega). Sequencing, restriction mapping, PCR and Southern blot analysis were used to determine the sizes of the introns. Partial digestions fractionated by pulse field gel electrophoresis (Bio-Rad) followed by Southern blot analysis using 5’ end labelled insert-end specific oligonucleotides were used to determine the size and restriction map of the second intron. Nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger et al. 1977), either manually or using an automated DNA sequencer (Applied Biosystems). The nucleotide sequence data were analyzed by DNASIS (Amersham Pharmacia Biotech). Consensus sites for the binding of transcription factors were searched for in the Transcription Factor Data Base using the Sequence Analysis software package, Version 8.0 (Genetics Computer Group, Inc.). The BLASTN 2.0.5 program (www.ncbi.nlm.nih.gov/gorf/wblast2.cgi) was used to identify homologous 5’ sequences in the human COL15A1 and mouse Col15a1 genes.

4.1.3. Generation of deletion constructs for Col15a1 promoter analysis (I)

To identify 5’ sequences of importance for the regulation of Col15a1 expression, a series of six 5’ deletion constructs containing the genomic region spanning from -135 to ~-7900 bp were cloned upstream of the luciferase reporter gene. The constructs were obtained as follows. For the construct named M2, a 3696 bp BamHI-fragment (-3654 to +42) derived from the genomic λ clone was inserted into the BglII site of the pGL2-Basic Vector (Promega) upstream of a luciferase reporter gene. To create four truncated 5’-flanking sequences containing a common 3’ end (+42), the BamHI subclone M2 was digested with restriction enzymes. To obtain the construct M3, the plasmid M2 was first digested with MscI (position -2926) and SmaI, which makes a cut within the vector polylinker. The linear DNA was then religated. Cleavage of M2 with SnaI (position -2064) and SmaI and religation resulted in construct M4. To create construct M5, the plasmid M2 was digested with the restriction enzymes SpeI (position -657) and NheI within the vector polylinker and religated. Cleavage of M2 with SaeI (position -135), which cuts sites within both the promoter sequence and the vector polylinker, followed by religation, gave construct M6. To clone the longest construct (M1), a 7.2 kb SpeI-restriction fragment (about ~7900 to −657), derived from the genomic λ-clone, was ligated to SpeI and NheI-digested M2 plasmid. Thus the deletion constructs used in promoter analysis consisted of the following fragments: M1, about −7900 to +42; M2, −3654 to +42; M3, −2926 to +42; M4, −2064 to +42; M5, −657 to +42; and M6, −135 to +42. In the case of the human promoter fragments, the same constructs were used as described earlier (Hägg et al. 1998).
4.1.4. Construction of the targeting vector for Col15a1 inactivation (II)

A genomic clone including the first two exons, about nine kb of 5’-flanking sequences and five kb of the second intron of the Col15a1 gene, was isolated from a mouse 129sv genomic library (Stratagene). To construct a targeting vector, a 10.2 kb fragment containing exons 1 and 2, 4.5 kb of the second intron and a 5.5 kb of the 5’-flanking region was subcloned into the pSP72 vector (Promega). A PCR fragment containing a loxP sequence with novel XbaI sites to allow ligation and deletion of a genomic SpeI-site after cloning was created by PCR and inserted into the SpeI site existing about 900 bp upstream of the first exon. A selection marker gene cassette (the neomycin resistance gene and the herpex simplex virus tymidine kinase gene), flanked by loxP sites and containing a novel EcoRI site just upstream of the 5’ loxP sequence, was released from the plasmid and inserted into an ApaI site 120 bp downstream of the second exon. All the parental plasmids containing the loxP sequences and the selection marker genes were constructed by Reinhard Fässler (Max-Planck-Institut, Martinsried, Germany).

4.1.5. Construction of the targeting vector for Col18a1 inactivation (III)

A genomic clone corresponding to exons 17 to 38 of the Col18a1 gene was isolated from the mouse 129sv genomic library (Stratagene), and a 12 kb NotI / KpnI fragment was used to construct a replacement vector. A selection gene cassette containing the phosphoglyceratekinase gene promoter, the neomycin resistance gene and a polyadenylation signal was ligated into a SalI site to disrupt the reading frame within exon 30.

4.1.6. Nuclease S1 protection assay (I)

The transcription initiation sites of the Col15a1 gene were determined by nuclease S1 protection analysis. Total RNA from mouse heart and kidney isolated by guanidium isothiocyanate-chloroform-phenol extraction (Chomczynski & Sacchi 1987), the materials for which were generous gifts from Ritva Ylikärppä (University of Oulu, Oulu, Finland). The genomic probe (755 bp PstI-PvuII fragment: nt -666 to +89) was 5’-end-labelled with T4 polynucleotide kinase and [γ-32P] ATP (3000 Ci/mmol, Amersham Pharmacia Biotech). The double-stranded probe (8 x10^4 cpm) was hybridized to 40 and 20 µg of total RNA from mouse kidney and heart, respectively, in the presence of 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl and 1mM EDTA at 60°C for 16 h. After hybridization, 300 µl of buffer (280 mM NaCl, 50 mM sodium acetate, pH 4.5, 4.5 mM ZnSO4) was added and the mixture was digested with 800 units of S1 nuclease (Boehringer, Mannheim) at room temperature for 20 min. The protected fragments were analyzed on a 6% polyacrylamide gel. Twenty microgrammes of yeast tRNA was used as a negative control. The sizes of the protected fragments were determined by comparison
with parallel runs of \( \alpha^{32}\)SdATP-labelled DNA sequencing reactions (Sanger et al. 1977). The major start site was designated as +1 in the Col15a1 sequence.

4.1.7. Northern blot hybridization (II, III)

Northern blot hybridization was used to study the expression of type XV collagen mRNAs in the homozygous mutant mice. Total RNAs were isolated as described above and Northern blotting was performed (Sambrook et al. 1989). The filters were hybridized with a \( \alpha^{32}\)P-labelled mouse type XV collagen cDNA probe encoding exons 7-10, and a human \( \beta\)-actin probe (Clontech) was used as a control.

To analyze the consequence of insertion in the Col18a1 allele, total RNAs were prepared using a modified guanidinium thiocyanate method (Chirgwin et al. 1979), separated by ultracentrifugation for overnight in 5.7 M cesium chloride and precipitated in 50% isopropanol. Northern blots were hybridized with the cDNA probe corresponding to the 3’ untranslated and endostatin regions of the mRNA. A cDNA probe corresponding to glyceraldehyde-3-phosphate dehydrogenase mRNA was used as a positive control.

4.2. Cell culturing

4.2.1. NIH/3T3 cell culture, transfections and luciferase assays (I)

NIH/3T3 cells obtained from the American Type Culture Collection were routinely cultured at 37°C in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 10% calf serum (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 4.5 g/L glucose (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 100 units/ml penicillin (Gibco BRL) and 50 \( \mu \)g/ml streptomycin (Gibco BRL) and adjusted to contain 1.5 g/L sodium bicarbonate (Gibco BRL). The cells were transiently transfected with the non-liposomal FuGENETM 6 Transfection Reagent (Boehringer Mannheim), according to the manufacturer’s protocol. Briefly, cells at 80% confluence were transfected with 2 \( \mu \)g of the various luciferase deletion constructs mixed with 6 \( \mu \)l FuGENETM 6 Transfection Reagent and harvested 48 h after transfection. Luciferase activity was determined from cell extracts using the luciferase assay system (Promega). To normalize transfection efficiency, total DNA was extracted from each sample and dot-blotting was performed. The nitrocellulose membrane was hybridized with a specific probe corresponding to a fragment of the luciferase reporter gene, and densitometry scanning of the autoradiograms was performed with the Bioimage scanning software (Millipore). The results were expressed as relative luciferase activity. The pGL2-Basic vector and pGL2-Control vector (Promega) were used as negative and positive controls, respectively.
4.2.2. Embryonic fibroblast cell culture and mitogenic inactivation (II)

The embryonic fibroblast cell culture was established as described (Joyner 1993). Briefly, cells from embryos at 14.5 days post coitum derived from a transgenic mouse line expressing a neo-resistance gene (Department of Biochemistry, University of Oulu, Oulu, Finland) were cultured in a medium containing high glucose Dulbecco’s MEM (Biochrom KG Seromed), 1 mM sodium pyruvate (Gibco BRL), 10% fetal bovine serum (Gibco BRL), non-essential amino acids (Gibco BRL) and 2 mM Glutamax-I (Gibco BRL). They were grown to confluence, trypsinized and resuspended in the medium, whereafter the mitogenic ability was arrested with γ-irradiation (1500 rad for 30 min).

4.2.3. Embryonic stem cell culture (II)

R1-embryonic stem cells (Nagy et al. 1993) were cultured in the medium as above (4.2.2.), but supplemented with 20% fetal bovine serum (Gibco BRL), 0.1 mM β-mercaptoethanol (Sigma) and 1000 U/ml murine leukemia inhibitory factor (Gibco BRL). The ES cells were routinely cultured on a mitotically inactivated embryonic fibroblast cell layer and trypsinized every second day.

4.3. Generation of Col15a1−/− and Col18a1−/− mouse lines (II, III).

The Col15a1 targeting vector (100 µg) was linearized with XhoI, electroporated (800 V, 3 µF) into 4.2x10^7 R1-ES cells and cultured on embryonic fibroblast feeder cells. After selection, genomic DNA from G418 (400 µg/ml; Gibco BRL) resistant ES clones was digested with SpeI and analyzed by Southern blot hybridization with a 5’ external probe. Seven out of 140 clones were correctly targeted, having the selection marker genes (neo’ and HSI-tk) in the second intron of the Col15a1 gene and loxP sites flanking the first two exons. To create Cre-mediated deletions, 3.7x10^7 targeted ES cells were electroporated with 29 µg of the supercoiled Cre-plasmid (pIC-Cre, a gift from Dr. W. Müller, University of Cologne, Germany). Ganciclovir selection (2 µM, Syntex) was started one to four days after electroporation, depending on the plate. Cells were selected for five days and replated at the proper density if needed. The DNA from 32 surviving ES clones was digested with XhoI and EcoRI to identify Cre-mediated recombinations. Two clones shown by Southern blotting to have an inactivated Col15a1 allele were injected into blastocysts and implanted in pseudopregnant mice. Both produced highly chimeric mice, which were bred with both C57Bl/6J and 129sv females to produce mice of mixed and inbred genetic backgrounds, respectively. Heterozygous F1-mice were mated to obtain the homozygous null mice.

In the case of Col18a1 targeting, J1 ES cells were transfected with the linearized replacement vector by electroporation and selected in medium containing G418 as described (Li et al. 1992). The genotypes of the G418-resistant clones was analyzed by EcoRI digestion and Southern blotting with an external probe. One positive ES cell clone
with a homologous recombination event was identified among 188 ES clones screened. The correctly targeted clone with an interrupted Col18a1 allele was injected into blastocysts to generate chimeric mice. The founders were then bred with either C57BL/6J or 129sv/J mice to generate 129sv inbred or F1 hybrid heterozygous mutant mice, respectively. The F1 heterozygous mice were further backcrossed with C57BL/6J mice for 15 generations to generate the C57BL/6J inbred knockout lines. After the initial Southern blot characterization, PCR was used for subsequent genotyping of mice with oligonucleotides 5'-TAGAGCTGAATAACACCTG-3', 5'-CCTCAGTCTGAAACCCG-3' and 5'-CAGCGCATCGCCTTCTAT-3'.

4.4. Physiological studies

4.4.1. Exercise experiments (II)

In the first exercise experiment four mutant and seven wild-type male mice, aged 7-13 weeks, ran on a motor-driven treadmill with 6° uphill tracks at a speed of 10.5 m x min⁻¹ for four hours. After two hours of running there was a 20-min rest period during which the animals had free access to pelleted food and water. 48 hours after the cessation of exercise, the animals were sacrificed together with unexercised mutant (n=5) and wild-type controls (n=4). The soleus muscles from both hind limbs and the proximal part of the quadriceps femoris (MQF) muscle from the left leg were excised and frozen in liquid nitrogen for assays of β-glucuronidase activity. For histological analysis, including H&E, immunofluorescence stainings with antibodies against tenascin-C, dystrophin, type IV collagen and the laminin α2 chain, MQF from the contralateral leg was oriented under a microscope and frozen in isopentane cooled with liquid nitrogen.

In the second exercise experiment, 11 mutant male mice aged 27-34 weeks and 11 age and sex-matched wild-type mice were subjected to six hours of running with a 6° uphill inclination at the speed of 8.5 m x min⁻¹ with two 20 min pauses. 48 hours after the cessation of exercise, the animals were sacrificed together with unexercised mutant (n=7) and wild-type controls (n=7) and prepared for β-glucuronidase activity assays and histological analysis as described for the first exercise experiment.

4.4.2. Functional analyses of isolated and perfused hearts (II)

Two groups of female mice at ages of six (Col15a1⁻/⁻ n = 7; Col15a1⁺/⁺ n = 7) and twelve months (Col15a1⁻/⁻ n = 8; Col15a1⁺/⁺ n = 11) were studied. The hearts were prepared for perfusion analyses as previously described for the rat (Ruskoaho et al. 1986). Briefly, the mice were decapitated and after the aorta had been cannulated above the aortic valve, retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer (pH 7.4, equilibrated with 95% O₂ / 5% CO₂ at 37°C, and containing salts (mmol/L): NaCl 113.8, NaHCO₃ 22.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.1, CaCl₂·(2H₂O) 2.5 and
glucose 11.0). The hearts were perfused with a peristaltic pump (Minipuls 3, model 312, Gilson) at a constant flow rate of 2.5 mL/min. They were stimulated (9 V, 0.5 ms) with a Grass stimulator (model S88, Grass Instruments) using an electrode placed on the right atria to increase heart rate to the level of 400 beats/min. Isoproterenol (Sigma) was infused via an infusion pump (Secan PSA 55, Sky Electronics S.A.).

The isometric force of contraction was measured by cutting the left atrium away and inserting an empty plastic balloon on the left ventricle through the mitral valve. The balloon was then filled with 50% ethanol to give an end diastolic pressure of 2-3 mmHg. The intraventricular pressure inside the balloon was recorded with a transducer (Isotec, Hugo Sachs elektronik). Heart rate was determined from the changes in intraventricular pressure. Variations in the perfusion pressure arising from changes in coronary vascular resistance were measured with a pressure transducer (model MP-15, Micron Instruments) situated on a side-arm of the aortic canula. The data were analysed and recorded on an IBM PC-compatible computer using Ponemah data acquisition software (Gould Instrument System Inc.). The first derivatives of the intraventricular pressure, +dP/dT and -dP/dT, in millimetres of mercury per second, were recorded as a measures of contractility and relaxation, respectively.

There was a 20-minute stabilisation period before infusion with the vehicle was started. After 10 minutes control time, the experiment was started with infusion of the first dose of isoproterenol. Infusion was stopped after 30 seconds, when the maximal response had been observed. Then, after a 10-min equilibration period, the next dose was given. Meanwhile, vehicle was infused into the hearts to keep the flow rate constant. The doses of isoproterenol were from 0.01nmol/L to 800 nmol/L. The contractile response to each dose was calculated as a ratio of the maximal level of contractility to the basal level before isoprenalin.

**4.4.3. Cyclic AMP measurements (II)**

Hearts from 18-month-old wild-type (n=5) and null mice (n=6) were perfused as mentioned above. Five minutes after the 60-second isoproterenol (0.1 nmol/L) infusion the left ventricles were frozen in liquid nitrogen and stored at -80°C. Cardiac samples were homogenized with 6% trichloroacetic acid at 4°C and centrifuged at 2000g for 15 minutes. The supernatants were collected and washed with 5 vol of water-saturated diethyl ether. The extracts were lyophilised and the cAMP content was measured by radioimmunoassay according to the manufacturer’s protocol (Amersham International).

**4.4.4. Analysis of markers of cardiac injury (II)**

After the second exercise protocol the left and right ventricles were separated and the left ventricles cut into three pieces for histological, mRNA and biochemical analysis. The possibility of cardiac injury was studied by comparing the extent of apoptosis, the activities of matrix metalloproteinase 2 (proMMP-2) and β-glucuronidase and the mRNA
levels of atrial natriuretic peptide (ANP) between the unexercised controls and the exercised mice. H&E staining was used to document overall histopathological changes.

4.4.4.1. TUNEL-staining (II)

DNA fragmentation (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling, TUNEL assay) was detected from cryostat sections stained with the In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer’s protocol. For quantitative analysis, the mean number of TUNEL-positive nuclei was counted in four sections on different depths in each sample.

4.4.4.2. ANP mRNA analysis (II)

For ANP mRNA analysis, a cDNA reaction was performed according to the manufacturer’s protocol (Gibco BRL), after which ANP mRNA levels were measured by quantitative RT-PCR analysis with an ABI 7700 Sequence Detection System using TaqMan chemistry and the forward and reverse primers GAAAAGCAAAGGCTGCTGTGAGGCTCTG and CCTACCCCGAAAGCAGCT, respectively. 70-bp amplicon was detected using the bifunctional fluorogenic probe 5’-Fam-TCGCTGGCCCTCGGAGCCT-Tamra-3’. The ANP mRNA expression levels were normalized to the levels of 18S mRNA quantified in the same samples using the forward and reverse primers TGGTTGCAAAGCCTGAAACTTAAAG and AGTCAAAATTAAGC CGCAGGC, respectively. The probe for the 18S amplicon was 5’-Vic-CCTGGTGTTG CCTTCCTCCTCA-Tamra-3’.

4.4.4.3. Preparation of muscle samples for biochemical assays (II)

A frozen muscle sample was placed in buffer (1:10 w/v in homogenization buffer: 0.2 M NaCl, 0.1% TritonX-100, 0.02 M Tris) and homogenized on ice. 50 µl of muscle homogenate was taken for the β-glucuronidase activity assay. For zymography, the homogenate was centrifuged for 30 min at 15000 g at 4°C and the supernatant was removed and stored at –70°C. In the case of the cardiac samples, homogenization was performed by hand using a glass probe. The homogenate was transfer to Eppendorf tube, 50 µl being reserved for the β-glucuronidase assay and the rest being centrifuged for 20 minutes at 13000 rpm for zymography.

4.4.4.4. ProMMP-2 activity (II)

ProMMP-2 activity was measured by zymography. 7.5% running gels containing 1 mg/ml gelatin were over laid with 4% stacking gels. The samples (muscle homogenates containing 50 µg protein mixed with a 1/1 volume of sample buffer: 0.4 M Tris, pH 6.8;
2% SDS; 20% glycerol and 0.03% bromphenol blue) were loaded into the gel and electrophoresis was carried out first at 16 mA for 1 hour and then at 24 mA until the dye front ran off the gels. The gels were first incubated for 30 min in a solution containing 2.5% Tween80 and 50nM Tris, pH 7.5, and then at 37°C for 18 h in a solution containing 50 mM Tris, pH 7.5, 5mM CaCl$_2$ and 10 mM ZnCl$_2$. Gelatinase activity was revealed by negative staining with Coomassie Brilliant. Purified proMMP-2 was used for identifying the enzyme activity. The degree of digestion was quantified by densitometry and area analysis.

4.4.4.5. β-glucuronidase activity (II)

β-glucuronidase activity was measured in a muscle homogenate. Briefly, 450 µl of 0.1M acetate buffer (pH 4.2) was added to 50 µl muscle homogenate. After five minutes of preincubation at 37°C, 250 µl of substrate (5mM p-nitrophenyl-βD-glucuronidase, Sigma) was added, and incubated overnight at 37°C. The reaction was stopped by adding 1.5 ml of cold glycine buffer (0.1M pH 10.8), followed by centrifugation at 3000 rpm for 10 minutes, after which β-glucuronidase activity was calculated based on absorbance at 420 nm.

4.4.5. Assay of endostatin in plasma (III)

Blood was collected by heart puncture from 3-4-month-old wild-type, heterozygous and homozygous Col18a1$^{-/-}$ mice and plasma concentrations of endostatin and endostatin-like fragments of collagen XVIII were determined using a competitive enzyme-linked immunoassay (Accucyte, CytImmune Sciences, Inc.).

4.5. Histological studies

4.5.1. Light microscopy (II, III)

The muscle samples were oriented under a microscope, frozen in isopentane cooled with liquid nitrogen and stored at -80°C. Ten µm sections were stained by routine H&E staining protocol. The histopathological stage was studied at ages ranging from 3 to 84 weeks. Muscles studied were: soleus, gastrocnemius, quadriceps femoris, triceps brachii, diaphragm and muscles from the neck and back. Other tissues were prepared and placed in 10% phosphate-buffered (pH 7.0) formalin (FF-Chemicals) and fixed in RT overnight. The fixed tissue samples were embedded in paraffin, sectioned and stained with H&E by standard methods.

For histological examination of the eye vasculature, mice were sacrificed on postnatal days 0.5, 4, 8, 16 and 24, and the eyes were enucleated and fixed in phosphate-buffered
formalin (10%, pH 7.0, FF-Chemicals) overnight at room temperature and embedded in paraffin by routine methods. The eyes were then sectioned from the surface of horizontally oriented globes under the plane of the optic nerve head. The H&E stained sections were observed under a light microscope and the number and location of the hyaloid capillaries in the primary vitreous body (vasa hyaloidea propria) and around the developing lens (tunica vasculosa lentis) were evaluated. For quantitative evaluation, the mean number of vessels was counted in at least eight sections on different planes for each horizontally oriented eye.

### 4.5.2. Immunohistological stainings (II, III)

For indirect immunofluorescence stainings frozen tissue samples were sectioned (5 µm) and fixed in precooled ethanol for 10 min at –20°C. After washing in 1xPBS, the samples were blocked by incubating the sections with 1% BSA in PBS for 60 min at room temperature. The sections were then incubated with appropriately diluted primary antibodies overnight at 4°C, washed thoroughly with PBS, stained with TRITC or FITC-conjugated secondary antibodies for 30 min, extensively washed with PBS, mounted (Immu-mount™, Shandon Inc.) and examined under an epifluorescence microscope.

Tissues for immunohistochemical detection were fixed in PFA fixative (4% PFA in phosphate buffer, pH 7.4) overnight at 4°C and embedded in paraffin by routine methods. The samples were sectioned, treated with Proteinase XXIV (Sigma), blocked with 5 or 10% goat serum in PBS and stained with appropriately diluted primary antibodies. Biotinylated IgG (Sigma) were used as secondary antibodies and detected with peroxidase-labelled streptavidin and DAB reagent (BioGenex). The immunostained sections were counterstained with methylgreen.

### 4.5.3. Electron microscopy (II, III)

For ultrastructural examinations of the cardiac and skeletal muscle, lung and cerebellum, pieces of tissue of size about 1 mm³ were prepared and pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and post-fixed in 1% osmium tetroxide. The specimens were dehydrated in acetone and then embedded in Epon LX112. The semi-thin sections were stained with toluidine blue, and the thin sections were cut with a Reichert Ultracut E-ultramicrotome (Reichert-Jung), stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope (Philips CM100).

Eyes from ten-day-old and adult Col18a1⁻/⁻ and wild-type mice were pre-fixed at room temperature for 1-2 hrs in 1.25% paraformaldehyde and 0.03% picric acid in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide at room temperature for 1 hr, stained en bloc in 1% uranyl acetate for 30 min and embedded in Epon (Embed 812). Semi-thin sections were stained with toluidine blue, while ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined in a 1200EX JEOL electron microscope.
4.5.4. Immunoelectron microscopy (III)

Immunostaining of type XVIII collagen for electron microscopy was performed by prefixing the eyes in 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hr and washing in 0.1 M cacodylate buffer. Immunolabelling was performed en bloc by immersing the samples in primary antibody against the NC11 domain of type XVIII collagen (Marneros AG, Keene DR, Fukai N, Deckers M, Hansen U, Löwik C, Bruckner P, Olsen BR, unpublished), washed with PBS and then immersed in goat anti-rabbit 1 nm gold conjugate (Amersham), diluted 1:3, and washed with PBS. The 1 nm gold particles were enhanced in some instances using the Nanoprobe GEEM gold enhancement kit. Briefly, tissue samples in buffer were chilled on ice, incubated on ice for 15 min in complete enhancement solution and then warmed quickly to 25°C and incubated for 5 min. After rinsing in ice-cold PBS followed by 0.1 M cacodylate buffer, the tissues were fixed in 1.5% glutaraldehyde and 1.5% paraformaldehyde containing 0.5% tannic acid in 0.1 M cacodylate buffer. This was followed by fixation in 1% osmium tetroxide and embedding in Spurr’s epoxy. Ultra-thin sections were contrasted with uranyl acetate and lead citrate prior to evaluation using a Phillips 410LS electron microscope.

4.5.5. Whole-mount staining of the developing blood vessels of the retina (III)

Mice were sacrificed on postnatal day 4 and their eye globes were placed in PFA fixative (4% paraformaldehyde in PBS, pH 7.2). To visualize the developing retinal vessels, the eye globes were first incised along the level of the ora serrata to remove the lens, iris diaphragm, cornea and hyaloid vasculature under a dissection microscope. The retina was exposed by dissecting the surrounding choroid and pigment epithelium, and the retinas were fixed in PFA fixative for 1 hr at room temperature. After 1 hr of incubation in methanol at –20°C, the retinas were treated for two hours in a blocking buffer (50% FCS and 1% Triton-X100 in PBS) at room temperature and subsequently incubated with primary antibodies (rabbit anti-mouse type IV collagen polyclonal antibody, Chemicon International Inc., 1:200 in 10% FCS, PBS) at room temperature and subsequently incubated with primary antibodies (rabbit anti-mouse type IV collagen polyclonal antibody, Chemicon International Inc., 1:200 in 10% FCS, PBS) overnight at 4°C. The retinas were washed 4x5 min with PBS, incubated for 3-4 hrs with secondary antibodies (CY3-conjugated polyclonal swine anti-rabbit antibodies, Amersham Inc., 1:300 in 10% FCS, PBS), washed 4x5 min with PBS, laid on a microscope slide, mounted with Immumount (Shandon) under coverslips, and examined under an epifluorescence microscope as a flat mount preparation.
4.6. Statistics (II, III)

The non-parametric Mann-Whitney $U$-test was used to compare the means of the cardiac injury markers (II) and the mean numbers of intraocular vessels (III). Student's $t$-test was used for the other comparisons.
5. Results

5.1. Characterization of the mouse type XV collagen gene (I)

5.1.1. Isolation and characterization of mouse Col15a1 genomic clones

Genomic clones were isolated from libraries using PCR probes designed on the basis of human and mouse cDNAs or using fragments derived from previously found mouse genomic clones. Since no cDNA for the 5'-end of the mouse α1(XV) chain was available when the library screenings were initiated, human α1(XV) chain cDNA clones F-10, F-1.6 and XV-A were used as probes to obtain the 5'-end of the mouse Col15a1 gene. These interspecies cross-hybridization library screenings yielded five λ clones, two of which (MG-3.1 and MG-13.1) were characterized in more detail. These clones contained sequences for exons 3 to 9 and extended about 17 kb into the second intron.

As the first genomic library screenings using human cDNA fragments as probes did not yield clones covering the beginning of the Col15a1 gene, a 260 bp PCR-fragment LE-MXV5/6 corresponding to the first two exons and 5'-untranslated sequence of the mouse gene was used as a probe to screen a genomic λ DNA library. This resulted in isolation of the genomic clones MG5'-8 and MG5'-11, which were found to contain the first two exons and about 8 kb of the 5'-flanking region.

Screening with a mouse cDNA clone HM-29.2 representing the 3' portion of the mouse α1(XV) mRNA resulted in the isolation of six overlapping λ phage clones, four of which (MG-12, MG-14, MG-19 and MG-22) were further characterized. These covered the whole 3' end of the gene from intron 14 to exon 40 and contained about 15 kb of the 3'-flanking region.

To cover gaps in the genomic sequences between clones MG5'-11 and MG-3.1 (intron two) and between clones MG-13.1 and MG-14 (exons 10-14), a cosmid library was screened with three probes. The cosmid clone MG-15.2, containing exons 10–26, was obtained by screening with a 470-bp PCR fragment PHMXV26/47 corresponding to exons 10-12. Rescreening of the cosmid library with the genomic SacI-fragments HP3'
(the 3' end of clone MG5'8) followed by screening with 3.18S2 (the 5' end of clone MG-3.1) then yielded clone MGII-1, covering the gap in the second intron in the previously isolated λ clones.

5.1.2. Exon-intron structure of Col15a1

The isolated genomic clones covered the entire Col15a1 gene, 8 kb of the 5'-flanking regions and 15 kb of the 3'-flanking regions. Characterization of these clones revealed that the mouse gene for the α1 chain of type XV collagen is quite large, containing 40 exons and covering approximately 110 kb of genomic DNA. All the exons, intron junctions and introns of reasonable size were sequenced, together with approximately 5 kb of the 5'-flanking region. Partial digestions and pulse field gel electrophoresis was used to determine the size and SacI restriction enzyme sites of the second intron, while the sizes of the rest of the introns were obtained by PCR and Southern blot analysis. The exons, including 5' untranslated sequences, vary in size between 36 and 548 bp and the introns between 89 and about 38000 bp. The first exon contains 121 bp, with a 109 bp untranslated sequence. The exon-intron boundaries (AG-exon-GT) confirm the consensus sequence for intron splicing, except that the normal donor site following exon 6 (exon-GT) is replaced by exon-GC. The coding information is not evenly distributed in the Col15a1 gene, since the introns at the 5' end of the gene are in general larger in size than those at the 3' end. The first 12 exons of the Col15a1 gene were found to encode the N-terminal non-collagenous domain. Exon 13 is a junction exon at the beginning of the collagenous sequences, and exons 13 to 34 cover the collagenous sequences with multiple interruptions, in which exon 34 is a junction exon between the end of the collagenous sequences and the beginning of the NC8 domain. Exons 14, 15, 18, 21, 22, 24, 25 and 30 code for purely collagenous sequences, their sizes being 63 bp, 36 bp, 36 bp, 36 bp, 63 bp, 36 bp, 54 bp and 81 bp respectively. Interestingly, two short collagenous exons which are found in the human COL15A1 gene were absent from the mouse gene.

5.1.3. Nuclease S1 protection assay and the 5'-flanking region of Col15a1

The transcriptional start sites for the Col15a1 gene were identified by a S1 nuclease protection assay using total RNA extracts from mouse kidney and heart. Three major sites were identified within 108 - 110 bp upstream of the first codon (ATG), with three minor ones located in the same region of 9 nucleotides. The sizes of the protected fragments were determined by comparison with parallel runs of DNA sequencing reactions. The same pattern of transcription start sites was obtained regardless of whether total RNA from heart or kidney was used.

Sequence analysis of the mouse α1(XV) promoter, first intron and first two exons (nucleotides -1004 to +395) revealed several potential binding sites for previously described transcription factors. The 5'-flanking region of the mouse Col15a1 gene was
found to lack TATA and CAAT boxes in a position typical of functional binding sites. Instead, the Col15a1 promoter has a high G+C content and a high frequency of CpG dinucleotides.

5.1.4. Comparison of mouse and human type XV collagen genomic sequences

The overall structure of the mouse Col15a1 gene was found to be well conserved relative to that of the human COL15A1 gene, although the mouse Col15a1 gene contains 40 exons, which is two less than the human counterpart. Moreover, the genomic area encoding the end of the N-terminal non-collagenous domain was markedly different from its human counterpart. Comparison of the genomic structures indicates that mouse exon 10 (267 bp in size vs. 150 bp in the corresponding human exon) encodes a mouse-specific 41-residue sequence which does not show homology to any known polypeptide. In addition, the mouse exons 9 (129 bp) and 11 (129 bp) are clearly different in size from the corresponding human exons (153 and 144 bp, respectively) and do not show sequence homology. No sequences homologous with human exons 12 or 13 could be found in the mouse intron gene. Otherwise the overall exon size pattern of the genes was similar, with 33 exons being identical in size.

To characterize the 5’-regulatory sequences and to identify putative conserved promoter regions that could potentially be involved in the regulation of Col15a1 expression, we isolated and sequenced the first two exons and the 5’-flanking sequences of both the mouse Col15a1 gene (from -5084 to +441) and the human COL15A1 gene (from -5429 to +368). Sequence comparisons showed four conserved domains within the proximal promoter and also in sequences further upstream, corresponding to mouse -36 to +74 (a cross-species identity of 86%), -90 to –133 (84%), -905 to –957 (81%) and -2067 to –2540 (77%) sequences and named H1, H2, H3 and H4 respectively.

5.1.5. Deletion constructs and Col15a1 promoter analysis

To study the importance of different 5’ regions for the activity of the Col15a1 promoter, transient transfections of the mouse fibroblast cell line NIH/3T3 were performed using six 5’ deletion promoter-luciferase reporter gene constructs.

Based on the luciferase activities, the shortest fragment of the mouse promoter (M6: -135 to +42 bp) used here contained the necessary elements for gene expression, as compared with the pGL2-basic vector used as a negative control. The level of expression was increased approximately three-fold when more distal upstream fragments (up to -657 and -2064 bp) were added to the front of the luciferase gene. Transfection analysis with the M3 construct (-2926 bp) revealed a marked decrease in luciferase activity, suggesting the existence of a silencer-like element between positions -2064 and -2926 bp. The addition of further upstream sequences (M2: -3654 and M1: ~-7900 bp) resulted in a
~2.5-fold increase in luciferase expression, suggesting the presence of one or more positive elements in the region -2926 to -3654 bp.

To compare the functional properties of the human and mouse Col15a1 promoters and to define putative conserved domains involved in the regulation of gene expression in both species, human 5'-deletion constructs were transfected into mouse NIH/3T3 fibroblasts. Quantitatively similar results were obtained with human promoter constructs and mouse promoter constructs, except that the longest human 5' fragment (-3598 to +27), which contained the 3' end of the most distal homology domain (H1), had no repressive effect.

5.2. Type XV collagen-deficient mice (II)

5.2.1. Generation of a mouse strain lacking type XV collagen

The Cre-loxP method was selected for inactivating the type XV collagen gene. First ES cells with a modified Col15a1 allele were generated by homologous recombination, resulting in a Col15a1 gene with selection marker genes (neo' and HSV-tk) in the second intron and loxP sites flanking the closely spaced first and second exons, which encode the first 33 amino acid residues of the 1367-residue polypeptide. To generate Cre-mediated deletions, a Cre expression plasmid was electroporated into targeted ES cell lines. As expected, two types of Cre-mediated recombination allele were observed: loxP-flanked alleles (conditional alleles) and inactivated alleles lacking the first two exons, the start codon and the transcription start sites. Two ES clones derived from Cre-transfected ES cell lines with an inactivated Col15a1-allele were injected into blastocysts and inserted in pseudopregnant mice. Germline transmission was obtained from both of these clones.

5.2.2. Mice lacking type XV collagen are viable and fertile

Northern blot hybridization was used to study the expression of type XV collagen mRNAs in the homozygous mutant mice. The deletion completely abolishes Col15a1 gene expression and no mRNA for type XV collagen was detected in the tissues. Deficiency with respect to this collagen type was confirmed throughout the tissues by immunostaining with antibodies against the C-terminal NC domain.

Despite the complete lack of type XV collagen, the mice displayed no obvious alteration in phenotype, were fertile and had a normal life span. Genotyping of 345 offspring from heterozygous intercrosses showed that 21.5% were of the wild-type, 53.9% were heterozygous and 24.6% were homozygous for the null allele. No compensatory changes were observed in the level of expression of the homologous type XVIII collagen mRNAs as analyzed by Northern blotting or immunohistological studies.
5.2.3. Focal areas of histopathological changes characteristic of myopathic disorders

Histological screening of tissues from Col15a1−/− mice revealed changes in the skeletal muscle, including muscle cell degeneration, macrophage infiltration, increased regeneration and variation in fibre size ranging from mild to moderate, with atrophic and split muscle fibres. The histological changes were first detected at 13 weeks of age and were seen more clearly after 26 weeks of age, indicating that the changes were progressive. The changes were focal, with the number of degenerative fibres in the samples ranging from none to a few necrotic ones. The histological changes were more frequent in the back and paraspinal muscles than in the other muscle groups analyzed, but none or only a fraction of the cells in a given muscle sample were affected. Two-thirds of the mutant mice aged over six months displayed these muscle abnormalities, the majority of the samples being mildly affected. No clear signs of fibrosis were observed in H&E and van Gieson stainings. Antibodies against BM components (laminin α2 chain, tenascin-C and type IV collagen) and dystrophin showed a normal pattern of immunostaining. No histological abnormalities at the light microscopy level were observed in any of the major organs studied, except in the skeletal muscles.

5.2.4. Col15a1−/− mice are vulnerable to exercise-induced muscle damage

The histopathological signs of muscular disorder led us to study whether type XV collagen deficiency increases the sensitivity of muscle cells to damage following acute physical exercise. Mice were put to run on a motor-driven treadmill in two separate exercise experiments, the first under milder loading conditions and the second with a heavier physical load. Both the mutant and wild-type mice maintained the two exercise protocols without signs of serious exhaustion or behavioural avoidance of running. The first protocol did not cause any marked muscle injury in the wild-type mice, whereas the second exercise session caused marked damage to the wild-type mice, too.

The histopathological findings, based on the evaluation of H&E-stained sections, showed that the mutant runners had more degenerative fibres and more intensive inflammation than the wild-type runners. The integrity of the BM zone appeared to be maintained well in the mutant mice when evaluated from the immunostainings for laminin α2, type IV collagen and the subsarcolemmal protein dystrophin.

The activity of muscle β-glucuronidase, an established quantitative marker of muscle cell injury, increased statistically significantly in both the soleus and quadriceps femoris muscles of the Col15a1−/− mice in both experiments by comparison with the respective unexercised mice or exercised wild-type mice. This was true whether the results were expressed in absolute or relative terms. No significant differences in the basal level of β-glucuronidase activity were found between the mutant and wild-type mice.
5.2.5. **Ultrastructural changes in the heart and skeletal muscle capillaries**

Although light microscopy did not reveal any conspicuous changes compared with the controls, electron microscopy showed abnormalities encompassing the microvessels and their endothelium. Where the capillaries in the wild-type mice were round and had a wide lumen, some of those in the mutant mice were irregular in shape with intensively folded endothelial membranes. Some of the endothelial cells were degenerated and swollen, had a pale cytoplasm with only a few cell organelles and bulged into the vessel lumen. In some cases the endothelial cells were shrunken, showing a thin, electron-dense structure. These changes were focal and the incidence of the capillary and endothelial defects varied between samples, ranging from a few abnormal microvessels to more than 50% affected capillaries. The capillary defects were found in both the heart and skeletal muscle samples, although they were more frequent in the heart. No changes were noted in the vascular BM, and no endothelial cell degeneration or changes in the capillary structure could be seen in the wild-type mice. The heart specimens containing swollen endothelial cells also showed focal ischaemic changes in the cardiac myocytes, such as intracellular oedema and vacuolisation. No degenerative endothelial cells or changes in the capillary structure were observed in the lung and cerebellum in the Col15a1-/- mice.

5.2.6. **Heart response to cardiovascular stress**

To test the hypothesis that the abnormalities in capillary morphology observed in the mutant mice could have some consequences for blood flow and lead to pathological changes after cardiovascular stress, left ventricle samples were prepared after the second exercise experiment, and the possible cardiac injury was studied by assessing known markers of cardiac injury, including the extent of apoptosis, the activities of β-glucuronidase and proMMP-2 and the mRNA levels of ANP. Following exercise, statistically significant increases in the number of TUNEL-positive nuclei and in the activities of β-glucuronidase and proMMP-2 were detected in the Col15a1-/- mice. The exercise also induced highly increased ANP mRNA levels in two null mice out of eleven, although this was not statistically significant on average (P=0.06). No changes were observed in the wild-type individuals. It should be noted that the basal proMMP-2 level was lower in the null mice than in the controls.

5.2.7. **Diminished cardiac responses to β-adrenergic stimulation**

Cardiac function was studied using isolated perfused hearts from Col15a1-/- mice and wild-type mice aged six months and one year. The developing pressure as an index of cardiac contractility was compared at the basal level and after β-adrenergic receptor stimulation with increasing doses of isoproterenol. The basal contractility in the mutant hearts did not differ significantly from that of the wild-type mice, but the response to
isoproterenol at concentrations of 0.01, 0.1 and 1 nmol/L showed significantly smaller changes in the null mice.

Since cAMP is known to mediate the positive inotropic effect of β-adrenergic receptor stimulation as a second messenger, the tissue levels of cAMP was measured. A somewhat diminished decrease in the left ventricular cAMP response five minutes after the administration of 0.1 nmol/L isoproterenol was observed in the null mice compared with the wild-type mice (5.42±0.72 and 7.55±1.37 pmol/mg, respectively, mean±SEM, \( P=0.098 \)).

5.3. Location of type XVIII collagen in the eye (III)

Immunostaining with antibodies to the N-terminal NC domain of type XVIII collagen was seen in the Bruch’s membrane of the embryonic mouse eye and in the inner limiting membrane. The staining continued into the pial BM of the developing brain, while staining of the inner limiting membrane continued in the BM of the VHP. Staining was also seen in the BM of vessels in the TVL and in the lens capsule. In the mature eye, immunostaining showed reactivity in the iris BMs, lens capsule, inner limiting membrane and Bruch’s membrane.

Type XVIII collagen was localized by immuno-electron microscopy to the BMs of the eye, including Bowman’s membrane of the cornea, the BMs of the pigment epithelial cells of the iris, the inner limiting membrane and Bruch’s membrane. Gold labelling in Bowman’s membrane was localized almost exclusively to the matrix side of the lamina densa. Moreover, the gold particles were distributed in clusters at regular intervals along the BM. A similar clustering, but with less regularity, was seen in the iris and the inner limiting membrane. In all three of these anatomical structures, the clusters appeared to coincide with fibrillar structures in the adjacent collagenous matrix. Along the inner limiting membrane, the labelling was clearly restricted to regions where vitreous fibrils are in close proximity to the inner limiting membrane. The clustering of gold particles was particularly striking in Bruch’s membrane, along the lamina densa of both the pigment epithelial BM and the capillaries. In tangential sections, the clusters of gold particles were distributed in an almost uniform lattice, and appeared to coincide with collagen fibrils in the connective tissue separating the pigment epithelial and endothelial BMs. Robust labelling was seen on both sides of the lamina densa along the pigment epithelial cells, so that this BM differs from all other BMs examined in this respect.

5.4. Type XVIII collagen-deficient mice (III)

5.4.1. Targeted inactivation of Col18a1

Characterization of a 17 kb genomic clone isolated from a mouse genomic DNA library resulted in the identification of exons 17 to 38 of the Col18a1 gene. A SalI site within
exon 30 was used to insert a neo' cassette to generate a replacement vector, as in this position it would interrupt all variant forms of collagen XVIII. Homologous recombination was confirmed by genomic Southern analyses and by PCR.

Northern blot analyses of RNAs prepared from embryonic and adult tissues of homozygous mutants failed to reveal detectable levels of α1(XVIII) mRNA. In contrast, RNAs prepared from wild-type embryos and adult livers showed high levels of α1(XVIII) expression, and RNAs from heterozygous embryos showed about half the levels of wild-type mice. Furthermore, determination of circulating levels of collagen XVIII-derived proteolytic fragments in plasma of wild-type, heterozygous and homozygous animals showed no detectable endostatin or endostatin-like fragments in the homozygous null mice. The heterozygotes had levels of 23.4±2.7 ng/ml, i.e. 50% of those of the wild-type animals, 44.9±7.5 ng/ml.

No gross abnormalities were detected by inspection of the homozygous and heterozygous mutant embryos or adult mutant animals. The animals were identical in size and appearance between the genotypes. Similarly, mating of the homozygotes indicated no reduction in reproductive capacity. Genotyping of 355 offspring of heterozygous intercrosses showed that 25.1% were of the wild-type, 49.0% were heterozygous and 25.9% were homozygous for the null allele. Examination of the gross anatomy and light microscopy of several organs revealed no abnormalities in the mutant mice. Since collagen XVIII is localized in vascular and epithelial BM zones, particular attention was paid to the vascularity of tissues and the distribution of other BM components, but no morphological differences between the wild-type and homozygous mutant mice were detected in organs such as the heart, lungs, liver and kidney. Examination of the eye, however, showed striking differences between the homozygous and the wild-type/heterozygous animals in the regression of the hyaloid vessels in the vitreous body after birth and in the postnatal outgrowths of retinal vessels.

5.4.2. Delayed regression of the hyaloid vessels in the vitreous body

The vessels attached around the lens capsule, the tunica vasculosa lentis (TVL), and the vasa hyaloidea propria (VHP) along the inner limiting membrane of the retina in the vitreous body were examined in the homozygous mutant and wild-type mice on postnatal days 0.5, 4, 8, 16 and 24. On postnatal day 0.5 there was no difference in the number of vessels between the two types of mice, but at all subsequent days examined the homozygous mutant mice had significantly more vessels in the vitreous body than wild-type mice. At day 4 the VHP was clearly dissociated from the retinal surface and had started to show signs of regression in the wild-type mice, remaining in about half of them on day 8, and being practically gone by postnatal day 16. No VHP was seen in the vitreous body at day 24 in the wild-type mice. By contrast, the VHP in the homozygous mutant animals was still present near the retina on days 4 and 8, and persisted on day 16. Most samples from the homozygous mutants showed no VHP by day 24, but some sections still showed numerous enlarged vessels in the vitreous body. This delay in regression of the VHP in the homozygous Col18a1° mice was evident from the quantitative data obtained by counting vessel profiles in sections from wild-type and
mutant mice. No differences were observed in the number and regression of vessels in the TVL, however.

5.4.3. Delayed and abnormal outgrowth of retinal vessels

Examination of the retinal vessels by immunofluorescent staining of whole mounts with collagen IV antibodies on postnatal day 4, showed poor or irregular growth of the capillaries in the retina of the Col18a1⁻/⁻ mice. Comparing 18 wild-type eyes with 55 Col18a1⁻/⁻ eyes, the latter showed poor or no outgrowth of vessels, focal areas of denser growth and irregular growth. 44% of the Col18a1⁻/⁻ samples showed no or very few short vessels in the retina by day 4, and 40% showed some vessels, but less than in the wild-type eyes. Vessel outgrowth was comparable to that in the wild-type samples in 16% of cases, and focal areas of unusually dense capillary growth were seen in 20%.

5.4.4. Ultrastructural changes in the the vitreous body

The ultrastructure along the inner limiting membrane at the periphery of the vitreous body revealed changes in the Col18a1⁻/⁻ mice. Collagenous fibrils were observed in close proximity to this membrane in the wild-type eyes, and an abundant presence of gold-labeled antibodies to type XVIII collagen was seen in areas where the fibrils appeared to “insert” into the membrane, whereas the majority of sections of Col18a1⁻/⁻ eyes had no fibrils visible in the peripheral regions of the vitreous body and areas of fibril “insertions” into the inner limiting membrane were difficult to find.
6. Discussion

6.1. Structure and conservation of the type XV collagen gene

Isolation and characterization of the mouse gene for the α1 chain of type XV collagen (Col15a1) showed it to be about 110 kb in length and to contain 40 exons. To find evolutionally conserved and potentially important genomic areas, this gene was then compared with its human counterpart. Its general organization was found to be highly similar to that of its human homologue, with 33 exons being identical in size and having a high nucleotide sequence homology, but the number of exons differed by two and the genomic area encoding the end of the N-terminal non-collagenous domain showed marked divergence from that of the human gene. Sequence analyses revealed that due to the lack of two exons and a codon divergence in one exon, the mouse α1(XV) chain contains seven collagenous domains whereas the human equivalent contains nine. This may suggest that the two short collagenous domains and the end of the NC1 domain are likely to be of lower functional importance than the conserved regions. The exons of the C-terminal non-collagenous domain show the highest homology of coding sequences, over 84%. This genomic region comprising exons 38-40 encodes the endostatin domain and shows homology percentages ranging from 80 to 87% at the nucleotide level with the human COL15A1 gene, suggesting the functional importance of this domain. The sequences encoded by exons 35 and 36, corresponding to the trimerization region, show high sequence conservation, 85-87%, while exon 37, encoding the hinge region (Sasaki et al. 1998), shows somewhat lower homology, 78%.

The region of the mouse Col15a1 gene encoding collagenous sequences comprises 21 exons (exons 13-34). Out of the eight exons encoding solely collagenous sequences, exon 25 is 54 bp in length, as is characteristic of fibrillar collagen genes, whereas the sizes of exons 15, 18, 21 and 24 (36 bp), and exons 14 and 22 (63 bp) are similar to the triple-helix coding exons of the non-fibril-forming collagen genes (Chu & Prockop 1993; Sandell & Boyd 1990; Vuorio & de Crombrugghe 1990).

Analysis of the 5'-flanking region of the mouse gene revealed several properties typical of widely expressed and housekeeping genes, some of them similar to those noted in the human COL15A1 promoter region (Hägg et al. 1998). The Col15a1 gene lacks
TATA and CAAT boxes in positions typical of functional binding sites (Maniatis et al. 1987), has several transcription start sites and a high G+C content and a high frequency of CpG dinucleotides. Functional analysis of the mouse promoter identified cis-acting elements for both positive and negative regulation of Col15a1 gene expression in mouse NIH/3T3 cells. In the light of the hypothesis that conserved sequences are important for the regulation of gene expression, and on the assumption that mouse transcription factors can bind to human as well as mouse sequences, we transfected mouse fibroblasts with both human and mouse luciferase reporter gene constructs. The effects of the deletion constructs from both species on the level of expression of the reporter gene in the mouse fibroblasts were similar. The highest expression levels being measured with constructs del2 and M4, containing three homologous domains, H2, H3 and H4. A striking decrease in transcription activity was observed using the mouse M3 construct, which contained 5' sequences up to -2962, including the H1 domain. The addition of further upstream mouse sequences (-2926 and ~-7900) resulted in a two-fold increase in luciferase expression relative to the M3 construct, suggesting the presence of positive elements in this region.

6.2. Skeletal myopathy and cardiovascular defects in Col15a1-/- mice

Despite the wide occurrence of type XV collagen in BMs throughout the body, mice lacking in it are viable and fertile. Histological examinations of Col15a1-/- skeletal muscle nevertheless revealed focal changes characteristic of myopathic disorders, including the presence of degenerative fibres, increased numbers of muscle cells with central nuclei and variations in fibre size. These changes appeared at the age of three months and became more apparent in older mice, suggesting a progressive disorder. Muscle diseases and their molecular mechanisms are known to show wide diversity, and crucial roles have been indicated for ECM molecules and their cell membrane receptors in maintaining the structural integrity of muscle fibres, notably the components of the dystrophin-associated glycoproteins-laminin α2-axis (Hack et al. 2000), the integrin α7 subunit (Mayer et al. 1997) and type VI collagen (Jöbsis et al. 1996). According to structural hypothesis, disrupted linkage between the cytoskeleton and the matrix leads to sarcolemmal instability and muscle cell necrosis (Campbell 1995). Type XV collagen occurs widely in the BM zone and the adjacent fibrillar matrix, including the endomysium of skeletal muscle (Myers et al. 1996, Hägg et al. 1997b, Muona et al. 2001). The increased fragility of muscle fibres in the null mice does not appear to be caused by any major defects in the BM, however, since antibodies against known BM components (type IV collagen and laminin α2) showed normal, uninterrupted staining around degenerative as well as non-affected muscle fibres. C-terminal fragments of type XV collagen interact in vitro with certain BM and microfibrillar components (Sasaki et al. 2000) and it has been localized by immunoelectron microscopy to the outermost layer of the lamina densa of skeletal muscle and to the interstitial collagen fibres near the BM (Sormunen R, Muona A, Pihlajaniemi T, unpublished). Thus the prominent changes seen in the type XV collagen-deficient muscle fibres could reflect a defect in linkage between the muscle cell BM and the surrounding fibrillar matrix. A similar function has been proposed for type VI collagen in muscle (Kuo et al. 1997).
Mice manifesting muscle phenotypes respond differently to acute exercise, suggesting variations in the aetiology of exercise-induced muscle cell damage (see section 2.4.6). A single episode of acute physical exercise is known to cause damage to the unaccustomed skeletal muscle (Komulainen & Vihko 1998), and is shown to induce focal lesions in muscle fibres, leading to cell necrosis (Armstrong et al. 1983, McNeil & Khakée 1992, Komulainen et al. 1998). The lack of type XV collagen did not affect the overall running performance of the mice in our protocols. It however did result in more prominent muscle damage when assessed on histological and biochemical criteria, which suggests mechanical causes for the cell degeneration observed in the Col15a1−/− mice. Focal muscle fibre degeneration can be caused by local ischaemia (Mäkitie & Teräväinen 1977) and in the light of the present data potential microcirculatory involvement in occasional fibre degeneration in Col15a1−/− mice cannot be excluded. Their increased sensitivity to exercise-induced muscle damage is nevertheless more likely to be caused by a defect in the connective tissue around the muscle fibres, since numerous experiments suggest that the initial defect causing post-exercise fibre injury is mechanical in origin, leading to secondary changes such as an elevated intracellular Ca2+ concentration, and finally to cell necrosis, although exact mechanism involved is not known. The focal appearance of exercise-induced injuries mimics ischemic damage, but there is no evidence that muscle fibre degeneration is primarily caused by capillary alterations (Peeze Binkhorst et al. 1989). A neurogenic component may be the cause of the atrophic fibres observed in histological evaluation, although the lack of fibre type grouping, the observation of fibre de/regeneration and increased susceptibility to exercise-induced muscle damage suggest a primary defect in the ECM of muscle fiber. Type XV collagen is known to be located in the BM zones of peripheral nerve fibres, however, and ultrastructural examination has shown occasional segmental demyelination of the intramuscular nerve axons in the null mice (Eklund L, Sormunen R, Pihlajaniemi T, unpublished). Thus type XV collagen deficiency may play a role in Schwann cell and BM interactions, causing focal myelin degeneration. Further research would be needed to characterize the role of type XV collagen in myelination and to assess the functional consequences of this finding.

It was of special interest whether the lack of type XV collagen would affect blood vessel formation. Despite the antiangiogenic role of type XV collagen-derived endostatin (Ramchandran et al. 1999, Sasaki et al. 2000), we could not observe any abnormalities in the number of vessels. Instead, type XV collagen appeared to play a role in the integrity of the microvessels, since its deficiency was found to lead to an apparent collapse of the capillary wall in the heart and skeletal muscle, resulting in various degrees of narrowing or obstruction of the capillary lumen and endothelial cell degeneration and swelling. Morphologically similar degenerative changes in capillary endothelial cells have been observed in experimental models for the ischaemic (Armiger & Gavin 1975) and reperfused (Ward & McCarthy 1995) myocardium and in patients with small vessel disease (Mosseri et al. 1991), microvascular angina, hypertrophic cardiomyopathy or dilated cardiomyopathy (Suzuki et al. 1995). Those previous observations suggest that the endothelial cell degeneration and swelling in Col15a1−/− mice may be caused by impaired microvascular perfusion and ischaemic damage to the endothelium. Immunostaining studies (Muona et al. 2001) have indicated that while type XV collagen is associated with many capillaries in adult mice, including those in the heart and the
skeletal muscle, there are some tissues, including the mature lung and brain, in which it is not detected around the capillaries. The fact that the lung and brain capillaries of the null mice were normal in structure and no endothelial cell degeneration was observed in them further confirms that the defects seen in the heart and skeletal muscle capillaries are due to a lack of type XV collagen.

Our exercise protocols were optimised for studying skeletal muscle injury, and the timing for analysis could cause limitations for the markers used to study cardiac injury, since the maximal responses in the expression of MMP-2 (Cleutjens et al. 1995) and ANP (Hama et al. 1995) are reached later than 48 hours after acute cardiac injury and the apoptotic effects earlier (Kajastura et al. 1996). Interestingly, the basal MMP-2 activities were found to be significantly lower in the Col15a1−/− mice than in the wild-type ones. Since MMP-2 is known to be expressed by endothelial cells (Lewalle et al. 1995), this could be suggestive of an endothelial cell defect and coincide with the ultrastructurally identified endothelial cell degeneration.

As the organization and function of the heart as a continually contracting muscle differs from that of skeletal muscle, acute exercise is not likely to lead to similar injuries to those affecting the latter. The abnormalities in the heart microvasculature observed at the morphological level will most probably cause marked ischaemic-like damage only upon loading. This is the case with young mice lacking δ-sarcoglycan, where the primary causes of the heart phenotype is thought to be a perturbation in vascular function. In both δ-sarcoglycan (Coral-Vazques et al. 1999) and type XV collagen-deficient mice acute exercise caused cardiac injury before the development of apparent cardiomyopathy. Furthermore, the preservation of the histological integrity of the heart tissue in the Col15a1−/− mice supports the hypothesis that a certain degree of vascular dysfunction may be required to reach the ischaemic threshold necessary to induce myocardial necrosis, as proposed by Corel-Vazquez et al. (1999).

Reduced responsiveness to β-AR stimulation is associated with chronic heart diseases (Post et al. 1999) and with ageing (Lakatta 1999). Hearts suffering from chronic diseases have shown multiple changes in β-AR-mediated events, including the expression and function of β-adrenergic receptors, G-proteins, adenylyl cyclases and G-protein receptor kinases (Post et al. 1999). Results obtained with a pacing-induced animal model suggest that the down-regulation of β1-AR receptors occurs at an early stage in the development of heart failure (Kiuchi et al. 1993). In humans, reduced β1-AR mRNA levels (Engelhardt et al. 1996) and β-receptor density (Fowler et al. 1986) are also detected in a mild form of cardiac dysfunction, indicating that down-regulation of β-AR receptors is not restricted to severe or advanced heart disease. Moreover, histological analysis of cardiac tissue from volume-overloaded pigs indicates that decreased responsiveness to β-AR stimulation can occur without degenerative changes such as inflammation or fibrosis (Hammond et al. 1992). It is not known at present, however, whether the changes in the β-AR system are causes or consequences of heart dysfunction. The present isolated perfused hearts of Col15a1−/− mice showed decreased responses to a β-AR agonist, which is a result of one or more changes in the β-adrenergic-mediated signal transduction system.

Microvasculature defects have been shown to be involved in the initiation and progression of heart failure and cardiomyopathy in some modes of human heart disease (Gavin et al. 1998, Liu et al. 1999), and microcirculation abnormalities have been
demonstrated earlier in Syrian hamsters suffering from cardiomyopathy (Factor et al. 1982) together with a decreased response to isoproterenol, indicating changes in β-AR signalling pathways (Feldman et al. 1990). The desensitisation of β-AR signalling observed in Col15a1−/− mice is a hallmark of heart failure, and the potential chronic microcirculation defect may be a factor contributing to cardiac dysfunction, as also suggested in the cardiomyopathic Syrian hamsters (Factor et al. 1982).

In view of its collagenous primary structure, its location in the extracellular space and the consequences of the loss of its function, it could be assume that type XV collagen functions as a structural component which is needed to stabilize cells with the surrounding connective tissue, at least in the skeletal muscle and microvessels. Data also suggest that a lack of type XV collagen will cause damage to the heart in connection of induced cardiovascular stress. It is attractive to speculate that this deficiency may cause mild cardiac dysfunction, detectable first as a diminished inotropic response to isoproterenol, before any clear morphological changes emerge. Furthermore, the sedentary life-style of laboratory mice may prevent the development of severe heart failure in Col15a1−/− mice. Interestingly, these changes mimic early or mild heart disease with respect to features such as decreased inotropy and impaired response to exercise. The microvessel defects are more pronounced in the heart than in skeletal muscle and are accompanied by ischaemic changes in the endothelial cells and adjoining cardiomyocytes. While the skeletal muscle defect appears to be due to defects between the muscle fibre and surrounding ECM, it is possible that the heart phenotype is due to impaired microcirculation.

6.3. Type XVIII collagen is needed for the normal development of the eye

Surprisingly, type XVIII collagen null mice are viable and fertile and do not manifest any obvious phenotypic consequences. Thus, although this collagen is a ubiquitous component at the BM zone, it is not essential for the normal development of the mice. In the light of the proposed function of type XVIII collagen-derived endostatin in angiogenesis, it was decided to attempt careful monitoring of vessel formation and regression in the eye. The postnatal rodent eye provides a unique organ for studying various aspects of angiogenic dynamics, including the sequential characterization of angiogenesis from avascularity to the mature vascular plexus in the retina and changes related to vascular regression and remodelling of the hyaloid vessels.

The hyaloid vascular system (HVS) is transiently present in the developing mammalian eye and it is important during the growth and maturation of ocular structures, whereafter it regresses in a developmentally coordinated manner. The stimuli for this regression are mainly unknown, but apoptosis and tissue macrophages are likely to be involved (see section 2.5.2.). The vasa hyaloidea propria (VHP) persist in type XVIII collagen null mice beyond the time when they have completely disappeared in wild-type mice, indicating that collagen XVIII/endostatin plays a role in the normal regression of the hyaloid vasculature in the vitreous body. Persistence of the (HVS) has been observed in mice carrying a transgene that allows elimination of macrophages in the eye (Lang &
Bishop 1993), which suggests that macrophages and collagen XVIII/endostatin could cooperate in the normal regression of hyaloid vessels. Moreover, endostatin has been reported to induce apoptosis of vascular endothelial cells (Dhanabal et al. 1999a, Dixielius et al. 2000). It could be proposed, therefore, that macrophage-induced regression of VHP may be mediated by endostatin released from type XVIII collagen by macrophage-derived proteolytic enzymes. This hypothesis would predict that elimination of either the macrophages or type XVIII collagen would prevent regression of the hyaloid vasculature, and this is what is observed in the experiments reported by Lang and Bishop (1993) and in the present study.

In addition persistent HVS has been found in some other genetically engineered mice, domestic animals and also in humans (see section 2.5.3.). In most of these cases its presence is associated with abnormal proliferation of fibrovascular tissues posterior to the lens and the development of cataract (Goldberg 1997). In contrast, no fibrovascular proliferation or cataract formation was detected in the Col18a1−/− mice. This may be due to the transient nature of the defect, since most of the VHP disappear with time also in the Col18a1−/− mice, or it may suggest that the phenotype is restricted only to the blood vessels of these mice. Interestingly, there also appear to be developmental defects in the vascularization of the eyes of angiopoietin-2 knockout mice. Angiopoietin-2 is a secreted growth factor that is assumed to be of importance for vascular remodelling, and consistent with this the null mice show delayed regression of the HVS and abnormal growth of the retinal capillaries (Yancopoulos et al. 2000). The similarities in the eye phenotype suggest that endostatin and angiopoietin-2 may be involved in the same cascade of events during remodelling of the eye vasculature.

The role of the HVS is gradually taken over by the developing retinal vasculature. The retinal vessels in mice develop postnatally, in a process driven by hypoxia-induced VEGF expression in the neuroglial cells (Alon et al. 1995, Stone et al. 1995, Benjamin et al. 1998). Any alteration in VEGF expression will cause abnormalities in vascular outgrowth. Exposure to hyperoxia has been shown to repress VEGF expression (Alon et al. 1995, Pierce et al. 1995, Stone et al. 1996), and thus the persistence of large hyaloid vessels on the anterior retinal surface in the collagen XVIII null mice would lead to higher local oxygen levels than in wild-type eyes and possibly prevent the normal up-regulation of VEGF. The variability in retinal vascular patterns among individual mutants could be a direct consequence of the variability in the number and precise location of the persistent vessels in the vitreous body. Even in mutant mice, where the VHP eventually do disappear, the retinal vessels may be abnormal, since it has been demonstrated that a return to normoxia following a brief period of hyperoxia leads to an abnormal burst of VEGF production and an abnormal pattern of vessel outgrowth (Yancopoulos et al. 2000). In fact, the retinal vasculature was not totally lacking in some of the type XVIII collagen null mice but presented abnormal growth patterns.

On the other hand, our current results do not totally rule out the possibility that the abnormalities observed in the retinal vasculature may represent the primary defect and that the persistence of VHP is a secondary consequence of the null allele. Recent data suggest that the NC1 domain of type XVIII collagen has a role in regulating cell mobility that has been conserved from C. elegans to vertebrates (Ackley et al. 2001, Kuo et al. 2001). More specifically, C. elegans has a gene homologous with those for the vertebrate type XV and XVIII collagens, and lack of the endostatin domain results in defective
migration of neuronal cells and various others (Ackley et al. 2001). Normal development of the retinal vasculature is preceded by the migration of astrocytes and endothelial cells (see section 2.5.4.). Moreover, Bishoff et al. (1983) have demonstrated experimentally that normal regression of the HVS may be dependent on development of the retinal vasculature. If the presence of type XVIII collagen during eye development is important for cell migration, a deficiency in this collagen could lead to an abnormal vascular pattern in both the retina and the hyaloid vessels.

The delayed regression of hyaloid vessels on the surface of the retina in type XVIII collagen-deficient mice is in contrast to the normal disappearance of the vessels on the posterior surface of the lens. This suggests that the regression of vessels within the TVL and the regression of VHP are controlled by different mechanisms. It has been shown, for example, that apoptosis of the endothelial cells within the TVL is triggered by a rapid thickening of the posterior lens capsule that separates the endothelial cells from VEGF-producing cells within the lens (Mitchell et al. 1998).

It has been suggested that the type XVIII collagen located in the BM region of the blood vessels is a local regulator of angiogenesis-related proteolysis (Felbor et al. 2000). A release of proteolytic enzymes by stimulated endothelial cells would lead to cleavage within the hinge region of type XVIII collagen and release of endostatin. This would in turn have a negative effect on the endothelial cell activities, causing reduced angiogenesis. The abnormalities seen in the VHP in type XVIII collagen null mice are consistent with this hypothesis. The local regulatory role of type XVIII collagen cannot be a major, rate-limiting one, however, since most vascular systems in the null mice develop normally.

The human counterpart to collagen XVIII null mice has recently been described. Patients with the recessively inherited Knobloch syndrome have a splice site mutation which causes premature termination of the short form of collagen XVIII (Sertié et al. 2000). The disease is characterized by various eye changes and occipital encephalocele (Knobloch & Layer 1971). In patients with Stickler, Wagner, and Marshall syndromes, mutations in COL2A1 or COL11A1 cause abnormalities in the vitreous body and retina, leading to high myopia, vitreoretinal degeneration and retinal detachment (Mundlos & Olsen 1997, Annunen et al. 1999), abnormalities similar to those seen in Knobloch syndrome. It is possible that structural changes in the vitreal matrix as a direct result of a lack of collagen XVIII may cause the eye findings associated with the Knobloch syndrome, in the same way as the abnormalities in the Stickler and Marshall syndromes are caused by mutations in COL2A1 and COL11A1.

Electron microscopy pointed to a reduced number of vitreous collagen fibrils along the inner limiting membrane in sections from Col18a1-/- eyes. In the wild-type mice, the secondary vitreous body begins to form postnatally, between the inner limiting membrane and hyaloid vessels, compressing the VHP centrally around the hyaloid artery and towards the lens (Bischoff et al. 1993, Ito & Yoshioka 1999). It was noted during the dissection of postnatal eyes for whole-mount staining that the VHP appeared to have adhered more tightly to the retina in the Col18a1-/- eyes than in the wild-type eyes, and VHP profiles were seen in close association with the retinal surface in sections of the former. This indicates defects in vitreous formation that would most likely affect the structure of the vitreal matrix, as observed by electron microscopy. On the other hand, the localization of type XVIII collagen in regions where collagen fibrils are ‘inserted’ into the
inner limiting membrane, combined with the changes in the fibrillar collagen matrix of the vitreous body along the inner limiting membrane in the Col18a1−/− eyes, suggests that type XVIII collagen may be important for the connection between the collagenous matrix of the vitreous body and the inner limiting membrane of the retina. Type XVIII collagen in this location may primarily function as a component of an anchoring complex operating between the collagen fibrils and the inner limiting membrane.

Abnormalities in the regression of the fetal intraocular vasculature in man cause a disorder associated with progressive ocular pathologies (PHTVL/PHPV), which results in severe secondary complications resembling the clinical features of Knobloch syndrome (e.g. early onset, cataract formation, myopia, progressive retinal detachment, macular abnormalities and dislocation of the lens), which may suggest a common pathological pathway. The persistent fetal vasculature involved in PHTVL/PHPV is difficult to observe ophthalmoscopically or angioscopically, and due to early opacification of the lens and fragmentiation of the eye PHTVL/PHVP can easily be misdiagnosed (Haddad et al. 1978, Goldberg 1997). The eyes of Knobloch patients are severely affected early in childhood and it may be difficult by means of standard ophthalmoscopic examinations to diagnose whether the fetal vasculature is present or not (Knobloch & Layer 1971, Czeizel et al. 1992, Seaver et al. 1993, Passos-Bueno et al. 1994). The number of Knobloch patients with proper ophthalmoscopic records is limited, and the lack of histopathological data on patients with this syndrome means that the exact pathological mechanism remains uncertain. There are two reports, however, describing clinical findings that are suggestive of the presence of fetal hyaloid vasculature (Seaver et al. 1993, Wilson et al. 1998) and one indicative of secondary changes related to inappropriate vascularization of the retina (Passos-Bueno et al. 1994).

Although it is not possible to compare the mouse and human eye changes directly, it seems that they are similar but not identical. The consequences in the eye are more dramatic in man, as witnessed by the occurrence of cataract formation or progressive retinal detachment. Characteristic features present in mice include certain iris abnormalities (Ylikärppä R, Eklund L, Sormunen R, Määttä M, Muona A, Fukai N, Olsen BR, Pihlajaniemi T, unpublished), which have not been reported in connection with the Knobloch syndrome (Knobloch & Layer 1971, Czeizel et al. 1992, Seaver et al. 1993, Passos-Bueno et al. 1994). In contrast, a diagnostic feature in the human disease is the presence of an occipital encephalocele (Knobloch & Layer 1971), whereas no abnormalities in the posterior part of the brain or skull were observed in the null mice.

### 6.4. Lack of the homologous type XV and XVIII collagens results in separate, mild phenotypes

The lack of either type XV or particularly type XVIII collagen result in unexpectedly mild phenotypes despite their wide occurrence in tissues and the important functions postulated for them. Diverse biological roles are also indicated for these closely related collagens. Type XV collagen has a structural function in skeletal muscle and capillaries, whereas type XVIII is needed in the developing vasculature of the eye. The Col15a1−/− phenotype is evident in tissues shown to be rich in type XV collagen, namely in the
skeletal muscle and heart (Hägg *et al.* 1997a, Muona *et al.* 2001), where there are lower levels of type XVIII collagen (Rehn & Pihlajaniemi 1995, Saarela *et al.* 1998). In the eye, type XV collagen is not found in the surface between inner limiting membrane and the vitreous body (Ylikärppä R, Eklund L, Sormunen R, Määttä M, Muona A, Fukai N, Olsen BR, Pihlajaniemi T, unpublished) that contains type XVIII collagen and was found here to be affected in *Col18a1*⁻/⁻ mice. The liver, however, which is rich in type XVIII collagen (Rehn & Pihlajaniemi 1995, Saarela *et al.* 1998) and essentially lacking in type XV (Hägg *et al.* 1997a, Muona *et al.* 2001), is normal in *Col18a1*⁻/⁻ mice. The mild phenotypes are apparently not caused by compensation, since double knockout mice lacking both of these collagens do not show prominent changes (Ylikärppä R, Eklund L, Sormunen R, Määttä M, Muona A, Fukai N, Olsen BR, Pihlajaniemi T, unpublished). 28 endogenous angiogenic inhibitors have been described in the literature reviewed by Cao (2001), and it is possible that multiple redundant and physiological compensatory mechanisms are responsible for the lack of any major angiogenic phenotype in the mutant mice. This could be exemplified in the present mice lacking type XV collagen, which were shown to have reduced MMP-2 levels. This may cause down-regulation of degenerative activity in the ECM. Angiogenesis requires proteolytic activities leading to degradation of the ECM and dissolving of the cell attachments prior to cell migration (Carmeliet & Collen 2000), and in mice lacking in the collagen XV-derived endostatin homologue the expected enhanced angiogenic phenotype could be counterbalanced by physiological compensatory mechanisms such as the suppression of certain proteolytic activities. It is also possible that the absence of these collagen genes from the earliest stages of development allows for compensatory responses in other genes involved in the angiogenic processes.

There are several lines of evidence to support the notion that endostatin plays a role in cell migration (Dhanabal *et al.* 1999b, Yamaguchi *et al.* 1999, Ackley *et al.* 2001, Kuo *et al.* 2001, Shichiri & Hirata 2001). It is possible that similar mechanisms may regulate the migration of neurogenic cells in *C. elegans* and endothelial cells in vertebrates. In addition, endostatin may also affect several other biological events that are important for angiogenesis, proposed functions that are summarized in Figure 6 (see also section 2.3.1.). The exact mechanisms responsible for the phenotype in the type XVIII collagen null mice still remain an open question.
Fig. 6. What are the mechanisms for the Col18a1−/− phenotype? 1, Proteolytic cleavage within the hinge region results in the release of monomeric endostatin and larger endostatin-containing fragments. Endostatin or trimeric parental forms may exert their biological functions via cellular receptors such as glypicans (2), certain integrins (3) and unknown receptors (4, ?). 2, Endostatin could mediate signals through a co-receptor complex in which glypicans serve as the low affinity receptors presenting endostatin alongside another, as yet unidentified high-affinity receptor. 3, Immobilized endostatin (on the right) supports endothelial cell survival and migration in an α5 and αv integrin-dependent manner, whereas soluble endostatin inhibits endothelial cell functions (on the left). 4, The trimeric NC1 domain and monomeric endostatin have opposite effects in C. elegans and in mammalian Matrigel cultures. This may be due to the ability of trimeric endostatin to bind and cluster cellular receptors to activate certain cell functions. 5, The trimeric NC1 domain has been found to bind strongly to certain BM proteins, including the laminin-1-nidogen complex. The proposed pro-migrational effect of endostatin may be due to an ability to interfere with cell attachment by binding to ECM molecules that are important for cell adhesion. 6, Angiogenesis requires proteolytic activities leading to degradation of the ECM and dissolving of cell attachments, and endostatin may also exert its antiangiogenic effect by suppressing the activities of certain proteolytic enzymes. 7, Endostatin has also been shown to cause changes in structures required for cell mobility and matrix invasion, including the disassembly of focal adhesions and the reorganization of the cytoskeleton (8). For references, see text.
7. Future perspectives

The results presented in this thesis indicate diverse biological roles for the closely related collagen types XV and XVIII and support the idea that type XV is a structural component of the extracellular matrix needed to stabilize capillaries and muscle fibres and is important for the proper functioning of the heart. These findings could be helpful in order to identify human diseases, both skeletal muscle myopathies and heart conditions, that are due to defects in the type XV collagen gene. Further studies are needed to reveal what changes in the skeletal muscle and heart of Col15a1−/− mice are caused by impaired perfusion or defects between the target cell and the surrounding extracellular matrix. This may be achieved by conditional inactivation of the gene in the endothelial cells of mice.

Type XVIII collagen was found to be important in the eye, although the exact mechanism behind the abnormalities was not fully understood. Further analyses using the mouse model to determine what are the primary and secondary defects present in the null mice would be important in order to understand the pathology of the related human eye diseases. It would be essential to explore the role of endostatin fragments versus entire type XVIII collagen molecules in the development of the eye. Furthermore, further analyses might reveal connections between the C. elegans cell migration defects and mouse and human abnormalities. In collagens a null allele and dominant negative mutation have been found to result in different phenotypes, and it is quite possible in the light of the complex multidomain structure of type XVIII collagen that there are other diseases in which this collagen is altered in addition to the Knobloch syndrome.

The lack of either type XV or type XVIII collagen was found to result in unexpectedly mild phenotypes in view of the wide occurrence of these collagens in tissues and the important roles postulated for them as angiogenic regulators. It remains to be seen whether the lack of both known endostatin sequences reveals any changes in the angiogenic phenotype under stress conditions, e.g. in tumor-induced vascularization or high oxygen-induced neovascularization. Type XVIII collagen has been of particular interest due to its potential antiangiogenic fragment, endostatin, which is currently being used in clinical trials, although our knowledge of its mechanisms of action is still in its infancy. Thus further analyses of potential interactions between the extracellular and cell membrane ligands that normally bind type XVIII collagen and the endostatin derived
from it will be important for defining the signalling cascades involved in its antiangiogenic properties.
8. References


Benjamin LE, Hemo I & Keshet E (1998) A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125: 1591-1598.


Gee SH, Blacher RW, Douville PJ, Provost PR, Yurchenco PD & Carbonetto S (1993) Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein,
dystroglycan, and binds with high affinity to the major heparin binding domain of laminin. J Biol Chem 268: 14972-14980.


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Rehn M & Pihlajaniemi T (1994) α1(XVIII), a collagen chain with frequent interruptions in the collagenous sequence, a distinct tissue distribution, and homology with type XV collagen. Proc Natl Acad Sci USA 91: 4234-4238.


