TYPE XIII COLLAGEN: REGULATION OF CARDIOVASCULAR DEVELOPMENT AND MALIGNANT TRANSFORMATION IN TRANSGENIC MICE
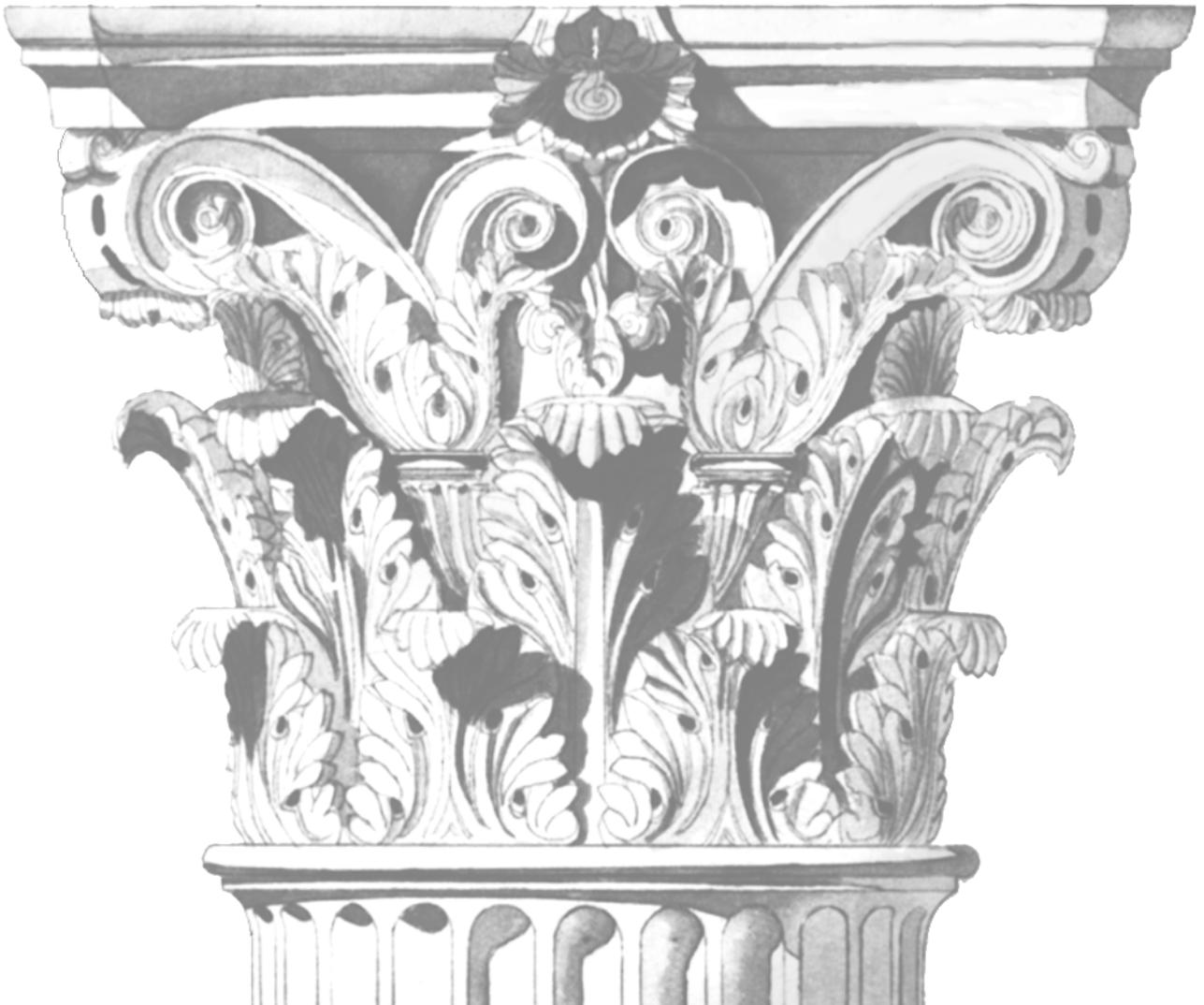
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Abstract

Type XIII collagen is a type II oriented transmembrane protein with a short intracellular domain, a single transmembrane domain and a large, mostly collagenous extracellular domain. Tissue localization and cell culture studies have implicated that it is involved in cell adhesion.

The spatio-temporal expression of type XIII collagen mRNA and protein during murine development is studied here. Type XIII collagen mRNAs were expressed at a constant rate during development, with an increase of expression towards birth. The strongest expression was detected in the central and peripheral nervous systems of the developing mouse fetus. Cultured primary neurons expressed this collagen, and recombinant type XIII collagen was found to enhance neurite outgrowth. Strong expression was also detected in the heart, with localization to cell-cell contacts and perinatal accentuation in the intercalated discs. Other sites of type XIII collagen expression included cartilage, bone, skeletal muscle, lung, intestine and skin. Clear developmental shifts in expression suggest a role in endochondral ossification of bone and the branching morphogenesis in the lung.

To elucidate the function of type XIII collagen transgenic mice were generated by microinjection of a cDNA construct that directs the synthesis of truncated $\alpha1(XIII)$ chains with an in-frame deletion of the central collagenous COL2 domain. This construct was thought to disrupt the assembly of normal type XIII collagen trimers. Expression of shortened $\alpha1(XIII)$ chains by fibroblasts derived from mutant mice was demonstrated, and the lack of intracellular accumulation in immunohistochemical analysis of tissues suggested that the mutant molecules were expressed on the cell surface. Transgene expression led to developmental arrest and fetal mortality in offspring from heterozygous mating with two distinct phenotypes. The early phenotype fetuses were aborted by day 10.5 of development due to a failure in the fusion of the chorion and allantois membranes and subsequent disruption in placentation, while the late phenotype fetuses were aborted by day 13.5 of development due to cardiovascular and placental defects. Furthermore, it was shown that the heterozygous mice that were initially of normal appearance and bred normally had an increased susceptibility to develop T-cell lymphomas and angiosarcomas later in life.

The results presented here increase the evidence that type XIII collagen is involved in cell adhesion, with several important tasks during development. A role of type XIII collagen in malignant transformation of certain mesenchymal cell populations is also implicated.

Keywords: collagen, transgenic mice, cardiogenesis, angiogenesis, placentation, malignant transformation, fetal development, fetal death

Acknowledgements

Umeå, October 2001   Malin Sund

Abbreviations

αx(a)	collagen polypeptide; x: number of chain; a: number of collagen
aa	amino acid
BM(Z)	basement membrane (zone)
BSA	bovine serum albumin
bp	base pairs
C-	carboxy-
cDNA	complementary DNA
COL	collagenous domain
dpc	day post coitum
kb	kilobases
kDa	kilodalton
mRNA	messenger RNA
N-	amino-
NC	noncollagenous
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
(Q)RT	(quantitative) reverse transcriptase
SDS	sodium dodecyl sulphate
SSC	citrate-buffered saline
wk	week of gestation
X (in Gly-X-Y)	any amino acid
Y (in Gly-X-Y)	any amino acid

List of original papers

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals, and on some unpublished data

I   Sund M, Väisänen T, Kaukinen S, Ilves M, Tu H, Autio-Harmainen H, Rauvala H & Pihlajaniemi T (2001) Distinct expression of type XIII collagen in neuronal structures and other tissues during mouse development. Matrix Biol 20: 215-230.

II   Sund M, Ylönen R, Tuomisto A, Sormunen R, Tahkola J, Kvist A-P, Kontusaari S, Autio-Harmainen H & Pihlajaniemi T (2001) Abnormal adherence junctions in the heart and reduced angiogenesis in transgenic mice overexpressing mutated type XIII collagen. EMBO J 20: 5153-5164.

III   Sund M, Tuomisto A, Tahkola J, Savolainen E-R, Autio-Harmainen H & Pihlajaniemi T (2001) Increased incidence of T-cell lymphomas and angiosarcomas in transgenic mice overexpressing mutated type XIII collagen. Manuscript.

Contents

1 Introduction

The extracellular matrix is a complex system of a myriad of proteins that support and surround the cells of multicellular organisms. The traditional view of it is that it functions as a mere supporting substance but numerous studies in recent years have indicated that it also takes part in and controls cellular events during development, differentiation and cell growth. This active role requires that the extracellular matrix should either directly interact or modulate the function of membrane receptor that mediate signals inside the cells, signals that will in turn affect gene expression and cell motility, division and growth. The signals may also subsequently lead to modulation of the extracellular matrix by the cells. Thus interactions between the matrix and the cells enable the complex steering of events required for the development and maintenance of complicated multicellular organisms.

Type XIII collagen is a type II oriented transmembrane protein that consists of a short intracellular domain, a single transmembrane domain and a large, mostly collagenous extracellular domain. Its function was unknown at the time when work on this thesis was initiated. The development of transgenic technologies has made the study of genes and their functions possible in model systems such as mice. By generating gene mutations in animal models, the role of a single gene in the organism can be elucidated and ultimately these models can serve as disease models for human pathological states, enabling subsequent analysis of the disease mechanisms and allowing therapeutic interventions to be tested.

The goal of the present work was to generate a transgenic mouse strain that expresses mutant type XIII collagen and to study the consequences of the mutant protein in the mouse. As results indicating an important role for type XIII collagen during fetal development started to evolve, the characterization of type XIII collagen expression during normal murine development became a necessary part of this work. The results presented in this thesis show that type XIII collagen is abundantly expressed in several tissues in the developing mouse, its importance during development being further underlined by the fetal lethality of mice expressing a mutant form of the molecule, which leads to developmental defects in the cardiovascular and placental systems. A role for type XIII collagen in processes involving growth control is also implicated, as expression of a mutant form of this protein causes the development of malignancies in certain

mesenchymal cell populations. These results imply that type XIII collagen is a protein involved in the interaction between the matrix and the cells.

2 Review of the literature

2.1 The collagen family of proteins

The collagens are a large family of proteins with variable functions in the generation and maintenance of the extracellular matrix of multicellular organisms, and the proteins of this family account for approximately one third of the total protein mass in vertebrates (Brown & Timpl 1995). Collagens are characterized by repeats of the amino acids Glycine-X-Y in their sequences, which enables the formation of a collagen triple helix from three polypeptides or α-chains, as the smallest amino acid, glycine, fits perfectly into the centre of the collagenous molecule. The X and Y positions in the collagen sequence are frequently occupied by proline and hydroxyproline, which increase the stability of the triple helix (van der Rest & Garrone 1991). Up to now 19 types of collagen have been described and the α-chains for their polypeptides are known to be encoded by more than 30 genes. However, at least four additional collagen α-chains are currently under characterization (Myllyharju & Kivirikko 2001). As several other proteins containing collagenous sequences are also known, the collagens are currently viewed as a family of proteins that contain varying stretches of collagenous sequences and are structural components of the extracellular matrix (Brodsky & Shah 1995, Hulmes 1992, Myllyharju & Kivirikko 2001, Pihlajaniemi & Rehn 1995, Prockop & Kivirikko 1995, van der Rest & Garrone 1991, Vuorio & de Crombrugghe 1990).

The collagens are classically divided into fibrillar and non-fibrillar types depending on their ability to form fibrils as a macromolecular structure. The fibrillar collagens (types I, II, III, V and XI) are characterized by long uninterrupted collagenous sequences that enable the arrangement of the molecules into highly ordered quarter-staggered fibrils. The members of the heterogeneous non-fibrillar group, on the other hand, contain disruptions in their collagenous sequences and are arranged in supramolecular structures of several kinds. The further division of the non-fibrillar collagens into the following subgroups is based on their structural or functional properties; network-forming collagens (types IV, VIII and X), fibril-associated collagens (types IX, XII, XIV, XVI and XIX), beaded filament-forming collagen (type VI), collagen of anchoring fibrils (type VII), transmembrane collagens (types XIII and XVII) and the subfamily of types XV and

XVIII collagens (Prockop & Kivirikko 1995). The fibrils formed by the fibrillar collagens give the necessary tensile strength to the major connective tissue structures of the body and are the most abundant protein components of structures such as bone, cartilage and tendons, while the non-fibrillar collagens not only perform structural functions but are also involved in several other tasks, functioning as filtration barriers or being involved in cell adhesion or in morphogenetic remodeling (Hägg *et al.* 2001, Lin *et al.* 2001, Miner & Sanes 1994, Myllyharju & Kivirikko 2001).

2.2 Collagens with a transmembrane domain

The small transmembrane subfamily of the non-fibrillar collagens currently consists of two members, type XIII and type XVII. These molecules are characterized by a hydrophobic domain at one end of the protein, enabling binding into the cell membrane. Other transmembrane proteins containing collagenous sequences have been described, but as these are not structural components of the extracellular matrix they are not regarded as collagens (Pihlajaniemi & Rehn 1995). Some of these proteins will nevertheless, be discussed later due to their structural similarities to type XIII collagen.

2.2.1 Type XIII collagen

2.2.1.1 Structure and chromosomal localization

Type XIII collagen is a transmembrane protein that consists of three collagenous domains (COL1-3) surrounded and separated by non-collagenous domains (NC1-4) (Hägg *et al.* 1998, Pihlajaniemi *et al.* 1987, Pihlajaniemi & Tamminen 1990, Tikka *et al.* 1988, Tikka *et al.* 1991). Its gene is large, the human and mouse genes being estimated to be 140kb and 135kb in size, respectively. The human type XIII collagen gene contains 41 exons and the mouse gene 42 exons (Kvist *et al.* 1999, Tikka *et al.* 1991). An unusual feature of type XIII collagen is the complex alternative splicing that its transcripts undergo, affecting 10 exons of the gene encoding for sequences in the COL1, NC2, COL3 and NC4 domains of the protein. Although several collagens other than type XIII are subjected to alternative splicing this protein is one of the few in which the collagenous domains undergo splicing (Pihlajaniemi & Rehn 1995). The possible combinations of splice variants number 1024, but the analysis of mRNAs from tissues and cells suggests that some combinations are found more frequently than others, and certain combinations of exons have not been observed at al (Juvonen *et al.* 1993, Juvonen & Pihlajaniemi 1992, Peltonen *et al.* 1997, Pihlajaniemi & Tamminen 1990, Tikka *et al.* 1988). Questions that still remain to be answered concern how the splice variants assemble into type XIII collagen molecules, what the role of alternative splicing is, and what the possible functional differences between the resulting splice variants may be.

The structures of the cDNAs and genes for both human and mouse type XIII collagen have been characterized and the genes located to chromosome 10 in both species (Hägg *et al.* 1998, Kvist *et al.* 2001, Pihlajaniemi & Tamminen 1990, Shows *et al.* 1989, Tikka *et al.* 1991).

2.2.1.2 Biosynthesis of type XIII collagen

A hydrophobic sequence in the N-terminal part of type XIII collagen combined with the lack of a classical signal peptide first indicated a potential membrane location for the molecule, and its cell membrane location was subsequently demonstrated by immunoprecipitation of biotinylated type XIII collagen from surface-labelled HT-1080 cells, immunofluorescence staining and subcellular fractionation of membranes (Hägg *et al.* 1998).

Although type XIII collagen has been found to be expressed in a variety of tissues, the expression level of this protein is quite low. To circumvent the problems this causes for extraction of type XIII collagen from tissues for more thorough characterization of the protein, a recombinant protein was produced in a baculovirus expression system and used to show that this collagen type assembles into stable triple helical homotrimeric molecules (Snellman *et al.* 2000a). Interestingly, a portion of the protein was found to be shed from the cells in the baculovirus system, an effect that could be inhibited by a furin protease inhibitor (Snellman *et al.* 2000b). Type XIII collagen has also been found to be shed into the media by cultured keratinocytes (Peltonen *et al.* 1999). Whether the observed shedding from the cell membrane also takes place *in vivo* and is related to the physiological functions of this collagen is still unknown.

Experiments with the type XIII collagen produced in the baculovirus expression system proved that its cellular topography is of the type II membrane orientation, so that the short N-terminal portion is located intracellularly and the large portion C-terminal portion extracellularly (Snellman *et al.* 2000b) (Figure 1). C-terminal deletion variants were able to form stable triple helical molecules, whereas variants containing N-terminal deletions could not. This indicated that chain recognition and association takes place in the N-terminal region of the α1(XIII) chains, with triple helix formation thus taking place in an N- to C-terminal direction, opposite to that described for the classical collagens (Snellman *et al.* 2000b, Prockop & Kivirikko 1995). Interestingly, type XIII collagen contains a conserved sequence in the N-terminal region close to the transmembrane domain that is also found in several of the other membrane proteins containing collagenous domains, such as type XVII, MARCO and EDA-A1 (Snellman *et al.* 2000b). This sequence may thus be relevant to the association of the three polypeptides that enables trimer formation in these membrane-bound proteins (Snellman *et al.* 2000b).
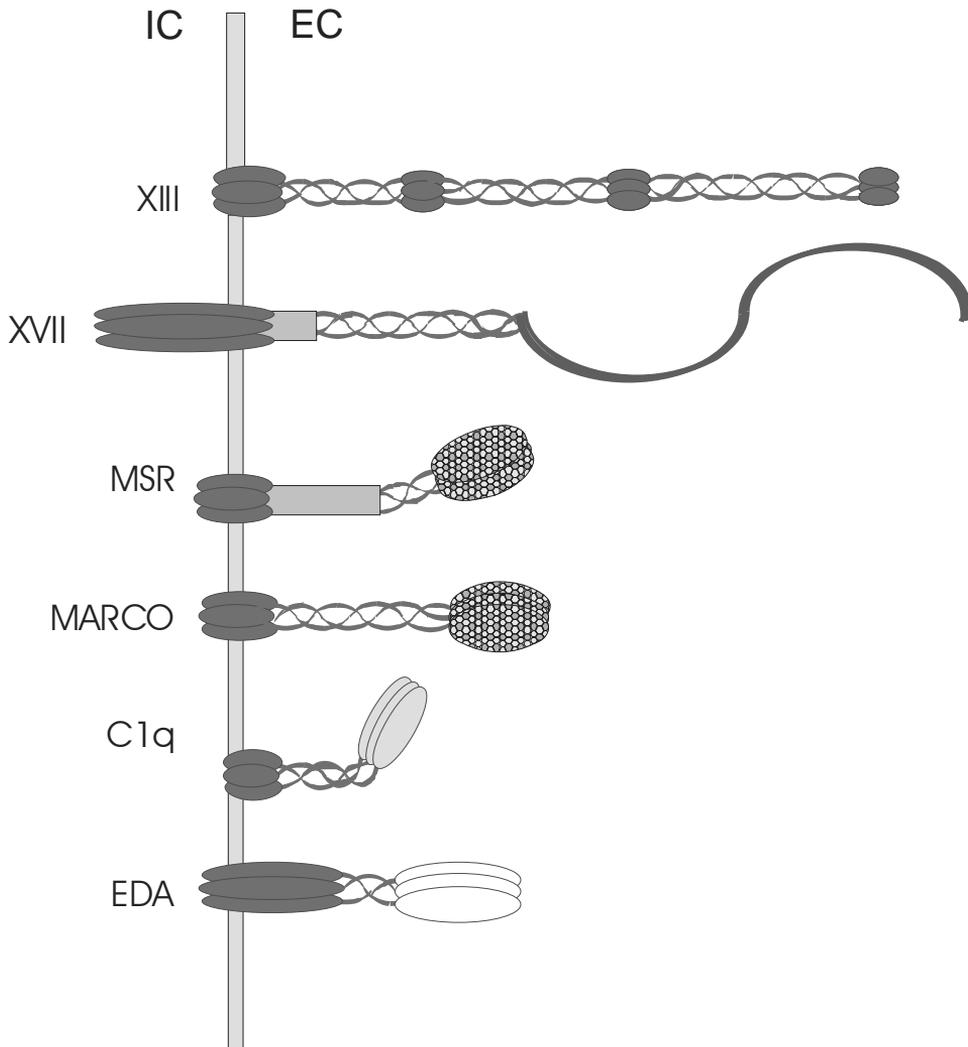
Fig. 1. Comparison of the structures of membrane proteins with collagenous domains. Collagenous domains are denoted by triple helices, globular domains by circles and other rod-like domains by boxes. The figure is compiled and modified from the references mentioned in the text (Elomaa *et al.* 1995, Hirako *et al.* 1996, Knobel *et al.* 1975, Mikkola *et al.* 1999, Resnick *et al.* 1996) and reproduced with permissions from the editors.

2.2.1.3 Cellular and tissue localization of type XIII collagen

The localization of type XIII collagen in cultured cells has been studied by indirect immunofluoresecence with antibodies generated against the protein. In all the cell types studied it colocalized with vinculin and talin in the focal adhesion plaques (Hägg *et al.* 2001), which are specialized adhesive structures used by cells when adhering to the

matrix or culture dish. The localization of type XIII collagen mRNA and protein in tissues has been studied by *in situ* hybridization of human tissues and by means of antibodies in both human and murine tissues. Type XIII collagen mRNAs were found to be expressed in tissues derived from all three embryonic germ layers, such as endodermally derived intestinal epithelia (Sandberg *et al.* 1989), epidermally derived skin (Sandberg *et al.* 1989), placenta (Juvonen *et al.* 1993) and nervous system (Sandberg-Lall *et al.* 2000), and most abundantly, mesodermally derived tissues such as cartilage, bone and muscle (Sandberg-Lall *et al.* 2000, Sandberg *et al.* 1989). Immunofluorescence analysis detected the protein in a number of adhesive structures that can be considered to be the tissue equivalents of focal adhesion plaques. Type XIII collagen was found at sites of cell-matrix interaction such as the myotendinous junctions in skeletal muscle and at sites of cell-cell interaction such as the intercalated discs of cardiac muscle and cell-cell contacts within the retina of the eye (Hägg *et al.* 2001, Sandberg-Lall *et al.* 2000), implying a role in cell adhesion.

2.2.1.4 Ligands and function of type XIII collagen

The ligands of type XIII collagen are unknown, but recent results indicate that the recombinant protein interacts strongly with the I domain of α1 integrin (Nykvist *et al.* 2000), raising the possibility of interactions both between adjacent cells or laterally within the membrane plane of one cell.

We have recently described the phenotype of a transgenic mouse strain, ColXIIIα1$^{n/n}$, produced by homologous recombination, that lack the cytosolic and transmembrane domains of type XIII collagen. The mice are viable and fertile, but display a progressive myopathy with increasing age due to abnormalities in basement membrane and muscle cell interaction, with ruptures and irregularities in the basement membrane structure. The phenotype of the ColXIIIα1$^{n/n}$ mice indicates a role for type XIII collagen in stabilizing the basement membrane zone, at least in some tissues (Kvist *et al.* 2001). Interestingly, the mutant molecules are secreted and can apparently take over the functions of the ectodomain of type XIII collagen to a great extent.

2.2.2 Type XVII collagen

The second membrane-bound collagen, type XVII, is a type II oriented transmembrane protein characterized by a large extracellular portion containing multiple collagenous domains with several interruptions. The intracellular domain is much larger than that of type XIII collagen (Giudice *et al.* 1991, Giudice *et al.* 1992, Hopkinson *et al.* 1992, Li *et al.* 1993, Pihlajaniemi & Rehn 1995) (Figure 1). It has been shown in rotatory shadowing experiments to have a globular domain, a rod-like domain and a flexible tail, most likely representing the intracellular domain, the largest collagenous domain and the C-terminal end of the protein, respectively (Hirako *et al.* 1996) (Figure 1).

Type XVII collagen is located in the hemidesmosomes at the epithelial cell-matrix junctions in skin, lung and cornea (Aho & Uitto 1999, Giudice *et al.* 1991, Li *et al.* 1991,

Michelson *et al.* 2000). Interestingly, this collagen also appears to be shed from the cell membrane through furine-mediated proteolysis, as has been shown for cultured keratinocytes (Schacke *et al.* 1998).

Recent studies have shown that type XVII collagen interacts both/either with the extracellular portion of α6 integrin and/or the intracellular portion β4 of this hemidesmosomal integrin (Hopkinson *et al.* 1998, Borradori *et al.* 1997, Niessen *et al.* 1996). Other findings indicate interactions with keratin 18 (Aho & Uitto 1999) and the 230-kDa bullous pemphigoid autoantigen, which is involved in the binding of the hemidesmosome proteins to intermediate filaments (Hopkinson & Jones 2000). The ectodomain of type XVII collagen is the target for autoantibody production in the skin blistering diseases bullous pemphigoid, herpes gestationis and cicatricial pemphigoid, and the protein was originally characterized as the BPAG2 or BP180 antigen (Labib *et al.* 1986). Mutations of the gene have been found to cause a blistering disease of the skin, generalized atrophic benign epidermolysis bullosa, which further underlines the importance of this collagen for hemidesmosome-mediated cell-matrix adhesion (Pulkkinen & Uitto 1998). The phenotype of patients with mutations in the type XVII collagen gene or with autoantibodies against this molecule indicates a role in epidermal-dermal cell adhesion, although the possibility has been raised of a potential role in signal conduction, as the large intracellular portion of the molecule contains several sites that may be modified by phosphorylation (Hopkinson *et al.* 1992, Li *et al.* 1993).

2.3 Other membrane proteins with collagenous domains

Several known transmembrane proteins contain collagenous sequences but have not been named as collagens. Another common denominator of these molecules is the rare type II orientation in the cell membrane (Pihlajaniemi & Rehn 1995). One group of these molecules is formed by proteins that are involved in host defense of cells against attacks from foreign substances. These are the group of class A macrophage scavenger receptors (MSR), MARCO and type I, II and III MSRs, and also the C1q subcomponent of the first component of the serum complement system. Due to their function in host defense these molecules are called the defence collagens (Kaul & Loos 1993, Tenner 1999, Yamada *et al.* 1998). Ectodysplasin is a recently described type II oriented membrane protein with a short collagenous domain that has an important role in epithelial morphogenesis (Ezer *et al.* 1999). Ectodysplasin is not classified as a defense collagen, but belongs to the family of tumor necrosis factor molecules (Mikkola *et al.* 1999).

2.3.1 The macrophage scavenger receptors and MARCO

The group of class A macrophage scavenger receptors (MRS), involving type I, II and the recently described type III, are trimeric splice products of a single gene and contain six distinct domains; an intracellular portion, a transmembrane portion, a spacer domain, an α-helical coiled-coil domain, a collagenous domain and the scavenger receptor cystein-rich (SRCR) domain (Kodama *et al.* 1990, Rohrer *et al.* 1990, Yamada *et al.* 1998). The

type II receptor has a short carboxy-terminal domain instead of the SRCR and the type III receptor contains a truncated form of the SRCR domain (Ashkenas *et al.* 1993, Gough *et al.* 1998). Additionally, the type I and II receptors were found in rotatory shadowing experiments to consist of two rod-like domains that could bend from a hinge region with an angle varying between 20° to 180°, and in the case of the type I receptor a globular domain was observed at one end of the molecule. The rod-like domains most likely represented the coiled-coil domain and the collagenous domain and the globular domain the SRCR domain at the C-terminus of the type I receptor (Resnick *et al.* 1996). The type I and II MSRs are constitutively expressed by most resident macrophages, including alveolar and peritoneal macrophages, Kupffer cells of the liver and the perivascular macrophages surrounding the arterioles (MATO cells) of the brain (Yamada *et al.* 1998). These receptors are able to scavenge a large number of polyanionic substances and are consequently thought to be involved in the accumulation of foam cells in the artherosclerotic plaques of vessel walls, in the amyloid-β protein deposit in the brain in Alzheimer's disease and in host defense against bacteria (Krieger & Herz 1994, Araki *et al.* 1995, El Khoury *et al.* 1996, Paresce *et al.* 1996, Dunne *et al.* 1994, Hampton *et al.* 1991, Christie *et al.* 1996, Krieger & Herz 1994, Yamada *et al.* 1998). Besides scavenger activity, these proteins are also involved in adhesion of cultured macrophages to the culturing dish, as was demonstrated by reduced adhesion in antibody perturbation analysis and of macrophages from mice deficient for the MSRs (Fraser *et al.* 1993, Suzuki *et al.* 1997).

MARCO is a macrophage receptor that is expressed on a subset of macrophages at sites of strong through flow of blood in the spleen, lymph nodes and lung, and thus this protein is thought to have a role in surveillance of the blood and clearance of bacteria (Elomaa *et al.* 1995). It is structurally related to the type I MSR, but its collagenous domain is longer and it lacks the α-helical coil-coiled domain (Elomaa *et al.* 1995) (Figure 1.). MARCO has a broad ligand binding capacity, which is thought to be mediated by the SRCR domain (Elomaa *et al.* 1998), and MARCO has more recently been shown to bind acetylated LDL, bacteria and unopsonized environmental particles, (Elomaa *et al.* 1995, Palecanda *et al.* 1999) so that it must be involved in host defense.

2.3.2 The complement subcomponent C1q

The first component of the classical serum complement, C1, contains a subcomponent C1q. This first forms a complex with the proenzymes C1r and C1s to form C1, thus initiating the complement cascade at relevant sites. C1q is a glycoprotein of about 460 kDa and consists of the N-terminal membrane-bound signal peptides, a collagenous domain of about 80 residues and a C-terminal globular non-collagenous domain (Pihlajaniemi & Rehn 1995) (Figure 1). C1q has been shown to reside in the plasma membrane of the guinea pig and human macrophages, most likely through its signal peptide (Kaul & Loos 1993, Kaul & Loos 1995), although a secreted form of the protein also exists (Kaul & Loos 1995). Since C1q is formed of six trimeric molecules, each C1q molecule consists of 18 polypeptide chains. These heterotrimers are composed of an A, B and C chain, each of which is encoded by an individual gene. The six C1q trimers have

been shown by electron microscopy to form a structure resembling a bouquet of tulips, in which the collagenous domain forms the stalks and the C-terminal non-collagenous domain the bulbs (Knobel *et al.* 1975, Thiel & Reid 1989). Ligand binding through the C-terminal globular domain of C1q has been shown to take place when binding to both immune complexes (Hughes-Jones & Gardner 1979) and non-immune ligands such as DNA (Jiang *et al.* 1992a) but the collagenous domain is also implicated in ligand binding as it has been shown to bind to decorin (Krumdieck *et al.* 1992), serum amyloid P component (Ying *et al.* 1993), C-rective protein (CRP) (Jiang *et al.* 1992b), DNA (Jiang *et al.* 1992a) and several polyanionic substances similar to the MSRs (Acton *et al.* 1993). The binding of C1q to its ligands usually causes activation of the complement system. C1q has also been found to have functions outside the complement system, as it is a ligand for various proteins and is involved through these in other cellular responses (Eggleton *et al.* 1998). A role for C1q in mediating and directing correct immune responses is implied by the fact that transgenic mice lacking the gene for A chain, and thus having no detectable C1q protein, display increased mortality from severe glomerulonephritis caused by the accumulation of immune complexes and the defective clearance of apoptotic bodies in the kidneys (Botto *et al.* 1998). These mice also display increased levels of autoantibody production, a feature typical of human subjects with mutations of the C1q genes, which is associated with the development of systemic lupus erythematosus (SLE) (Botto *et al.* 1998, Morgan & Walport 1991).

2.3.3 *The ectodysplasin family of proteins*

The ectodysplasin-A (EDA) family of proteins contains type II oriented transmembrane proteins that, like type XIII collagen, undergo complex alternative splicing of the mRNA transcript, generating molecules that differ in their C-terminal regions but leaving the intracellular and transmembrane domains intact (Ezer *et al.* 1997, Ezer *et al.* 1999, Kere *et al.* 1996). The phenotype of the spontaneous mouse mutant strain *tabby* is caused by mutations in the ectodysplasin gene, and thus the mouse EDA gene is often referred to in the literature as the tabby gene (Ta) (Ferguson *et al.* 1997, Srivastava *et al.* 1997). The ectodysplasin proteins contain a short intracellular domain, a single transmembrane domain, a short collagenous domain and a domain with TNF homology (Ezer *et al.* 1997, Mikkola *et al.* 1999) (Figure 1). So far several structurally different protein variants have been described in both man and the mouse. EDA-O, the original protein described, contains only a short collagenous repeat of four residues, while the longest forms, the EDA-A/Ta-A variants, contain an additional collagenous repeat of 23 residues. Splice variants named EDA-B/Ta-B, EDA-C/Ta-C, EDA-D, EDA-E and EDA-F, containing only the short collagenous repeat, have also been found by RT-PCR analysis, but it is not yet known whether these transcripts result in biologically functional proteins (Bayes *et al.* 1998, Mikkola *et al.* 1999). The EDA gene transcripts have a broad tissue distribution and are involved in epithelial cell morphogenesis (Kere *et al.* 1996, Mikkola *et al.* 1999, Montonen *et al.* 1998). Mutations of the EDA gene causes the heritable X-linked disease hypohidrotic ectodermal dysplasia (Kere *et al.* 1996), and the phenotype is similar in the *tabby* mouse with mutations of the EDA/Ta gene (Ferguson *et al.* 1997, Srivastava *et al.*

1997). The phenotype caused by the EDA mutations indicate its importance in ectodermal organogenesis, as patients have abnormal development of the sweat glands, hair and teeth (Ferguson *et al.* 1997, Kere *et al.* 1996), but EDA has also been shown recently to be involved in cell-matrix adhesion (Mikkola *et al.* 1999).

2.4 Collagen expression during fetal development

The spatio-temporal expression patterns of most collagens during development have been extensively studied and several of the proteins and mRNAs are known to be widely expressed during fetal development. The expression patterns observed in fetuses are broader than those in the corresponding mature tissues, indicating possibly different functions from those described for adult tissues. Most of the fibrillar collagens are clearly involved during development in generating the structural properties that are typical of them in the adult organism, but the genes for the α1 chains of type II and XI collagens display expression of alternate forms during early development that are likely to be involved in tissue morphogenesis (Lui *et al.* 1995b, Sandell *et al.* 1991, Sandell *et al.* 1994, Liu *et al.* 1993, Myllyharju & Kivirikko 2001). The same is the case with the heterogenous group of non-fibrillar collagens, many of which appear to be involved in similar tasks during development as in mature tissues although some display unexpected tissue distributions during development, such as type VIII collagen, which is involved in cardiac morphogenesis (Iruela-Arispe & Sage 1991), and type XIX collagen, which is widely expressed in fetal tissues, whereas only a few adult tissues express this collagen (Sumiyoshi *et al.* 1997). Results regarding the spatio-temporal expression patterns of collagens during human and/or murine fetal and embryonic development and details of the developmental stages analyzed, the methods used and the corresponding references are summarized in Table 1. When no references could be found to human or murine development results from other species are shown.

Table 1. Expression of collagens during fetal development

Collagen Type	Species	Stage	mRNA	Protein	Tissues	Reference
Fibrillar collagens						
α1(I)	Human	15-18 wk	IS, N		Bone, perichondrium, connective tissues	Sandberg & Vuorio 1987
α1-2(I)	Mouse	8.5-17.5 dpc	IS		Mesoderm, connective tissues sclerotomes & dermatomes, bone	Niederreither *et al.* 1995
α1(I)	Rat	19 dpc	IS, N, SB		Heart; valves, perivascular cells, fibroblasts	Carver *et al.* 1993
α1(II)	Human	15-18 wk	IS, N	IH	Resting & growth zone cartilage, inner ear; cartilage & connective tissue	Sandberg & Vuorio 1987, Khetarpal *et al.* 1994
α1(II)	Mouse	9.5-18.5 dpc	IS, RP		Chondrogenic tissues & cartilage, notochord, heart, epidermis, brain, inner ear, eye; retina, sclera, cornea, conjunctiva, lens, iris, vitreous	Cheah *et al.* 1991, Savontaus *et al.* 1997
α1(II), A&B	Human	22-44 dpc 4-24 wk	IS, RP, N		A; chondrogenic tissue, ganglial cells, tooth bud, adrenal cortex, gonad, ectodermal ridge A&B; mesenchymal cells, notochord, cartilage, bone, vertebral disc, skeletal & heart muscle, skin, brain, lung, kidney, liver, intestine	Sandberg *et al.* 1993, Lui *et al.* 1995a, Krengel *et al.* 1996
α1(II), A&B	Mouse	9.5-18.5 dpc	IS, N		A; prechondrocytes, ganglial cells, mesenchyme, somites, epithelium, intramembranous bone B; chondrocytes	Sandell *et al.*1991, Sandell *et al.* 1994
α1(III)	Human	15-18 wk	IS, N		Blood vessels, skin, synovial & connective tissue	Sandberg & Vuorio 1987
α1(III)	Mouse	8.5-17.5 dpc	IS		Like type I, except bone	Niederreither *et al.* 1995
α1(III)	Rat	19 dpc	IS, N, SB		Like type I	Carver *et al.* 1993
α2(V)	Mouse	9.5-16.5 dpc	IS		Bone, perichondrium, tendon, joints, heart valves	Andrikopoulos *et al.* 1992
α2(XI)	Human	15-19 wk	IS, RP, N		Cartilage, bone, kidney, skin, muscle, intestine, liver, brain, lung	Sandberg *et al.* 1993
α1-3(XI) α2(V)	Human	15-24 wk	RP		Cartilage, calvaria, bone, tendon, meninges, kidney, lung, skeletal & heart muscle, brain	Lui *et al.* 1995b
α1(XI)	Mouse	11.5 dpc-	IS		Cartilage, bone, neuro-epithelium, heart valves, tongue, intestine, otic vesicle	Yoshioka *et al.* 1995

Table 1. Continued.

Collagen Type	Species	Stage	mRNA	Protein	Tissues	Reference
Non-fibrillar collagens						
α1-6(IV)	Human	15-18 wk		IH	Kidney; α1-2 in all BM, α3-6 BM of distal tubuli & glomeruli	Lohi *et al.* 1997
α1-2(IV)	Mouse	5, 12.5 & 16.5 dpc	IS, N	IH	Parietal endoderm, BM of blood vessels, epithelial & mesenchymal cells, kidney; BM of glomeruli and tubules	Adamson & Ayers 1979, Thomas *et al.* 1993, Laurie *et al.* 1989
α1-3(VI)	Mouse	7.5-16.5 dpc	IS, N, RT	IH	Subepidermal & cephalic mesenchyme, blood vessels, lung, gut, muscle, heart & heartvalves, meninges, vertebrae, vertebral discs, perichondrium & periosteum	Dziadek *et al.* 1996, Marvulli *et al.* 1996,
α1(VII)	Human	19 wk		IH	Skin; epidermal-dermal BMZ	Ryynänen *et al.*1992
α1(VIII)	Mouse, chicken	11.5-18.5 dpc 19-		IH, IB	Cephalic mesenchyme, lung, capillaries, thymus, heart; myoblasts, subendocardial Cells, cardiac jelly	Sage & Iruela-Arispe 1990, Iruela-Arispe & Sage 1991
α1(VIII)	Cow			IH	Descement's membrane, sclera, meninges, periosteum & perichondrium, spinal cord, optic nerve	Kapoor *et al* 1988
α1(IX) S&L	Mouse	10.5-18.5 dpc	IS, N, RP, RT		S; eye, heart L; cartilage, lung, liver	Liu *et al.* 1993
α1-3(IX)	Mouse	9.5-18.5 wk	IS, N, SB, RP, RT		α1-3: chondrocytes α1 & α3: periosteal cells	Perälä *et al.* 1997
α1(X)	Human			IH	Hypertrophic cartilage	Nerlich *et al.* 1992
α1(X)	Chicken	15-18 dpc	N	IH, W	Hypertrophic cartilage	Lu valle *et al.* 1992
α1(XII) A&B	Mouse	9.5-18.5 dpc		IH	Perichondrium & periosteum, bone, tendon, ligaments, dermis meninges, cornea, blood vessels A; early developmental stages B; late developmental stages	Oh *et al.* 1993, Bohme *et al.* 1995
α1(XIII)	Human	15-18 wk	IS		Bone, cartilage, epidermis with appendages, intestine, skeletal muscle	Sandberg *et al.* 1989
α1(XIII)	Mouse	9.5-18.5 dpc	IS, N, QRT	IH	Central & peripheral nervous system, heart & skeletal muscle, cartilage, bone, lung, intestine, skin	Sund *et al.* (I)
α1(XIV)	Chicken	27-39		IH	Skeletal muscle	Tono-Oka *et al.*1996
α1(XV)	Mouse	9.5-18.5 dpc		IH	Capillaries, muscle, all BMZs	Muona *et al.* 2001
α1(XVI)	Mouse	9.5-18.5 dpc	IS	IH	Heart, smooth & skeletal muscle, kidney, intestine, ovary, testis, eye, lung, brain	Lai *et al.* 1996
α1(XVIII)	Mouse	9.5-18.5 dpc	N	IH	All BMZs	Saarela 1998
α1(XIX)	Mouse	11.5-18.5 dpc	RT, QRT		Limb, vertebrae, brain, tail, kidney, calvaria, lung, muscle, skin, intestine	Sumiyoshi *et al.* 1997

Abbreviations: IH= immunohistochemistry, IS= *in situ* hybridization, N= Northern blot analysis, QRT= quantitative RT-PCR, RP= RNAse protection, RT= RT-PCR, SB= slot blot analysis, W= Western blot analysis

2.5 The extracellular matrix and the nervous system

The importance of the extracellular matrix and of cell-matrix and cell-cell adhesion in the development of both the peripheral (PNS) and the central nervous (CNS) systems has been established through both *in vivo* and *in vitro* experiments (De Arcangelis & Georges-Labouesse 2000, Novak & Kaye 2000, Müller *et al.* 1995, Letourneau *et al.* 1994). The extracellular matrix of the nervous system is rich during developmental events such as differentiation, survival and migration of neurons and also in axonal and dendritic elongation and synaptic formation, but is substantially reduced in the mature tissue (Letourneau *et al.* 1994, Venstrom & Reichardt 1993). A number of matrix molecules, such as fibronectin, vitronectin, tenascin, thrombospondin, several laminins, collagens and proteoglycans, which are also found in the extracellular matrix of other tissues, are expressed in the nervous system. It is believed that the glial cells produce most of the extracellular matrix in the nervous system, although some molecules are also produced by neurons (Letourneau *et al.* 1994, Venstrom & Reichardt 1993). Some matrix molecules, such as S-laminin, agrin and F-spondin, have initially been found in the nervous system and are quite specific to this site (Letourneau *et al.* 1994). Many of the classical cell-matrix and cell-cell receptors, such as those of the integrin, cadherin and immunoglobulin families, are expressed on both neurons and glial cells (Müller *et al.* 1995, Venstrom & Reichardt 1993, De Arcangelis & Georges-Labouesse 2000). Another important group of cell-bound receptors in the matrix and other cells, consists of the membrane-bound proteoglycans (Müller *et al.* 1995). The traditional view of the extracellular matrix is that it functions as an adhesive scaffold to which neurons can adhere during migration and elongation but it is now clear that the matrix also has anti-adhesive tasks, regulates the information brought to neurons by sequestering different molecules and takes part in signalling events. Signals from the extracellular matrix are thus transduced through the membrane-bound receptors and finally modulate the same intracellular second messenger pathways as are used by growth factors, hormones and neurotransmitters (Letourneau *et al.* 1994, Venstrom & Reichardt 1993).

2.6 Transgenic mouse models for collagen gene function

With the development during the last decade of technologies that enable the manipulation of genomes in model animals, this approach has increasingly been adopted for studying the functions of genes. The goal is to generate models that can be of help for elucidating the pathological processes of diseases and also allowing testing of therapies. Transgenic animals are also useful for studying the physiological role of gene products. Mutations in collagen genes are known to cause many severe heritable disorders in humans for which there are currently no known curative therapies (Kivirikko 1993, Myllyharju & Kivirikko 2001, Prockop & Kivirikko 1995). Several transgenic mouse strains with mutations in collagen genes have been generated and will be discussed in the following chapter. Even before the man-made mutations had been generated, however, mouse strains with spontaneous mutations of collagen genes were described. The mouse lines *oim, Dmm* and *cho* contain mutations of the collagen genes for α2(I), α1(II) and α1(XI), respectively,

and these have proved valuable for studying the human genetic disorders osteogenesis imperfecta, chondrodysplasia type III, Stickler syndrome and the lethal form of chondrodysplasia (Brown *et al.* 1981, Chipman *et al.* 1993, Li *et al.* 1995b, Pace *et al.* 1997, Seegmiller *et al.* 1971).

2.6.1 *Transgenic mice expressing mutant collagen α-chains*

The first wave of transgenic mice was generated by the microinjection technique, where a transgene construct harboring the mutation is injected into fertilized mouse oocytes, with subsequent random incorporation into the genome of the embryo (Aszodi *et al.* 1998, Hogan *et al.* 1986). The transgene construct is thus expressed in the mouse together with the endogenous molecule. A disadvantage of this technique is that the site of integration cannot be controlled and thus it is possible that the integration of the transgene may cause inactivation of a gene which could interfere with the analysis of the phenotype of the mouse. The phenotype must therefore be found in several independent transgenic lines in order for the observed changes to be considered as caused by the mutation. Another problem is the genetic analysis of the mice. Several copies of the transgene are usually integrated, and the levels of expression of the transgene may thus vary between the lines and between the individuals within a line, all of which blurs the concepts of heterozygosity and homozygosity.

Microinjection technology has proved to be valuable in collagen research, however, the as the mutant α-chains usually associate stochiometrically with the endogenous normal α-chains. This leads to defects in the formation of the triple helical collagen molecules, as trimers containing mutant molecules are either degraded or are not functionally normal, causing a dominant negative effect (Jacenko *et al.* 1993, Kivirikko 1993, Prockop & Kivirikko 1995) (Figure 2). In fact, for some collagens the phenotypes of mice generated by the microinjection technique are far more severe than those of mice with a targeted inactivation of the same gene (Jacenko *et al.* 1993, Kwan *et al.* 1997, Rosati *et al.* 1994), and they often mimic the consequences of collagen gene mutations observed in human diseases (Myllyharju & Kivirikko 2001). Mouse strains that express mutant forms of collagen and the observed phenotypes are listed in Table 2.

Expression of mutated collagen α-chains

Disturbance of triple helix formation   Conformational change of collagen molecule

Normal α-chain

Truncated α-chain

Normal α-chain

Procollagen suicide

Abnormal fibrils
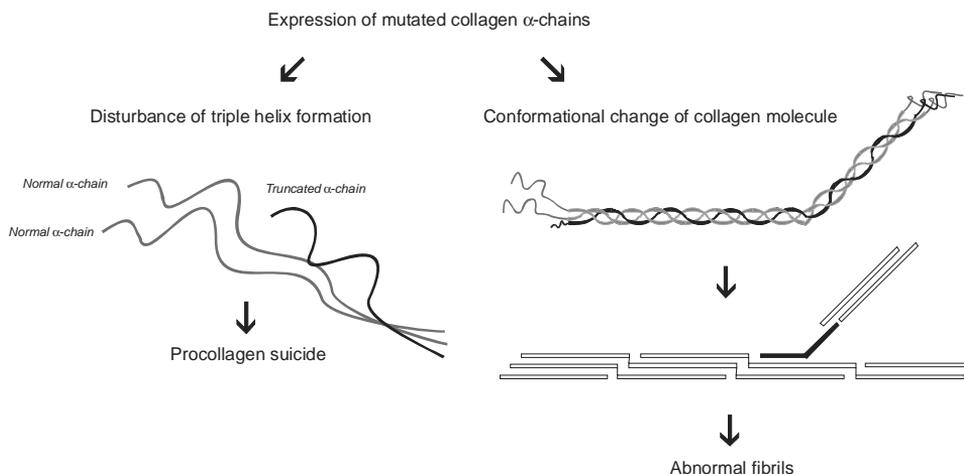
Fig. 2. Dominant negative effects of collagen mutations. A mutation can cause a defect in triple helix formation leading to procollagen suicide. If the mutant α-chain can be incorporated into the collagen molecule, the mutation may cause abnormalities in the structure of the fibril or other supramolecular structures, leading to functional defects. The figure is modified from that presented in reference Prockop & Kivirikko 1995, and is reproduced here with permission from the editor.

2.6.2 *Transgenic mice with targeted mutagenesis of collagen genes*

The microinjection technique was followed by the development of methods that made the targeted mutations of genes possible (Seidman 1999). This method based on homologous recombination of DNA has made it possible to direct the mutation to the right locus in the mouse genome and subsequently avoid the major disadvantages of the microinjection technique. The development of gene targeting made it possible to generate mice with inactivation of genes, i.e. knockout mice. A recent evolution in this technology is the generation of specific gene activation at certain time points or selected tissues only, i.e. conditional mutagenesis. Also possible, but so far less used, is the generation of point mutations and other minor mutations, excluding large deletions, by targeted mutagenesis (Seidman 1999). Several collagens have been mutated by targeted inactivation of their genes, the phenotypes of the resulting transgenic mice being listed in Table 2.

Table 2. Transgenic mouse models generated for collagen genes

Collagen type	Dominant negative mutation	Targeted mutation	Phenotype	Reference
Fibrillar collagens				
α1(I)	Insertion		Fetal lethal, vascular defect (H), bone defect, hearing loss (L)	Jaenisch *et al*. 1983, Bonadio *et al*. 1990
α1(I)	Gly to Cys		Lethal, respiratory distress, bone defect	Stacey *et al*. 1988
α1(I)	In-frame deletion		Perinatal lethal, respiratory distress, bone defect (H), fibrils with reduced collagen (L)	Khillan *et al*. 1991, Pereira *et al*. 1993
α1(II)	In-frame deletion		Perinatal lethal, respiratory distress, severe chondrodysplasia (H)	Vandenberg *et al*. 1991
α1(II)	Gly to Cys		Perinatal lethal, respiratory distress, severe chondrodysplasia (H)	Garfalo *et al.* 1991
α1(II)	In-frame deletion		Perinatal lethal, respiratory distress, lethal chondrodysplasia (H)	Metsäranta *et al.* 1992
α1(II)	Over-expression		Perinatal lethal, respiratory distress (H), abnormal cartilage	Garfalo *et al.*1993
α1(II)		Gene inactivation	Perinatal lethal (H), endoskeletal defects, lack of bone marrow	Li *et al.* 1995
α1(III)		Gene inactivation	Viable (10%), short lifespan, blood vessel rupture, reduced fibrillogenesis (H)	Liu *et al.* 1997
α2(V)		Exon 6 deleted	Perinatal lethal (>95%), defects in ligaments, skin, eyes (H)	Andrikopoulos *et al.* 1995
Non-fibrillar collagens				
α3(IV)		Gene inactivation	Postnatal lethal, renal failure (H)	Cosgrove *et al* 1996, Miner *et al.* 1996
α3-4(IV)	Insertion		Postnatal lethal, early renal failure (H)	Lu *et al.* 1999
α1(VI)		Gene inactivation	Viable, myopathy (H&L)	Bonaldo *et al.* 1998
α1(VII)		Gene inactivation	Postnatal lethal, skin defects (H)	Heinonen *et al.* 1999
α1(IX)	In-frame deletion		Viable, mild dwarfism (H), osteoarthritis (H&L), eye defects (H)	Nakata *et al.* 1993
α1(IX)		Gene inactivation	Viable, cartilage degeneration (H)	Fässler *et al.* 1994
α1(X)	In-frame deletion		Viable, 25% perinatal lethal, skeletal deformities, growth plate compression, haematopoetic defects (H)	Jacenko *et al.* 1993, Gress & Jacenko 2000
α1(X)		Gene inactivation	Viable, 11% perinatal lethal, minor skeletal defects, haematopoetic defects (H)	Kwan *et al.* 1997, Rosati *et al.* 1994, Gress & Jacenko 2000
α1(XII)	In-frame deletion		Viable, disruption of periodontal and skin matrix structure	Reichenberger *et al.* 2000
α1(XIII)	In-frame deletion		Fetal lethal, cardiovascular and placental defects (H), tumor formation (L)	Sund *et al.* (II), Sund *et al.* (III)
α1(XIII)		N-terminus altered	Viable, progressive myopathy (H)	Kvist *et al.* 2001
α1(XV)		Gene inactivation	Viable, mild myopathy, cardiovascular defects (H)	Eklund *et al.* 2001
α1(XVIII)		Gene inactivation	Viable, eye defects (H)	Fukai *et al.* submitted*

(H) indicates high levels of transgene expression and/or homozygous for the mutation, (L) indicates low levels of expression and/or heterozygous for the mutation,
*Fukai N, Eklund L, Li Q, Oh SP, Tamarkin L, Li E, Pihlajaniemi T & Olsen BR: Lack of collagen XVIII/ endostatin results in angiogenesis and matrix abnormalities in the eye. Submitted.

2.7 Transgenic mice with developmental defects due to abnormal cell adhesion proteins

During development cell adhesion molecules are involved in cell differentiation, migration and tissue morphogenesis (DeSimone 1994, Gumbiner 1996, Hynes 1992, Lin & Bissell 1993, Takeichi *et al.* 1993). In order to further analyze the function of individual genes and proteins in the organism, several transgenic strains involving cell adhesion molecules have been generated. Some of the results have been very severe, causing pre-implantation and peri-implantation mortality of the embryo, as in the case of E-cadherin, β1 integrin, E-catenin and γ1-laminin (Fässler *et al.* 1994, Haegel *et al.* 1995, Larue *et al.* 1994, Riethmacher *et al.* 1995, Stephens *et al.* 1995, Smyth *et al* 1999), but other molecules shown previously to have central roles in early developmental processes surprisingly survived mutagenesis without any obvious defects or at least reached a much later stage of development than had been anticipated. Plausible explanations for this may be compensation by other molecules or overlapping functions between closely related genes (Hynes 1996). Also, the resulting gene mutation can sometimes cause an unexpected phenotype in the mouse compared with the disease mutations found in man or else a phenotype may be generated that is milder than excepted (Aszodi *et al.* 1998, Hynes 1996).

The phenotypes of transgenic mice with mutations of the molecules involved in cell adhesion are discussed in more detail in the following. The focus is on gene mutations that cause defects in placentation, cardiogenesis and angiogenesis and are thus similar to the phenotypes found in the type XIII collagen COL2 deletion mouse lines. Cell adhesion molecules are defined here as molecules involved in some portion of the functional unit of cell adhesion, i.e. either cell adhesion receptors or their extracellular matrix ligands and intracellular ligands (Gumbiner 1996).

2.7.1 Placentation and its defects in transgenic mice

Mammalian implantation and placentation involves a complex process whereby the developing embryo physically becomes linked to its mother. This is a prerequisite for continued pregnancy, as a vascular link must be formed between the embryo and the mother to allow continued development and growth of the fetus (Cross *et al.* 1994). Maternal tissue is invaded, maternal structures are morphologically modified and the rejection caused by the maternal immune defense system is circumvented in order to form the placenta which in turn enables the exchange of gases and nutrients during pregnancy (Aplin 1991, Cross *et al.* 1994). The complexities of these processes are underlined by the large number of human pregnancies that are spontaneously aborted in the early stages and the in the development of pre-eclampsia, a disease thought to be caused by defects in placental formation and function (Cross *et al.* 1994).

The development of a hemo-chorial placenta, where a direct contact is formed between the trophoblasts derived from the fetus and the maternal blood, is intended to enable the exchange of nutrients and gas (Cross *et al.* 1994). Although similar, the structures of the

placenta are not identical between species, and the principle described in the following is that of murine placentation. Briefly, as reviewed by Cross *et al.* 1994, the initial implantation of the embryo (4.5-6 dpc) is followed by a process in which the polar trophoectoderm forms the ectoplacental cone and subsequently becomes the spongiotrophoblast layer of the mature placenta. The extreme peripheral trophoblasts are transformed into trophoblastic giant cells, which form a distinct border to the maternal decidua of the mouse placenta. Before the formation of the mature placenta, initial nutritional adsorption (6-8 dpc) and placental function is accomplished by means of the parietal yolk sac, formed by the parietal endodermal cells, Reichert's membrane and the trophoblast giant cells. During gastrulation, this structure becomes lineated by mesodermal tissue, thus forming the visceral yolk sac, in which the first fetal vascular structures, the blood islands and the vitelline circulation, are formed. The development of the early placenta, i.e. the development of the yolk sac and the vitelline circulation, is dependent on the functioning of a number of molecules involved in cell adhesion. The early fetal lethality in mice that lack $\alpha5$ integrin or its ligand fibronectin, N-cadherin and focal adhesion kinase (FAK) is at least partly attributable to the disassociation of endodermal and mesodermal portions of the yolk sac (Furuta *et al.* 1995, George *et al.* 1993, Georges-Labouesse *et al.* 1996, Radice *et al.* 1997, Yang *et al.* 1993).

With increasing growth and development of the fetus (by 9 dpc), the demand for nutrients cannot be met by the yolk sac, so that the labyrinth trophoblast layer and the mature placenta are formed through the fusion of chorion and allantois. This layer enables the fetal vessels derived from the allantois to come into close contact with the maternal blood lacunae generated through the invasion of trophoblasts into the maternal structures, thus allowing effective nutrient and gas exchange to continue until birth (Cross *et al.* 1994). Several mouse strains with mutations affecting adhesive proteins display defects in the process of chorio-allantois fusion. Mice lacking either $\alpha4$ integrin or its counter receptor VCAM-1 display a defect in the fusion process, leading to abortion of the majority of the offspring (Gurtner *et al.* 1995, Kwee *et al.* 1995, Yang *et al.* 1995). In a subset of the VCAM-1 mutants the initial chorioallantoic fusion takes place but subsequent defects in the spread of the allantois over the chorion and minimal invasion of the trophoblasts cause defects in placental function and increased mortality (Gurtner *et al.* 1995). A subset of the FAK mutants that survive the above yolk sac defect also display a problem in chorio-allantois fusion (Furuta *et al.* 1995).

For the placenta to function properly, the invasion of the trophoblasts into the maternal structures needs to be thorough enough and the compartment in which the nutrient and gas exchange takes place correctly developed, with close interaction between the maternal and fetal vasculatures. In some transgenic mice generated for the study of cell adhesion molecules, the placental defect is found in the principal area of exchange, namely the labyrinth layer. Mice lacking $\beta3$ integrin, αv integrin and laminin $\alpha5$ all display either a compact or a thin labyrinth layer, which will most likely lead to functional defects and the increased mortality observed in the offspring (Bader *et al.* 1998, Hodivala-Dilke *et al.* 1999, Miner *et al.* 1998). In the case of $\beta3$ integrin, however, maternal intrauterine bleeding also affects the survival of the offspring (Hodivala-Dilke *et al.* 1999). Mouse mutants lacking plakoglobin were also observed to have small placentas upon dissection from the uterus, but this defect was not characterized further (Bierkamp *et al.* 1996)

2.7.2 Cardiovascular development and its defects in transgenic mice

The other major defects that can cause midgestational fetal mortality apart from placentation are disorders in the cardiovascular system. Even gross malformations in organ systems such as the kidneys, liver or CNS, tend to cause rapid perinatal rather than fetal lethality (Cross *et al.* 1994, Rossant 1996). The development of the cardiovascular system involves the complicated process of cardiogenesis and vascularization inherent in vasculogenesis and angiogenesis. In highly simplified terms, cardiogenesis takes place through the migration of two separate clusters of cells in the splancnic mesoderm to a midline position on the ventral side of the fetus, where they subsequently fuse to form a single tube of contracting epimyocardium lined by an endocardium and the unfused ends of the tube, forming the future inflow and outflow tracts of the heart. This is followed by asymmetrical elongation and looping, to form an S-shaped structure with one atrium and one ventricle, and septation, whereby the atria and the ventricles become separated by the endocardial cushion. The walls between the right and left sides develop through growth from the periphery towards the cushion tissue, thus forming the four-chambered structure of the mammalian heart, followed by trabeculation and compactation of the ventricles and envelopment of the heart by the epicardium (Gilbert 1997, Moorman & Lamers 1999). In vascularization, vasculogenesis involves the formation of blood vessels and blood cells directly from the mesoderm (Pardanaud *et al.* 1989, Cleaver & Krieg 1999), while in angiogenesis new blood vessels are formed by sprouting from earlier ones (Cleaver & Krieg 1999, Gilbert 1997, Sariola 1985, Wilson 1983).

Several cell-cell adhesion molecules and molecules involved in cell-matrix adhesion have displayed fetal lethality in transgenic mice due to defects in the formation of a functioning cardiovascular system. Mice lacking fibronectin, α5 integrin, N-cadherin and FAK all display severe mesodermal defects affecting cardiogenesis. Even though initial commitment to the cardiac lineage may take place in these mice, the subsequent formation of a functional tube-like heart is altered, and this combined with problems of vasculogenesis in the large vessels and the yolk sac vasculature leads to the death of the fetuses (Furuta *et al.* 1995, George *et al.* 1993, Georges-Labouesse *et al.* 1996, Radice *et al.* 1997, Yang *et al.* 1993). It is not known whether the fetuses succumb to cardiac defects or the previously described placental defects.

Some mice with mutations of cell adhesion genes initially display normal cardiogenesis, until the developing fetus becomes dependent on heart function, whereupon the defects become evident. Mice lacking the intercalated disc component vinculin form a tube-like heart that is unable to contract, leading to fetal lethality by day 10 dpc (Xu *et al.* 1998). Mice lacking plakoglobin display an absence of desmosomes (Bierkamp *et al.* 1996, Ruiz *et al.* 1996) in the myocardium. A fused form of desmosomes and adherens junctions is formed that evidently is not capable of establishing the required strength in the adhesions between the cardiomyocytes, and such fetuses subsequently die by 12.5 dpc (Bierkamp *et al.* 1996). In the VCAM-1 and α4 integrin mutants, detachment of the epicardium from the myocardium leads to blood leakage from the developing coronary vessels into the pericardial cavity (Kwee *et al.* 1995, Yang *et al.* 1995), and in the VCAM-1 mutants a less compact myocardial layer was observed as well (Kwee *et al.* 1995). A leaky heart due to holes in the myocardium was found in the mice lacking

perlecan, underlining the requirement for a stable basement membrane to ensure proper heart function (Costell *et al.* 1999).

Death due to ruptured blood vessels is observed in fetal transgenic mice with a retrovirus insertion in the first intron of the collagen α1(I) gene, leading to a complete transcriptional stop of the gene (Harbers *et al.* 1984, Lohler *et al.* 1984, Schnieke *et al.* 1983, Jaenisch *et al.* 1983), while mice with targeted inactivation of VE-cadherin gene or expressing a truncated molecule are aborted by 9.5 dpc as abnormal endothelial structures of the heart and blood vessels prevent further development (Carmeliet *et al.* 1999). Mice lacking αv integrin display not only placental defects but also defects in angiogenesis, with abnormally distended and leaky vessels in the central nervous system (Bader *et al.* 1998). Interestingly, half of the mice lacking α7 integrin also appeared to succumb during the intrauterine period, although no analysis of the cause of their death has yet been published (Mayer *et al.* 1997)

2.8 Tumor formation

Malignant transformation is a complicated process involving multiple steps. First, a single transformed cell clone manages to bypass its programmed death and grows independent of growth factors and growth restraint signals. These cells then escape the immunological surveillance of the organism and grow into a cell mass where the vasculature needed for continued growth is generated. The cell mass then breaks down the surrounding tissue and spreads to other parts of the body in the process of metastasis. Solid tumors are benign if they display only local growth, whereas malignant tumors are indicated by invasion of other tissues and metastatic growth. Lymphomas and angiosarcomas are malignant tumors of the peripheral lymphatic tissue and the endothelial cells, respectively (Franssila 1992, Robbins *et al.* 1999b).

2.8.1 *Lymphoma and angiosarcoma in man*

Malignant transformation of the lymphatic cell population is called leukemia if the transformed cells are leukocyte precursors of the bone marrow, the malignant cells are found in the bone marrow and they subsequently appear in the circulating peripheral blood as the disease progresses (Jandl 1987, Robbins *et al.* 1999a). A malignantly transformed lymphatic cell in a primary peripheral location is called a lymphoma, however, although the clinical distinction between the two is sometimes remarkably difficult to make (Jandl 1987, Robbins *et al.* 1999a, Teereenhovi & Sauvo 1992). Lymphomas are traditionally divided into Hodgkin and non-Hodgkin lymphomas (NHL) based on the occurrence of a neoplastic giant cell, the Reed-Stenberg cell, typical only of Hodgkin lymphomas. Approximately 20% of all lymphomas are of the Hodgkin type (Jandl 1987, Robbins *et al.* 1999a, Teereenhovi & Sauvo 1992). The most common location for both Hodgkin and non-Hodgkin lymphomas are the lymphatic tissues such as the spleen, thymus, lymph nodes, tonsils and Waldeyer's ring in the pharynx although

they are also found at more uncommon locations, of which the gastrointestinal tract is most frequently affected (Young 1999, Otter *et al.* 1989).

Several methods have been developed for classifying the highly diverse group of cancers making up the NHLs. The difficulties encountered in creation a classification system that would be useful both in clinical practice and for organizing the disease subgroups theoretically, can be considered to reflect the complexity of the immunological system and its malignancies. REAL (the Revised European-American classification of Lymphoid neoplasms) has become the most widely used model in Europe and the USA in the recent years (Harris *et al.* 1994), and the recently published World Health Organization classification of malignancies of the haematopoietic and lymphoid tissues is based on this (Harris *et al.* 1999). Basically, REAL divides malignancies into B- and T-cell-derived ones and further into those of precursor or peripheral cells. The vast majority of NHL lymphomas in humans, 80-85%, are of B-cell origin, while only a minority of these cancers, 15-20%, affect the T-cells (Jandl 1987, Robbins *et al.* 1999a, Teereenhovi & Sauvo 1992).

NHL was the sixth most common cancer form among men in Finland in 1995, and the fifth most common among women (Finnish Cancer Registry 1997). For unknown reasons, the incidence of NHL has been increasing from the 1960s onwards (Morgan *et al.* 1997). The known causes of the various forms of NHL include radiation, virus infections and environmental pollution (Jandl 1987, Robbins *et al.* 1999a, Teereenhovi & Sauvo 1992). A number of chromosomal alterations typical of different human NHLs have been described, most of which cause the continued expression of an oncogene or the lack of expression of a tumor suppressor gene, ultimately leading to malignant transformation of the lymphatic cell population (Jandl 1987). The chromosomal alterations can be used to evaluate the response to therapy and subsequently the prognosis for the disease (Jandl 1987).

Therapeutic interventions for lymphomas include chemotherapy in combinations with radiotherapy and/or bone marrow or peripheral stem cell transplantation, and biotechnologically engineered remedies have broadened the available therapeutic arsenal in the recent years. Even though progress has been made in this field and there are large differences in mortality rates between the disease subgroups, the number of patients with NHLs who are cured remains low (Jandl 1987, Robbins *et al.* 1999a, Teereenhovi & Sauvo 1992).

Angiosarcoma is a malignant transformation of endothelial cells in either blood vessels or lymphatic vessels. Tumors can be located in various tissues, with a preference for the skin, soft tissues, breasts and liver. The overall occurrence of sarcomas is rare, as these tumors make up about 1% of all new cancers recorded in Finland per year (Blomqvist & Asko-Seljavaara 1992, Jandl 1987). Chemical carcinogens such as arsenic and polyvinyl chloride (PVC) have been shown to be causes of hepatic angiosarcoma. The therapeutic interventions used consist of chemotherapy, which can be combined with radiotherapy in certain cases. Angiosarcoma, like all forms of sarcoma, is a disease with a poor prognosis and low survival rates (Blomqvist & Asko-Seljavaara 1992, Schoen 1999).

2.8.2 Lymphoma and angiosarcoma in transgenic mice

The malignant potential of several oncogenes has been verified in transgenic mice by expressing the oncogene under a promoter that directs the expression of the transgene to different cell populations (Adams *et al.* 1999, Adams & Cory 1991, Seldin 1995, Yee *et al.* 1989). Several mouse mutants have developed lymphomas, which, as in man, mostly tend to be of the B-cell type. Apart from the traditional oncogenes, T-cell lymphomas have also been observed in mice that overexpress a mutant form of the intracellular enzyme PI3-kinase (Borlado *et al.* 2000) and in a Notch-3 transgenic mouse, in which overexpression of the intracellular domain of the protein creates a situation of ligand-independent signalling (Bellavia *et al.* 2000).

Mice lacking the tumor suppressor gene p53 display both angiosarcomas and lymphomas (Donehower *et al.* 1992). Penetration of the tumor frequency was induced in the PI3-kinase mutant mice by the crossing into a p53 negative background, indicating partly overlapping pathways for these proteins (Borlado *et al.* 2000). Interestingly, the phenotype of the p53 transgenic mice is reminiscent of the human cancer-susceptibility Li-Fraumeni syndrome (Donehower *et al.* 1992).

Endothelial tumors have also been observed in mice that are chimeric for the expression of the middle T virus oncogene of the polyoma virus by retrovirus insertion and also in mice overexpressing the human herpesvirus-8 chemokine receptor (Williams *et al.* 1988, Yang *et al.* 2000).

3 Outlines of the present research

When the work for this thesis was initiated the cDNA-derived primary structure of type XIII collagen had been elucidated and some information existed on its tissue distribution existed. There was no information regarding its function, however. Previous work with several other collagen genes had indicated that overexpression of mutant collagen in transgenic mice can be used to mimic disease phenotypes and consequently yield information about the functions of these collagens. Thus the aim was to study type XIII collagen by generating transgenic mice that overexpress mutant molecules. Due to the necessity for type XIII collagen during mouse fetal development, the characterization of its expression during this period became a necessary and logical part of the work.

The expression of type XIII collagen during mouse development was characterized at the levels of gene expression and translation. The expression of type XIII collagen by neurons was analyzed and its effects on neurite outgrowth was established. Similarly, the effects of the overexpression of type XIII collagen with a 90 amino acid in-frame deletion mutation of the COL2 domain in transgenic mice were analyzed. The results obtained from the transgenic mice point to the importance of type XIII collagen for growth and differentiation both during fetal development and in the malignant transformation of certain mesenchymal cell populations.

4 Materials and methods

4.1 Experimental animals (I, II, III)

Mice of the NMRI strain were used to study type XIII collagen expression during fetal development. Neuronal cultures were established from the brains of fetuses from rats of the Wistar strain, and the COL2del transgenic founder mice were bred into the B6D2F1 hybrid strain in order to establish the transgenic mouse lines.

4.2 Preparation of tissues (I, II, III)

For the analysis of fetuses mice were allowed to mate overnight. Pregnancy was detected by the appearance of a vaginal plug the following morning and this time point was designated as day 0.5 of development. The fetuses were dissected from the pregnant females between days 8.5-17.5 of development. Lung and muscle tissues from 1-month-old mice and heart tissues from 15-day-old mice were collected for analysis. The following tissues were collected for RNA extraction and/or histological and immunohistochemical analysis: cartilage, skin, brain, heart, liver, lung, intestine, kidney, spleen, thymus, lymph nodes, skeletal muscle, uterus, placenta and tumor tissue. Samples for frozen sectioning were embedded in Tissue Tec® compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen, and those for paraffin sectioning were fixed in 10% buffered formalin and embedded in paraffin. The samples for RNA extraction were rapidly frozen in liquid nitrogen and stored at -70°C until used.

4.3 In *situ* hybridization (I, II)

A 720 bp fragment corresponding to nucleotides 1419-2139 of the mouse type XIII collagen cDNA (Hägg *et al.* 1998) was cloned into the vector sP72 (Stratagene, La Jolla, Ca) and used to generate probes for *in situ* hybridization. The clone JA-2 was linearized

by *Bam* HI and *Hind* III digestion to generate sense and antisense digoxygenin-11-UTP (Roche Molecular Biochemicals, Germany) labeled RNA probes, respectively, by *in vitro* transcription (Promega, Madison, Wi). Frozen sections 10 μm thick and paraffin sections 4μm thick were cut from day 11.5 and 16.5 whole fetal specimens and placentas onto Super Frost Plus slides (Menzel Gläser, Germany). The paraffin sections were allowed to dry for 1 hour at 50°C, dewaxed with xylene and dehydrated. Both the paraffin and frozen sections were initially fixed in 4% paraformaldehyde in PBS, washed and incubated in 0.2M HCl followed by washing in DEPC-H$_2$O. The frozen sections were deproteanized with 0.1μg/ml Proteinase K (Roche Molecular Biochemicals, Germany) in PBS with 1mM CaCl$_2$, and the paraffin sections with 10μg/ml proteinase K followed by washing in 0.3% glycine in PBS. The sections were acetylated in 0.25% and 0.50% acetic anhydride in 0.1M triethanolamine and balanced for 15 minutes in 10mM Tris-HCl, 10mM NaPO$_4$, 5mM EDTA and 300mM NaCl, pH 6.8. Prehybridization of the sections was performed with a hybridization mixture consisting of 10mM Tris-HCl, 10mM NaPO$_4$ pH 6.8, 5mM EDTA, 300 mM NaCl, 19mM DTT, 50% formamide, 10% dextran sulphate, 1x Denhardt's solution, 1 μg/ml yeast tRNA and 0.25 μg/ml herring sperm DNA, alone for 2 hours at 50°C in the case of the frozen sections and at 60°C for the paraffin sections, followed by hybridization with either sense or antisense probes (400ng/ml) at 50°C for the frozen sections and 60°C for the paraffin sections overnight. The sections were subsequently washed in 50% formamide for 15 minutes, 2XSSC for 15 minutes and in 0.1X SSC four times for 15 minutes at the hybridization temperature and 0.1X SSC for 5 minutes at RT. Immunological detection was performed by incubation of the slides in buffer 1 (0.1M Tris-HCl and 0.15M NaCl pH 7.5), three times for 5 minutes at RT, blocking for 30 minutes in buffer 1 containing 0.1% Triton X-100 and 2% fetal calf serum, incubation with alkaline phosphatase (AP)-conjugated anti-digoxygenin antibody diluted in buffer 1 containing 0.1% Triton X-100, 1% fetal calf serum and 1% blocking reagent (Roche Molecular Biochemicals, Germany). This was followed by washing in buffer 1 three times for 5 minutes, and incubation in 0.1M NaCl and 0.05M MgCl$_2$ pH 9.5 for 10 minutes at RT. The color reaction was performed using Fast Red tablets (Roche Molecular Biochemicals, Germany) according to the manufacturer's protocol and stopped by incubation in 10mM Tris-HCl and 1mM EDTA, pH 8.0, for 10 minutes, after which the slides were dipped in 0.1M Na-acetate-buffer, pH 5.2, counterstained with methyl green, washed in tap water and mounted with Kaiser's glycerol gelatin (Merck, Germany).

4.4 Primary cell cultures (I, II)

4.4.1 *Neuronal cultures and antibody detection of type XIII collagen in primary cultured neurons*

Hippocampi were dissected from 18-day-old rat fetuses and the cells were isolated by trituration in Hanks' balanced salt solution (HBSS) (GibcoBRL, Rockville, Md) (Brewer

et al. 1993). The neurons were washed with HBSS, suspended in Neurobasal medium (GibcoBRL, Rockville, Md) supplemented with 2% B27 (GibcoBRL, Rockville, Md), 25uM L-glutamic acid (Sigma Gmbh., Germany) and 1% L-glutamine (GibcoBRL, Rockville, Md), and plated at a density of 50 000 cells/well on 8-chamber slides (Nunc Lab-Tek, Denmark) coated with heparin binding growth associated molecule (HB-GAM) (20ug/ml) or laminin (20ug/ml). The neurons were cultured for 5 days, fixed with 4% paraformaldehyde-0.05% glutaraldehyde for 20 minutes, permeabilized with methanol for 5 minutes and incubated with type XIII collagen (Hägg *et al.* 1998) and neurofilament (Boehringer-Mannheim, Germany) antibodies for 90 minutes at room temperature, followed by incubation with TRITC-conjugated polyclonal goat anti-rabbit and FITC-conjugated polyclonal goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pa).

4.4.2 Neurite outgrowth assay

Microtitre wells (Greiner Gmbh., Germany) were coated with purified recombinant laminin (Sigma Gmbh., Germany), HB-GAM (Raulo *et al.* 1992), BSA and the ectodomain of recombinant type XIII collagen (Nykvist *et al.* 2000) at a concentration of 20 μg/ml overnight at +4°C. They were then washed twice with water and blocked with 10 mg/ml of BSA in Neurobasal medium for 2 hours at room temperature. Hippocampal neurons were plated onto the wells and cultured in Neurobasal medium for 4 days. The cells were fixed and stained with Toluidine blue and visualized by phase contrast microscopy.

4.4.3 Mouse fetal fibroblast cultures

Fetuses from heterozygote COL2del mating were dissected from the uterus on day 11.5 of development. The head, heart and liver were removed and the remaining tissue was cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Rockville, Md) supplemented with 10% heat-inactivated fetal calf serum and non-essential amino acids.

4.5 Northern analysis (I)

The Mouse Embryo Multiple Tissue Northern Blot (Clontech, Palo Alto, Ca) was prepared by gel electrophoresis of 2μg of mouse fetal poly (A)+ RNA isolated at different stages of development (days 7, 11, 15 and 17) and hybridized with a 489 bp *Pst* I cDNA fragment recognizing the NC1 domain of the mouse type XIII collagen molecule (Hägg *et al.* 1998). The RNA loading of the blot was controlled by hybridizing it with a probe recognizing β-actin. The hybridization and washing conditions suggested in the manufacturer's protocol were used.

4.6 RNA extraction and RT-PCR of endogenous and transgenic type XIII collagen mRNA (I, II, III)

Total RNA was isolated from several mouse tissues by homogenization in 4 M guanidine-isothiocyanate as previously described (Chomczynski & Sacchi 1987). For the reverse transcriptase (RT) reaction, 2.5 µg of total RNA and 150 ng of random oligohexameres (GibcoBRL, Rockville, Md) were annealed at 70°C for 10 minutes and then incubated on ice. The RT reaction with 200 units of the M-MLV RT enzyme (GibcoBRL, Rockville, Md) was carried out at 42°C for 50 minutes followed by treatment of the products with two units of RNAseH (GibcoBRL, Rockville, Md) at 37°C for 20 minutes. For the PCR reaction, 2 µl of the RT reaction product was used with the primers RTpcr1 5'-GATGCTGCCATTATAATCCACCATCTC-3'   and   RTpcr2   5'-CCTAAAGGGGAACAAAGTCAGACTGGC-3'. These generate a 567 bp fragment from the endogenous type XIII collagen transcript and a 297 bp fragment from the transgene transcript. The PCR was carried out with denaturation for 60 seconds at 94°C, annealing for 60 seconds at 67°C and extension for 60 seconds at 72°C for 30 cycles. The accuracies of the resulting PCR fragments were verified by Southern analysis, probing the blots with a 1.2 kb mouse type XIII collagen cDNA fragment generated by *Kpn* I and *Xba* I digestion of clone 3VPL6 (Sund M., unpublished data).

4.7 Real-time quantitative RT-PCR (I)

Fetal mouse total RNA was isolated as described above and 200ng of it used as a template for a reverse transcriptase reaction primed with oligohexameres. The RT reaction was performed using the Taqman® RT-PCR-Gold kit (Applied Biosystems, Foster City, Ca) according to the manufacturer's protocol, the conditions being 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C. The TaqMan® probe and primers used in the PCR reaction were designed with the Primer Express software (Applied Biosystems, Foster City, Ca) and recognize the NC4 domain of type XIII collagen. The amplicon synthesized was 66 bp in length and the sequence of the probe and primers were; NC4 TaqMan®   probe   5'-CCCGCTGGGAGAAGATGGCTTACC-3',   NC4For   5'-ACCTGGACTAGACGCCCCTG-3' and NC4Rev 5'-TTGTTCCAGCAGCCTTGGACT-3'. The fluorogenic NC4 TaqMan® probe was labelled with the FAM reporter dye at its 5' end and the TAMRA quencher dye at its 3' end, while the probe for the endogenous control recognizing 18S rRNA was labelled with the VIC reporter dye at its 5' end and the TAMRA quencher dye at its 3' end (TaqMan® Ribosomal RNA Control, Applied Biosystems, Foster City, Ca). The PCR reaction was carried out on triplicate samples of the cDNA using the TaqMan® Gold PCR reagent kit (Applied Biosystems, Foster City, Ca) with initiation for 2 minutes at 50°C followed by 10 minutes at 95°C and subsequently denaturation for 15 seconds at 95°C, with annealing and extension for 60 seconds at 60°C, for 40 cycles.

The generated PCR products can be quantified by assessing the accumulation of fluorescence from dyes released from the sequence-specific probes by the Taq

polymerase enzyme during the amplification in each PCR cycle (PE Applied Biosystems 1997). In the linear phase of the PCR reaction the total fluorescence rises over a pre-set fixed threshold level and the parameter C_T (threshold cycle) is defined as the PCR cycle number at which an increase in reporter fluorescence above the threshold level is first detected. The C_T values obtained were plotted against log [RNA] in samples of serially diluted 16-day fetal mouse total RNA (20, 8, 3.2, 1.3 and 0.2 ng/µl), and used to generate standard curves for both type XIII collagen and S18. The amounts of RNA in the samples were then estimated from the standard curves. The triplicate PCR runs were repeated for five-ten separate RT reactions and the results calculated as mean values of these.

4.8 Site-directed mutagenesis (II)

Point mutations were generated in the mouse type XIII collagen cDNA clone P40, which contains exons 2-41 of type XIII collagen but lacks exons 15 and 31, due to alternative splicing (Peltonen *et al.* 1997). Two separate point mutations were created in the cDNA using the Pharmacia U.S.E.-kit system (Pharmacia Biotech, Sweden). The point mutations both cause a disruption of the collagenous sequence by changing a Glycine to a Cystein in exon 24 of the COL2 domain and a Glycine to a Tryptophan in exon 32 of the COL3 domain. The sequence of the oligonucleotides used to generate the point mutation in the COL2 domain, and subsequently a new *Hind* III restriction site (Hindmut 1; new restriction site underlined and mutant base marked with an asterisk) were: Hindmut1 5'-CCAAAGGGAG<u>AAGCTT</u> GTGTTGATGGCC-3'   and   Hindmutrev1   5'-GGCCATCAACAC<u>A AGCTT</u>CTCCCTTTGG-3'. The oligonucleotides used to generate the point mutation in the COL3 domain, also generating a new *Hind* III restriction site (Hindmut 2; new restriction site underlined and mutant base marked with an asterisk) were: Hindmut2   5'-AGAAAGGAG<u>AAGCTT</u> GGGAGAAAGGCGA-3'   and Hindmutrev2 5'-TCGCCTTTCTCCC<u>A AGCTT</u>CTCCTTTCT-3'. The cDNA clones with the point mutations were named Hindmut1 and Hindmut2, respectively and used for subsequent cloning of the transgenic constructs.

4.9 Cloning of the type XIII collagen COL2 deletion construct (II)

The clone Hindmut1 was used for the subsequent generation of the COL2del transgenic construct. The new *Hind* III restriction site in exon 24 and a *Bam* HI restriction site in exon 18 were used to delete 270 bp of the COL2 domain. The *Bam* HI and *Hind* III digested ends were ligated to the oligonucleotides BHlink 5'-GATCAGGGCTATGGAGA-3' and BHlinkrev 5'-AGCTTCTCCATAGCCCTG-3'. Since only the last 34 bp of the 70-bp exon 2 are included in the Hindmut1 clone, the beginning of the exon was added by ligating genomic sequences 5' of the cDNA clone. A 250 bp genomic DNA fragment containing the 159 bp extreme 3' sequences of the first intron and the first 91 bp of the second exon was generated by PCR using the plasmid 3HA (Sund M., unpublished results) as a template and the primers: PCRmbeg 5'-GA<u>AGATCT</u>GTATGAACTGCCATGCTTTC-3'   and   PCRmend   5'-

CAGTTACATCCTGGAGACATCTTCGGGGC-3'. A *Bgl* II restriction site (underlined in the sequence) was introduced into the 3' sequences of the first intron for further cloning purposes. Digestion of the 250 bp fragment with the *Bgl* II and *Mae* III restriction enzymes created a 213 bp DNA fragment, which was ligated to the 5' end of the Hindmut1 cDNA clone. To drive the expression of the transgene, a 2.5 kb *Bgl* II-digested genomic DNA fragment containing 1 kb of promoter and 5' flanking sequences, the complete first exon and 0.8 kb of the first intron was further cloned to the 5' end of the cDNA. In order to obtain transcription termination and polyadenylation signals, the SV40 Poly-A DNA fragment was cloned to the 3' end of the cDNA. The sequences of the entire type XIII collagen COL2del construct were verified by manual sequencing. At a later stage, however, a base insertion was detected by automated sequencing at the 3' end of the cDNA, causing a shortening of the COL3 domain by six collagenous repeats and a lack of the NC4 domain.

4.10 Generation of transgenic mice (II)

The COL2del transgene construct was released from the vector by *Spe* I and *Sac* I digestion, purified using the GeneClean II kit (Bio101, France), size fractionated through a 20%-60% sucrose gradient system, ultracentrifuged overnight (30 000 rpm at 15°C) and used for microinjection. Fertilized oocytes from B6D2F1 hybrid females mated with B6D2F1 males were isolated as described (Hogan *et al.* 1986) and the COL2del construct was microinjected into their pronuclei and cultured overnight at 37°C. The surviving zygotes were implanted into the oviducts of NMRI pseudopregnant female mice and the born offspring were analyzed for incorporation of the transgene into their genomes.

4.11 Identification of COL2del transgenic mice (II)

DNA samples extracted from the tails of 14-day-old mice, yolk sacs of fetuses (10.5-15.5 dpc) or fetal tissues (8.5-9.5 dpc) were used to identify the transgenic mice. The tissue samples were incubated overnight at 55°C in Tris-EDTA-SDS (STE) buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl and 0.1% SDS) with 400 µg of Proteinase K (Boehringer Mannheim, Germany) followed by DNA isolation by isopropanole precipitation. Both PCR and Southern hybridization analysis were used to screen the DNA for transgene incorporation. The PCR primers used were: MutScreen2 5'-GGTTTACCGGGGCCTCCTGGACCAAAGGG-3' and MutScreen2rev 5'-GGCCTGCTTGTCCTGTCTCCCCTTTCTCC-3' under conditions of denaturation for 60 seconds at 94°C, annealing for 60 seconds at 60°C and extension for 60 seconds at 72°C for 30 cycles. For Southern blot analysis genomic DNA was digested with *Kpn* I and *Xba* I restriction enzymes and probed with a mouse type XIII collagen cDNA fragment as described in 4.6. The probe recognized a 1.2 kb transgene and a 5.7 kb endogenous gene fragment.

4.12 Analysis of transgenic mice (II, III)

4.12.1 Copy number and Western blotting

The copy number of the integrated COL2del transgene constructs was determined by densitometric analysis, comparing the intensity of the signal for the endogenous type XIII collagen and the transgene when hybridizing Southern blots of *Sph* I-digested genomic DNA from the transgenic mice with a 0.85 kb *Nar* I fragment of the genomic type XIII collagen clone P7 (unpublished results). The probe identifies a 6 kb fragment of the endogenous gene and a 3.5 kb fragment generated from the transgene.

To identify the protein derived from the mutant mRNA Western blotting was performed on cell extracts isolated from fetal mouse fibroblasts. The cells were extracted with a Triton lysis buffer and prepared for electrophoresis as previously described (Hägg *et al.* 1998). Western blot hybridization was performed under reducing and non-reducing conditions and the blots were hybridized with a type XIII collagen antibody recognizing the NC3 domain of the molecule (Hägg *et al.* 1998)

4.12.2 Immunohistochemistry (I, II, III)

For indirect immunofluorescence, 5μm cryosections of whole fetal, placental and tumor specimens were cut onto Super Frost Plus glass slides (Menzel Gläser, Germany). The samples were fixed in precooled methanol or acetone for 10 minutes at -20°C. Unspecific binding was blocked by incubation for 60 minutes at room temperature in 1% bovine serum albumin (BSA) in PBS, pH 7.3. When using a mouse monoclonal antibody on mouse tissues, 2% goat serum (Vectastain, Vector Labs, Burlingame, Ca) and a 1:50 dilution of anti-mouse IgGs (Dako A/S, Denmark) were added to the blocking solution. Primary antibody incubation was carried out overnight at +4°C using the following antibodies and dilutions in 1% BSA-PBS, pH 7.3: anti-type XIII collagen at a 1:50 dilution (Hägg *et al.* 1998), anti-type IV collagen at a 1:100 dilution (Chemicon, Temencula, Ca), anti-neurofilament at a 1:75 dilution (Neomarkers, Fremont, Ca), anti-β1 integrin, anti-CD31 and anti-CD34 at 1:75 dilutions (Pharmingen, San Diego, Ca), anti-vinculin at a 1:50 dilution (Sigma Gmbh., Germany), anti-cadherin at a 1:75 dilution (Zymed, San Fransisco, Ca) and anti-desmoplakin at a 1:50 dilution (Boehringer Mannheim, Germany). After washing in PBS, pH 7.3, CY3 and/or CY2-conjugated secondary antibodies at a 1:300 dilution in 1% BSA-PBS, pH 7.3 or TRITC and/or FITC conjugated secondary antibodies at 1:100 dilution, were added for 60 minutes in the dark. After incubation, the slides were washed in PBS, pH 7.3, mounted with Glysergel (Dako A/S, Denmark) and examined under an epifluorescence microscope or confocal microscope (Leitz Aristoplan, Germany). The control specimens were incubated with the secondary antibody alone and a peptide block was also used for the anti-XIII collagen antibody to ensure the specificity of the detection.

5µm paraffin sections were cut for the histological and immunohistochemical analyses, those for the basic histological analyses being stained with hematoxylin-eosin. The immunohistochemistry of cell lineage markers was performed with the Zymed Histomouse Kit using the AEC chromogen (Zymed, San Francisco, Ca), according to manufacturer's protocol. When necessary the specimens were pretreated by digestion with 0.1% trypsin in PBS for 10 minutes at room temperature and/or heat-induced epitope retrieval by boiling for 20 minutes in 0.01M citrate buffer in a microwave oven. For some antigens a further enhancement of the detection was achieved with the Renaissance TSA™-Indirect kit for immunohistochemistry (NEN Life Science, Boston, Ma). The antibodies and dilutions used were: anti-neurofilament at a 1:75 dilution (Neomarkers, Fremont, Ca), anti-CD31 and anti-CD34 at 1:75 dilutions (Pharmingen, San Diego, Ca), anti-PCNA at a 1:100 dilution (Santa Cruz Biotehnology, Santa Cruz, Ca) anti-desmin at a 1:50 dilution (Sigma, St Louis, Mo), anti-vimentin at a 1:50 dilution (Zymed, San Francisco, Ca), anti-factor VIII at a 1:75 dilution and anti-S100 at a 1:100 dilution (Dako, St Louis, Mo), anti-CD20, anti-CD68, anti-myeoloperoxidase, anti-cytokeratin 7, anti-cytokeratin 8 and anti-smooth muscle actin at 1:75 dilutions (Neomarkers, Fremont, Ca), and anti-CD45 and anti-CD3 at 1:50 dilutions (Oxford Biomarketing, England). To study apoptosis, the TUNEL (Terminal dUTP-biotin Nick End Labeling) assay (Boehringer Mannheim, Germany) was performed according to manufacturer's protocol.

4.12.3 Electron microscopy (II)

For electron microscopy, hearts were dissected from 10.5-day fetuses, and fixed in 2.5% glutaraldehyde and 0.1 M phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon LX112. Thin sections were cut with a Reihert Ultracut E-ultramicrotome (Reichert-Jung, Vienna, Austria) and examined on a Philips CM100 transmission electron microscope (Philips Export B.V., Eindhoven, Netherlands) using an accelerating voltage of 80 kV.

4.12.4 Morphometric analysis of mutant fetuses and placentas (II)

For morphometric analysis of vascular development, the areas of interest on both mutant and control specimen were photographed, ensuring that the region analyzed was as similar as possible between the samples. The number of capillaries or distance between the capillaries and the maternal blood lacunae was measured in 15 squares out of a grid 25-square grid of size $6.25cm^2$ superimposed on the photographs.

4.12.5 Blood count and morphology of peripheral blood cells (III)

Blood samples of 500µl from the orbital sinus from 12-month-old from mutant and control mice were drawn into tubes containing lyophilized EDTA (Terumo Medical Corporation, Elkton, Md) and analyzed by automated cell counting. The peripheral blood cells were evaluated morphometrically by analyzing blood smears on slides under a light microscope.

4.12.6 Flow cytometry (III)

Tumor tissues were homogenized in a Medimachine homogenizer (DAKO, Carpinteria, Ca), and the cells were counted and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, Ca) using the CELL Quest software (Becton Dickinson, Mountain View, Ca). Forward and side-scatter gating was used to exclude dead cells from the analysis. FITC or PE-conjugated monoclonal antibodies against CD2, CD4, CD8a and CD19 (Pharmingen, San Diego, Ca), CD3 (ε chain) and CD20 (Santa Cruz Biotechnology, Santa Cruz, Ca) were used. PE and FITC-conjugated rat and hamster IgG (Pharmingen, San Diego, Ca) were used as controls for immunofluorescence.

4.13 Statistical evaluation of data (I, II, III)

The Mann-Whitney u-test was used to analyze the statistical significance of differences in the rate of expression of type XIII collagen mRNA during development and the defects in angiogenesis in the type XIII collagen COL2 deletion transgenic mice. The statistical significance of the distortion of the expected distribution of genotypes and the occurrence of tumors were analyzed with the chi^2 test.

5 Results

5.1 Type XIII collagen mRNA expression during
mouse development (I)

The rate and pattern of the expression of type XIII collagen mRNAs during mouse development was analyzed by Northern blotting, quantitative RT-PCR and *in situ* hybridization.

In Northern analysis of a blot containing mRNAs from 7, 11, 15 and 17-day-old murine fetuses, low expression of type XIII collagen mRNAs was detected already at day 7 of development and a stronger signal was observed from day 11 onwards, with strongest expression at day 17 (Paper I, Figure 1).

To obtain a more detailed analysis of the rates of expression of type XIII collagen mRNAs at different stages in mouse development, real-time quantitative RT-PCR was performed. The reliability of this method has been demonstrated in several studies (Schoenfeld *et al.* 1998, Wang & Brown 1999, Winer *et al.* 1999). The results at each developmental stage were compared with the expression level at the earliest time point, day 9 of development. The mRNAs were found to be expressed at almost constant levels during development until day 17, where an approximately three-fold increase in expression took place. This statistically significant increase was well in line with the results of the Northern analysis (Paper I, Figure 2 and Table1).

In situ hybridization of digoxygenin-labelled type XIII collagen cDNA revealed abundant mRNA expression in cells of both mesenchymal and epithelial origin. At the earlier stage, day 11.5 of development, the strongest expression was observed in the neuroectoderm of the developing brain and neural tube and in the myocardium of the developing heart. At the later stage, day 16.5 of development, high expression was observed in the chondrocytes of the developing cartilage and in both the resting and proliferative regions of the developing bone. Only moderate expression was observed in the hypertrophic regions of the developing bone, however, signals were observed only in the periosteum of the ossified bone. During late development type XIII collagen mRNAs

were also found in the nervous system, with expression in the peripheral nerves and the brain cells. Both developing cardiomyocytes and the myotubes of the developing skeletal muscle expressed type XIII collagen mRNAs at moderate levels. The basal cells of the epidermis and the epithelium of the intestine and the lungs were also observed to express type XIII collagen mRNAs. Hybridization with the control sense probe resulted only in faint background staining (Paper I, Figure 3).

5.2 Type XIII collagen protein in fetal tissues (I)

The expression of type XIII collagen protein was studied by immunofluorescence detection. As previous studies using mature mouse and human tissues had revealed colocalization of type XIII collagen with vinculin and the β1 integrin subunit in several adhesive structures and type IV collagen along the basement membrane zone (Hägg *et al.* 2001), the localization of type XIII collagen during development was compared with the patterns for vinculin, the β1 integrin subunit and type IV collagen. Antibodies detecting endothelial (CD31 and CD34) and neuronal cells (neurofilament) were also used.

5.2.1 The developing nervous system

The strongest expression of type XIII collagen during mouse development was observed in the developing central and peripheral nervous system. Intense signals were already observed at day 10.5 of development in the neuroepithelium that lines the brain vesicles and in the neural tube. At later stages in development, signals were detected in fibre-like bundles, most probably representing maturing neuronal structures in the brain and spinal cord. A clear staining for type XIII collagen also emerged in the meninges with the maturation of the spinal cord and the brain. As type XIII collagen appeared to be localized in tissues of neural origin, the pattern was highly different from those found for type IV collagen and β1-integrin, which were both localized in the capillaries and the basement membrane structures of the mesenchyme surrounding the brain and spinal cord.

A very intense signal for type XIII collagen was also observed in the developing ganglia, where it was detected in the bodies of the ganglionic cells and in the peripheral nerve bundles that leave the ganglia. Both type IV collagen and β1-integrin were localized to the basement membrane structures surrounding the ganglia.

Intensive detection of the peripheral nerves was observed throughout development, resembling the pattern observed with a neurofilament antibody. Type IV collagen and β1-integrin antibodies, on the other hand, were localized to the epineural and perineural sheaths of the peripheral nerves (Paper I, Figure 4).

5.2.2 Developing heart

Type XIII collagen was already detected in the heart on day 10.5 of development. Initially the signal was located throughout the developing myocardium of both the ventricles and the atria, and as the heart matured, it became accentuated at the junctions between the cardiomyocytes, resembling β1-integrin, whereas type IV collagen was detected in the subendothelial basement membranes and capillaries. Type XIII collagen was also observed in the epicardium, but the endocardium was negative at all stages studied, as demonstrated by comparison of the signal for type XIII collagen with that for the endothelial marker CD31. In the adult mouse heart, type XIII collagen is located in the intercalated discs that join the cardiocytes (Hägg *et al.* 2001), and the maturing intercalated discs in the hearts of 15-day-old mice already displayed these signals, which then became more intensive as the intercalated disc structure matured and stabilized. Type XIII collagen appeared to colocalize with vinculin in the mature intercalated discs . in conventional light microscopy (Hägg *et al.* 2001), but the better resolution of confocal microscopy indicated that the two signals did not overlap in double detection (Paper I, Figure 5).

5.2.3 The developing skeleto-muscular system

As soon as the cartilages started to condense from the mesenchyme, a type XIII collagen signal could be observed, only in the chondrocytes initially, but spreading to the perichondrium surrounding the cartilage as it matured. Quite strong type IV collagen and β1-integrin signals were also observed in the perichondrium, but only faint ones in the chondrocytes themselves. The type XIII collagen signal remained strong in the hypertrophic and proliferative cartilage as endochondral ossification began, and as this proceeded it could also be observed around the bone spicules, although it was strongest in the periosteum surrounding the bone. Similarly, type IV collagen and β1-integrin were detected in the periosteum, but only low signals were seen in the regions of hypertrophic and proliferative cartilage.

Both type IV collagen and β1-integrin appeared before type XIII collagen in the skeletal muscle. At day 12.5 of development, when the former were detected surrounding the developing muscle fibres, the latter signal was still undefined. On day 15.5 type XIII collagen was observed surrounding the myofibres resembling the pattern observed in mature muscle (Hägg *et al.* 2001) and that for vinculin, type IV collagen and β1-integrin. In skeletal muscle from a 1-month-old mouse, type XIII collagen was accentuated at the myotendinous junction and colocalized with vinculin, being indistinguishable from that described for adult mouse muscle (Hägg *et al.* 2001) (Paper I, Figure 6).

5.2.4 Developing lung

Weak type XIII collagen signals were observed in the developing lung on day 12.5 of development, being located homogeneously in the parenchyma of the lung bud. As the branching events began, the collagen was upregulated in the junctions between the parenchyma and the branching primary and secondary bronchioles, resembling type IV collagen and β1-integrin although these molecules both precede type XIII collagen at this site. Interestingly, when lung tissue from a 1-month-old mouse was studied, type XIII collagen was not detected surrounding the bronchioles any longer and the pattern was different from that of type IV collagen, with strong basement membranes signals underlying the epithelium of the bronchioles and alveoli. In the developing and postnatal lung the parenchyma type XIII collagen appeared at all stages to be localized in the interalveolar septa beneath the alveolar epithelium (Paper I, Figure 7).

5.2.5 The developing intestine

Previous studies have shown that type XIII collagen is located on the basal sides of the mucosal epithelium in mature mouse intestine and in the myofibroblastic cells in the cores of the villi (Hägg *et al.* 2001). It was first detected faintly at day 14.5 of mouse development, surrounding the lumen of the developing mid-gut structures. The pattern was different from that of type IV collagen and β1-integrin, which are both localized to the basement membrane beneath the mucosal epithelium. With maturation of the intestine, the type XIII collagen became more abundant and was observed in the myofibroblastic cores of the villi of the small intestine and those lining the crypts of the colon. In addition, a strong accentuation of type XIII collagen in the basal and lateral sides of the mucosal epithelium could be detected, resembling β1-integrin, although lacking the intensive smooth muscle signal observed for this molecule (Paper I, Figure 8).

5.2.6 Developing skin

Type XIII collagen was localized to the dermal-epidermal junction in adult human skin (Peltonen *et al.* 1999), and its mRNAs were expressed by the epidermis during human fetal development (Sandberg *et al.* 1989). During mouse development, type XIII collagen is initially observed in the thin ectodermal layer covering the fetus, but as development of the skin proceeds, it becomes concentrated in the basal layers of the epidermis. This is different from type IV collagen, which is localized in the epidermal-dermal basement membrane and the capillaries and β1-integrin found in the dermis and the capillaries (Paper I, Figure 9).

5.3 Type XIII collagen in neuronal cells (I)

5.3.1 Type XIII collagen expression in primary cultured neurons

As type XIII collagen was found in the developing central and peripheral nervous systems (CNS and PNS), its localization was studied in neuronal primary cultures, which are generated from hippocamppal cells of 18.5-day rat fetuses and are enriched (>90%) in neurons but also contain small amounts of non-neuronal cells (Rauvala *et al.* 1988). A very intense signal for type XIII collagen could be observed in the neuron-like cells of the cultures, while the astrocytes were negative. The signal was located in both the neurites and soma of the neurons and colocalized completely with the neurofilament signal, indicating that the tissue signal was of neuronal origin (Paper I, Figure 10).

5.3.2 Effects of type XIII collagen on primary neuronal cultures

The effects of recombinant type XIII collagen protein on the behavior and morphology of the neuronal cells were studied by plating the cells in culture wells coated with the ectodomain of recombinant type XIII collagen (Nykvist *et al.* 2000, Snellman *et al.* 2000a), and comparing the results with those for cells plated on either laminin or HB-GAM (Raulo *et al.* 1992), which are both known to support the growth of neuronal cells (Rauvala 1989). Type XIII collagen induced neurite outgrowth formation (neurite outgrowth percentage 27%, standard deviation 1.3) in a similar manner to HB-GAM, with the outgrowths extending from individual neurons. The morphology of neurons grown on laminin is different, as the cells aggregate and neurite outgrowth takes place from the aggregates. Neurons grown on BSA were used as a negative control for the experiment (neurite outgrowth percentage 3,5%, standard deviation 2.5) (Paper I, Figure 11).

5.4 Type XIII collagen COL2 deletion transgenic mice (II)

To elucidate the function of type XIII collagen, transgenic mice that overexpress mutant type XIII collagen α-chains were generated by micro-injection of a cDNA construct (COL2del) harboring a 90 amino acid in-frame deletion mutation of the conserved COL2 domain (Paper II, Figure 1).

Ten founder mice were positive for the transgene by both PCR and Southern blot hybridization and nine of these displayed germ-line integration of the transgene, giving rise to nine separate transgenic lines. To evaluate the number of COL2del transgene copies in the genomes of the nine lines, a genomic DNA fragment recognizing the first exon of the type XIII collagen gene was used to probe Southern blots containing *Sph* I-digested genomic DNA from the lines. This probe recognized a 6 kb endogenous type XIII collagen gene fragment and a 3.5 kb transgene fragment. The number of transgene

copies, estimated by densitometric analysis, ranged from 2 to 20 in the different lines (Paper II, Table 1).

5.5 Expression of transgenic mRNA (II, III)

RT-PCR carried out on total RNA isolated from cartilage, skin, brain, lung, intestine, liver, spleen, kidney, heart, skeletal muscle and placenta of the heterozygous mice was used to analyze the expression pattern of the transgene. RT-PCR was subsequently also performed on RNA isolated from tumors in the transgenic mice. Specific oligonucleotide primers for use in the PCR amplification were chosen that bracket the COL2 deletion, allowing transgene and endogenous gene transcription to be distinguished in the same reactions.

The transgene was expressed in all nine lines in all the tissues studied and the pattern of its expression resembled that of the endogenous type XIII collagen gene. This indicated that most of the control sequences necessary for correct transcription were included in the promoter sequences used in the construct. Nevertheless, the level expression of the transgene was variable. In lines 17, 18, 23 and 26 the expression was highly similar to the endogenous product, while in lines 2 and 14 both similarities and differences were observed and in lines 32, 35 and 41 the expression was clearly lower than that of the endogenous gene. Interestingly, the number of transgene copies in the genome did not correlate with the rate of its expression (Paper II, Table 1).

All the mesenterial tumors analyzed expressed both transgenic and endogenous type XIII collagen mRNA at high levels, but expression of the transgene mRNA in the liver tumors was variable (Paper III, Figure 1).

5.6 Mutant type XIII collagen protein in the transgenic mice (II)

Western blotting of proteins isolated from cell lysates of mutant and control fetal mouse fibroblasts derived from fetuses was performed to verify that the COL2del transgene mRNA is translated to the truncated protein. A 90 kDa band that had a slightly faster mobility than the endogenous type XIII collagen was observed, indicating that the mutant mRNA was indeed translated (Paper II, Figure 2).

Mice from lines 17, 23 and 26, with a similar pattern of COL2del transgene and endogenous type XIII collagen gene RNA expression, were analyzed for the protein localization by immunohistochemistry with an antibody detecting the NC3 domain of type XIII collagen, which is present in both the mutant and endogenous protein. The same pattern was obtained in tissues from both transgenic and wildtype littermate mice, as previously described for fetal and adult mice (Hägg *et al.* 2001, Paper I). No signs of intracellular accumulation were observed, and the mutant protein was presumed to be correctly transported to the plasma membrane in the cells of tissues positive for the transgene RNA. Although expression of the mutant product was not expected to result in a readily identifiable increase in the immunosignal in the tissues, stronger signals were detected in the proliferating chondrocytes of bones undergoing endochondral ossification,

in the osteoblasts of the developing bones and in the capsule of the liver of the transgenic mice than in the controls, suggesting that the promoter sequences used to drive expression of the transgene were particularly active in these cells.

5.7 Fetal lethality of the type XIII collagen transgenic mice (II)

The heterozygous mice of several months of age initially expressed no overt phenotypes and were thus bred further in order to increase the load of mutant protein. When the genotype distribution of the offspring born from heterozygous mating was analyzed in the nine lines, it became evident that in four of these (lines 17, 23, 26 and 35) the expected Mendelian ratio of 75% transgene positive and 25% transgene negative mice could not be observed. The distribution of genotypes indicated that the distortion of the birth rates was caused by a lack of transgene positive offspring. This indicated a developmental defect, and thus pregnancies were terminations at days 9.5, 12.5 and 15.5 of development. At day 9.5 the offspring from the heterozygous mating displayed the expected distribution of genotypes, but during the following days an increasing number of fetuses were aborted and by day 15.5 the genotype distribution was similar to that seen at birth. Further pregnancy terminations narrowed the timing of the abortions down to days 10.5-13.5 of development. It became evident that the abortions take place at two distinct time points, allowing two separate phenotypes to be distinguished among the fetuses. It also was evident that more than 25% of the fetuses were lost, indicating that some of the heterozygous fetuses were also aborted (Paper II, Table 2).

5.8 Early phenotype fetuses (II)

Approximately half of the aborted fetuses were severely retarded, small and aborted by day 10.5 of development, making up the group of early phenotype fetuses. During dissection from the uterus a balloon-shaped sac was observed next to the fetus and a lack of connection to the placenta was evident. The sac was the allantois membrane, which by day 9 is fusing with the chorion membrane to form the chorioallantoic placenta (Cross *et al.* 1994). Thus a failure in the fusion of the chorion and allantois membranes was evident in the early phenotype fetuses, leading to a defect in the formation of a functioning placenta, which is necessary for fetal development to proceed (Rossant 1996). Histological analysis of control fetuses indicated that the labyrinth layer of the placenta had formed and both nucleated fetal blood cells and anucleated maternal blood cells were observed. The mutant placenta displayed a complete lack of formation of the fetal portion of the placenta and no fetal blood cells could be distinguished, although a single layer of trophoblast giant cells lined the maternal portion of the mutant placenta, indicating that the implantation of the fetus had initially occurred successfully. These giant trophoblast cells were observed in the TUNEL-assay to be undergoing apoptosis (Paper II, Figure 3).

5.9 Adherence junction defect in type XIII collagen
transgenic mice (II)

Those fetuses that overcame the problems of chorio-allantois fusion developed further but were also aborted by day 13.5. This phenotype with fetuses aborting at two separate stages was observed in all four transgenic lines (Lines 17, 23, 26 and 35). The mutant fetuses were small and pale compared to the transgene negative littermates, a weak, irregular heartbeat was observed during dissection from the uterus. Thus, where the hearts of the controls continued to beat for almost an hour after removal from the uterus, those of the mutant fetuses would stop beating within 10-15 minutes. The observed dysfunction of the heart and the marked paleness of the fetuses were suggestive of a cardiovascular defect.

In macroscopic and histological analysis the development of the four-chambered structure of the heart and its inflow and outflow tracts appeared to be intact in the mutants, and the three layers of the heart, the endocardium, myocardium and epicardium, had also developed. The myocardial layer nevertheless appeared to be thinner in histological analysis, and the trabeculation of the ventricles was reduced relative to the situation on the wild type animals. Electron microscopy showed clear differences in the structure of the myocardium, the adherence junctions being less electron-dense in the mutant hearts and detachment of myofilaments from them being evident. Interestingly, no defects in the desmosomes were observed in the specimen analyzed, and these junctions were indistinguishable from those of the control. No differences in the developing gap junctions could be observed between the mutant and control hearts (Paper II, Figure 4).

Immunohistochemical analysis with markers for the different junctions showed no differences in the desmosomal component desmoplakin between the transgenic mutant animals and their wild-type controls but examination of the adherence junctions with a pan-cadherin antibody revealed a disorganized pattern and reduced signal intensities in the mutant myocardium (Paper II, Figure 4).

5.10 Angiogenesis defect in type XIII collagen transgenic mice (II)

The development of the vasculature in the mutant fetuses was studied by immunohistochemistry with the endothelial markers CD31 and CD34. Vasculogenesis appeared to proceed normally in the mutants, as no defects in the formation of the large vessels could be observed. Defects in angiogenesis were evident, however, leading to a perturbation in microvascularization in some regions of the mutant fetuses. In the cranial region, the number of small vessels was reduced in the developing CNS and in the trigeminal ganglia, whereas the development of the microvessels in other parts such as the cephalic mesenchyme and the upper limb was normal. The immunohistochemical observations were verified by morphometric analysis of the number of capillaries, no statistically significant differences were observed in the limb and the area surrounding the internal carotid artery, whereas they were observed in both the developing CNS and the trigeminal ganglia (Paper II, Figure 5).

Less well-defined placental blood vessels were observed in the mutant placentas during dissection, and consequently the placentas of the late phenotype fetuses were also analyzed. The labyrinth layer of the placenta was thick and less vascularized in histological sections. The cell lineages of the placenta were studied with the endothelial marker CD34, the trophoblast marker cytokeratin and the decidual markers vimentin and desmin, and apoptosis was studied with the TUNEL assay and cell proliferation with the PCNA antibody. The fetal vessels showed less spreading in the labyrinth layer of the mutant placentas than in the control placentas (Paper II, Figure 6). Morphometric analysis of placentas from mutant and control fetuses indicated that the defect in the development of the fetal vascularization had led to a statistically significant increase in the average distance between the fetal vessels and the maternal blood lacunae (average distance 24.5µm (standard deviation 5.75µm) for the mutant and 6µm (standard deviation 1.5µm) for the control, p-value <0.001).

Cytokeratin, vimentin and desmin antibody detection revealed no defects in the development of the other compartments of the mutant placenta, but the TUNEL assay pointed to somewhat more numerous apoptotic cells in the mutant placentas, most likely secondary to the poorly developed vascularization. PCNA in the placentas did not indicate any changes in cell proliferation, nor did the localization of type XIII collagen in the labyrinth layers differ between the mutant and control placentas (Paper II, Figure 6).

5.11 Incidence of tumors in type XIII collagen transgenic mice (III)

The COL2del heterozygote mice, which were initially of normal appearance, were analyzed for tumor occurrence at 18 and 24 months age. Increased ratios of tumors in the gastrointestinal tract, the liver and the muscles were observed in the transgenic mice. Tumors were already observable at the earlier time point, and the tumor ratios in the transgenic mice were higher than in their wild-type littermate controls. Macroscopic tumors were found in 22.6% of the COL2del transgenic mice (n=195), whereas only 8.4% of the control mice (n=119) displayed tumors. Macroscopic analysis showed the majority of the tumors found in the transgenic mice to be located in the gastrointestinal tract (68.2%).Those in the intestine were compact, large and displayed growth at several locations in the mesenterium, suggestive of metastasis or multiple simultaneous locations for tumor development. In approximately 50% of cases the intestinal tumors were accompanied by massive splenomegalia. A significant number of highly vascularized tumors were also found in the liver and muscle (13.6%), with large necrotic areas. These tumors appeared to grow by directly infiltrating the surrounding tissue, as no signs of metastasis to other sites were observed (Paper III, Table 1).

5.12 Characterization of the tumors (III)

In histological analysis the tumors of the intestinal mesenterium were seen to be composed of small atypic lymphocytes, with the cells displaying multiple nucleoli, a coarse chromatin pattern and a high mitotic activity. Sections from the enlarged spleens

also displayed massive infiltration of lymphocytes, with complete destruction of normal splenic architecture. All the major organs, the lung, heart, kidney, liver and skeletal muscle, were analyzed for metastases, and in two cases tumor growth within the mucosal and muscular layers of the intestine was also observed, but neither macroscopic nor microscopic tumor growth could be found in the other organs analyzed. The tumors of the liver and the muscle were different, with formation of vascular structures destroying the hepatic tissue, large areas of necrosis and undifferentiated malignant tissue (Paper III, Figure 2).

To characterize the cellular origin, the expression of several markers for different cell lineages were studied. The intestinal tumors stained positive for CD45 and CD3, indicating that they consisted of lymphocytes of T-cell origin. Only a few cells that were positive for the B-cell marker CD20, the macrophage marker CD68 and myelopreoxidase as a marker of cells of myeloid lineage could be observed in the intestinal tumors. In double detection, the same cells expressed the T-cell marker CD3 and type XIII collagen. The results of FACS analyses of tumor lysates were well in line with the immunohistochemical characterizations as over 80% of the cells from tumor lysates were positive for the T-cell markers CD2 and CD3 and only a small proportion for the B-cell markers CD19 and CD20. In further FACS analysis the tumors were shown to consist of a mixed population of single positive mature T-helper (CD4+) and T-suppressor (CD8+) cells. The characterization of tumors from the liver showed that they were positive for CD34 and factor VIII, indicating that they were angiosarcomas of endothelial origin (Paper III, Figure 3).

To study the expression of the transgene, RT-PCR was carried out on total RNA isolated from the tumors found in five transgenic lines (Lines 17, 18, 23, 26 and 35). As the specific oligonucleotide primers used bracket the COL2 deletion, the transgene and endogenous gene transcription can be distinguished in the same reaction. All the lymphomas analyzed expressed both transgene and endogenous type XIII collagen mRNA, but the rate of transgene expression was much higher than that of the endogenous gene expression. The level of expression of the transgene was variable in the tumors of the liver, however, being similar to that of the endogenous gene in some whereas no transgene expression at all could be observed in others.

The localization of the type XIII collagen protein in the tumors was studied by immunohistochemistry in lines 17, 23, 26 and 35. The lymphomas were positive for type XIII collagen and no signs of intracellular accumulation were observed. The type XIII collagen signals observed in the tumors were of a much higher intensity than those in the normal lymphatic tissues (spleen, thymus and lymph nodes) of the COL2del transgenic mice and controls, suggesting that expression of the transgene was significant in the tumor cells. On the other hand, only weak type XIII collagen signals were seen in the tumors of the liver.

5.13 Blood counts of type XIII collagen transgenic mice (III)

As it is difficult to distinguish peripheral lymphomas and leukemias in histological analysis, the blood counts and the morphological appearance of the peripheral blood cells

were analyzed in 12-month-old COL2del and control mice. The blood counts did not reveal any differences in the numbers of blood cell types or in the haemoglobin content between the transgenic (tg) and control mice (con) (Hb con 132, SD 10.2 and tg 129, SD 6.3; WBC con 5.8, SD 2.5 and tg 5.4, SD 2.0; RBC con 9.0, SD 1.4 and tg 8.8, SD 1.7; PLT con 785, SD 276 and tg 759, SD 202), indicating that the lymphomas were most likely primary peripheral lymphomas. Blood lymphocytosis sometimes accompanies lymphomas, but it is always detected in leukemia, and the results thus indicate that the observed tumors are peripheral lymphomas.

6 Discussion

Type XIII collagen is a transmembrane protein characterized by a short intracellular domain, a single transmembrane domain and a large extracellular domain. Its function is so far unknown. Results regarding its localization in cultured cells and mature tissues indicate that it is involved in cell adhesion, where it could function as a receptor for molecules in the extracellular matrix or for molecules on adjacent cells (Hägg *et al.* 2001, Peltonen *et al.* 1997, Sandberg-Lall *et al.* 2000). Although the occurrence of type XIII collagen during mouse development often coincided with markers of adhesive structures and basement membranes, certain differences were observed, indicating that it is involved in developmental events associated not only with cell adhesion but also with signal transduction and cell migration.

This first thorough characterization of type XIII collagen expression during mouse development (Paper I) shows that it is already expressed at the early stages of development. Type XIII collagen mRNAs were detected by Northern analysis from day 7 of development onwards and by quantitative RT-PCR from day 9 onwards, with expression levels fairly constant initially but increasing markedly during the final stages of organogenesis, with initiation of the rapid fetal growth phase before birth. Localization of type XIII collagen mRNAs in fetal tissues by *in situ* hybridization revealed strong expression in the developing nervous system, the heart and skeletal muscle, the cartilage and bone and in the basal layers of the skin and the epithelia of the intestine and lung. The examination of type XIII collagen protein in fetal tissues by immunohistochemistry revealed very strong signals in the nervous system and the heart even during the early stages of organogenesis. In most other tissues the protein levels were initially quite weak but the increase in mRNAs during the later stages of development coincided with the observed accentuation of type XIII collagen in specific structures or developmental processes in a number of tissues, e.g. the branching morphogenesis of the lung, the maturing skeletomuscular system and the process of endochondral ossification in bone.

The most intensive type XIII collagen protein expression during mouse development was observed in the central and peripheral nervous systems. Previous immunohistochemical studies have localized type XIII collagen to the endoneural sheets of the peripheral nerves in adult mice (Hägg *et al.* 2001) and to the opticus nerve and retina in fetal human eye (Sandberg-Lall *et al.* 2000). The localization to the

neuroepithelium surrounding the brain vesicles and the neural tube was markedly different from that observed for type IV collagen and β1-integrin, but highly similar to that described for the neural marker neurofilament (Portier *et al.* 1993). Clear type XIII collagen signals were also detected in ganglionic structures and peripheral nerves throughout development. In subsequent immunohistochemical investigation of primary cultured neurons a very intense signal was observed in the neurites and the soma of neurons, while a small number of astrocytes in the culture remained negative. Furthermore, the ectodomain of recombinant type XIII collagen protein was found to induce neurite outgrowth and neuron network formation in a similar fashion to HB-GAM. Interestingly, no other collagen has been localized to neuronal cells (Brown & Timpl 1995). Thus type XIII collagen forms a unique member of the collagens, with functions in both the mature and the developing nervous systems (Hägg *et al.* 2001, Sandberg-Lall *et al.* 2000, Paper I). Previous studies have shown that several non-collagenous extracellular matrix proteins and receptors are involved in the development of the nervous system, in processes such as neural cell migration, axon growth and synapse formation and in the differentiation and development of the glial cell population (Venstrom & Reichardt 1993). Interestingly the I-domain of α1β1-integrin, one of the receptors described as mediating neurite outgrowth (Tomaselli *et al.* 1990, Tomaselli 1991), has recently been shown to bind the ectodomain of type XIII collagen with high affinity (Nykvist *et al.* 2000). Thus this collagen may interact with α1β1-integrin and support neuron-neuron interactions. Although cell-extracellular matrix interactions are widely accepted as regulating the development of the peripheral nervous system and other peripheral organs, little is yet known about the structure and function of the extracellular matrix in the brain. The present findings therefore suggests that interactions depending on collagen-type molecules should also be considered in the context of brain development and plasticity.

Type XIII collagen was already found in the heart on day 10 of development, and it became accentuated at sites of cell-cell contact with maturation of the myocardium. In the mature myocardium it is localized to the intercalated discs (Hägg *et al.* 2001) that mature during the perinatal period (Angst *et al.* 1997, Kostin *et al.* 1999). We observed it in the intercalated disc of hearts from 15-day-old mice and the signal became stronger with further maturation of the disc. Previous studies have indicated colocalization of type XIII collagen and vinculin in the intercalated discs (Hägg *et al.* 2001) but we were able to observe by confocal microscopy that these signals do not overlap in the nascent or the mature disc. Type XIII collagen was also found in the epicardium of the heart, but the endocardium was devoid of any signal at all stages studied. Maturation of the intercalated disc is essential for continued development of the conductive system and for supplying enough mechanical strength in the adhesions between the cardiomyocytes (Kostin *et al.* 1999, Smolich 1995) as the growing fetus becomes fully dependent on the cardiovascular system during late organogenesis (Rossant 1996). Adherence junctions and desmosomes precede the development of the disc, and since type XIII collagen is found in these structures, it may thus function in the disc maturation process.

Both the chondrocytes and the perichondrium in cartilage contains type XIII collagen, as shown previously by *in situ* hybridization in human fetal tissues (Sandberg *et al.* 1989). As endochondral ossification begins, strong type XIII collagen expression remains in the hypertrophic and proliferative chondrocyte regions, resembling the pattern of

expression for type X collagen (Prockop & Kivirikko 1995, Vuorio & de Crombrugghe 1990). Type XIII collagen is not as restricted to this area, however, as intensive expression was also observed in the periosteum and moderate expression in the areas surrounding the spicules of the newly formed bones.

Type XIII collagen mRNAs have been localized in previous *in situ* hybridization studies to the endomysium surrounding the muscle fibres (Sandberg *et al.* 1989). *In situ* hybridization of mouse fetal tissues showed the myocytes to contain type XIII collagen mRNAs, while immunofluorescence staining of the developing skeletal muscle gave signals in the area surrounding the muscle fibres, but there type XIII collagen was preceded by both type IV collagen and β1-integrin. During the perinatal period, type XIII collagen also emerged at the myotendinous junction where it is found in adult skeletal muscle (Hägg *et al.* 2001).

After branching of the lung had initiated, type XIII collagen became accentuated in the bronchioles of the developing lung, colocalizing with the basement membrane molecule type IV collagen and the extracellular matrix receptor β1-integrin. The type XIII collagen signal in the bronchioles was scarcely detectable after birth, however, and only weak expression remained in the parenchymal cells. Previous studies indicate that several other matrix molecules, such as fibronectin and its receptor α5β1 integrin, accumulate in the clefts between the branching regions during lung development (Roman & McDonald 1992, Buck *et al.* 1996, Gumbiner 1996) and, interestingly, recent results from our group indicate that recombinant type XIII collagen interacts with fibronectin (Tu H, Sasaki T, Snellman A, Göhring W, Timpl R & Pihlajaniemi T: The type XIII collagen ectodomain is a 150-nm rod and capable of binding to fibronectin, nidogen-2 and several other matrix molecules. Submitted). The similarity of the pattern of type XIII collagen to that of a number of matrix molecules implicated in lung development, together with the decrease in the postnatal period, suggests that it could be involved in lung branching morphogenesis. This does not apply to all tissues undergoing branching, however, as no comparable pattern was observed in the kidney.

Type XIII collagen also appears to be involved in cell-matrix adhesion in the developing skin and intestine, since the initially diffuse signal became accentuated towards the basal sides of the mucosal epithelium and the epidermal-dermal junction with maturation of these tissues. In the intestine, the pattern of type XIII collagen expression clearly differed from that of type IV collagen but resembled that of β1-integrin to some extent. The type XIII collagen signal in the skin differed from those of both type IV collagen and β1-integrin.

Previous *in situ* hybridizations of human tissues have suggested that some endothelial cells contain type XIII collagen RNAs (Sandberg *et al.* 1989), but this could not be verified for murine tissues here. The results obtained with the NC3-specific antibody used here were later verified with antibodies specific to the cytosolic portion of the NC1 (Tuomisto A., Väisänen T., Sund M. and Pihlajaniemi T., unpublished results). The discrepancy between the results obtained for human and mouse tissues can, however, be caused by differences of type XIII expression between species.

In order to elucidate the function of type XIII collagen in the mammalian organism the main project of this thesis was the generation of a transgenic mouse line that overexpressed type XIII collagen α-chains with a 90 amino acid in-frame deletion mutation of the conserved COL2 domain (Paper II). This large deletion was designed to

severely affect the function of type XIII collagen and thereby cause clear phenotypic changes in the transgenic mice. The shortened α1(XIII) chains were expressed in cultured fibroblasts derived from the mutant fetuses, and immunohistochemistry of the mutant fetal tissues did not reveal any signs of intracellular accumulation of type XIII collagen. Furthermore, the 90-amino acid in-frame deletion in the central portion of the molecule does not affect sequences known to be involved in the association of the α1(XIII) chains (Snellman *et al.* 2000b). Thus the mutant α1(XIII) chains are likely to associate stochiometrically with both mutant and endogenous normal α1(XIII) chains and be transported to the plasma membrane of cells. Previous studies of human disorders and of mouse models with respect to fibrillar collagens and some of the non-fibrillar collagens indicate that when a mutation does not involve the chain association sequences, mutant and normal chains associate stochiometrically, leading to the formation of abnormal collagen and a dominant negative phenotype (Jacenko *et al.* 1993, Kivirikko 1993, Prockop & Kivirikko 1995). It is therefore likely that cells from the mutant mice contain a mixture of type XIII collagen molecules including heterotrimers of mutant and normal chains, homotrimers of normal α1(XIII) chains and homotrimers of mutant α1(XIII) chains and that the mutant molecules are functionally abnormal, leading to the defects observed in the mutant mice.
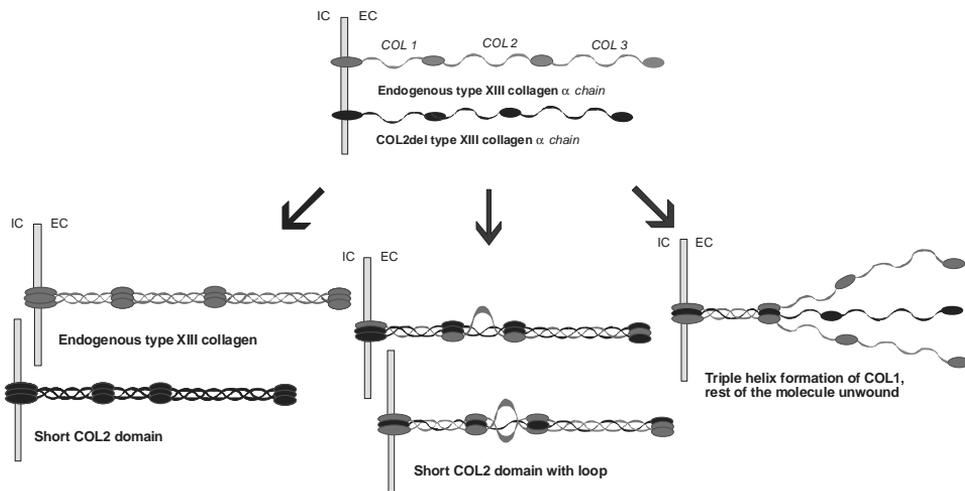
Fig. 3. Possible effects of overexpression of truncated type XIII collagen α-chains in the COl2del transgenic mouse model. As mutant α-chains can be thought to associate both with other mutant molecules and with endogenous type XIII collagen chains, mutant molecules of several different kinds may exist.

Expression of the truncated type XIII collagen α-chains in transgenic mice did indeed result in developmental arrest in some of the offspring from heterozygous mating between days 10.5 and 13.5 of gestation. No viable mice that were homozygous for the mutation were obtained, indicating recessive lethality as the load of mutant protein increased. As some of the heterozygous offspring were also lost, however, individual differences must exist in the actual transcription level of the mutant protein during development. The fetuses from heterozygous mating abort in two stages, approximately 50% in each stage.

The early phenotype fetuses were aborted by day 10.5 of development, as the chorion and allantois membranes failed to fuse, hindering the formation of a functioning placenta, which is critical for development to proceed (Rossant 1996).

The fetuses that were able to overcome this initial critical incident and formed a placenta constituted the late phenotype cases. These fetuses were also aborted by day 13.5 of development, however, due to cardiovascular defects. It can be speculated that the two distinct abortion time points could be due to differences in the localization and/or expression rates of the transgene between these fetuses.

During the mid-gestational period the fetus becomes dependent on its own blood circulation and a well functioning cardiovascular system for subsequent developmental processes to continue (Rossant 1996). The fact that type XIII collagen was observed to be strongly expressed in the developing heart and that the hearts of the late phenotype fetuses were beating poorly during dissection from the uterus suggests that expression of type XIII collagen is essential for normal development of the heart. Subsequent histologically and ultrastructural studies revealed hypoplasia of the myocardium of the ventricles, with reduced trabeculation and poorly developed adherence junctions between cardiomyocytes, leading to the detachment of myofilaments from these structures. The cardiomyocytes also displayed degeneration and contained less myofilaments, most likely a secondary effect caused by the lack of organization as the contact with the adherence junctions had failed. The desmosomes were indistinguishable from those of the control fetuses, however. Immunohistochemistry with a pan-cadherin antibody showed the structure of the adherence junction to be disorganized in the mutant, whereas the expression pattern of the desmosomal desmoplakin did not differ between the mutant and the control. Thus expression of mutant type XIII collagen α1(XIII) chains appeared to greatly impair the development of the adherence junctions in the heart.

As no localization of type XIII collagen to endothelial structures was observed in immunohistochemistry, it was very surprising to find reduced microvascularization in some regions of the mutants, such as the CNS and the trigeminal ganglion, whereas the number of capillaries in other regions, such as that surrounding the internal carotid artery and the upper limb of the same fetuses, did not differ between the mutant and wild-type animals. Alterations in the levels of expression of adhesive glycoproteins and integrins and of other proteins produced by the endothelial cells and the extracellular matrix surrounding them are nevertheless known to influence the ability of the endothelial cells to adopt migratory properties during angiogenesis (Lucinskas & Lawler 1994). Spatio-temporal analysis of type XIII collagen expression during mouse development showed that the CNS and ganglia are sites of strong expression, whereas the rate is lower in the mesenchyme of the head and the limb. Thus decreased angiogenesis was observed in the

mutant mice in tissues that normally express high levels of type XIII collagen and are also likely to express high levels of the mutant molecules. This effect may be mediated by the collagen receptor $\alpha1\beta1$ integrin, which is expressed both in large vessel and microvessel endothelial cells (Enenstein *et al.* 1992, Klein *et al.* 1993) and has recently been shown to interact strongly with recombinant type XIII collagen (Nykvist *et al.* 2000). The expression of altered type XIII collagen in some tissues could affect the binding or signal transduction between the endothelial cells and the surrounding extracellular matrix, causing a decrease in the ability of the endothelial cells to sprout and form new vessels during angiogenesis.

Even though the placentas of the late phenotype fetuses did develop, these were not entirely normal. The labyrinth layer where the exchange of nutrients and other substances takes place is well vascularized, with the fetal vessels and maternal blood lacunae in close contact (Cross *et al.* 1994). The labyrinth layer from the mutant type XIII collagen fetuses, however, appeared compact and poorly vascularized, with the fetal vessels showing defects similar to those previously observed in the late phenotype fetus. The localization of type XIII collagen in the human placenta has been previously studied by *in situ* hybridization (Juvonen *et al.* 1993), and the mRNAs were found to be expressed by fibroblastic stromal cells in the placental villi, developing endothelia, cytotrophoblastic cells and decidual cells. In immunohistochemical analysis of the mouse placenta, type XIII collagen was observed in the trophoblasts of the labyrinth and the spongiotrophoblastic layer but was not associated with the endothelia in either the control or mutant fetuses. Therefore, as type XIII collagen is quite strongly expressed by the placenta, it appears that the expression of the mutant protein in the placenta has the same effect on the angiogenesis of placental vessels as was observed in the CNS and the trigeminal ganglia.

A number of adhesion molecules have been either subjected to mutation or knocked out in transgenic mice in the recent years, and the phenotype of the present transgenic mice expressing mutant type XIII collagen bears a strong resemblance to those of some of the previous transgenic lines, such as those with targeted disruptions of the genes for plakoglobin (μ-catenin) and the focal adhesion molecule vinculin (Bierkamp *et al.* 1996, Ruiz *et al.* 1996, Xu *et al.* 1998) (Table 3). As the correct expression of adhesion molecules is important for several developmental processes, many of the mutations have led to mortality during fetal development due to cardiovascular and placental defects. The fetal lethality among the plakoglobin mice was caused by the formation of fused desmosome and adherence junctions in the heart, leading to cardiac failure (Ruiz *et al.* 1996). The mice lacking vinculin also died during the fetal period due to a dysfunctional heart (Xu *et al.* 1998). These results and those for the mice expressing mutant type XIII collagen underline the importance of the correct sequential formation of the cellular junctions in the heart for the formation of the intercalated discs, which are essential in order to generate of the necessary mechanical strength in the cell-cell adhesions of the myocardium (Angst *et al.* 1997). The type XIII collagen mutants also resemble to some extent the targeted inactivations of the receptor-counterreceptor pair $\alpha4$ integrin (Yang *et al.* 1995) and VCAM-1 (Gurtner *et al.* 1995, Kwee *et al.* 1995) (Table 3). These mutations are lethal during fetal development, with a similar two-stage abortion mode to that observed in the type XIII collagen mice. The cardiac phenotype of these mice is nevertheless due to detachment of the epicardium from the myocardium, which results in

defects in the formation of coronary vessels and leakage of blood from the heart. The N-cadherin transgenic mice die of cardiac defects on account of a failure in cell-cell adhesion, and they mice display dissociation of cardiomyocytes during early development, but other defects were also observed (Radice *et al.* 1997). The mice with a targeted inactivation of the αv integrin gene displayed both fetal and perinatal mortality. The offspring with fetal lethality have histological defects of the myocardium that are similar to those of the type XIII collagen mice, and in addition they have an abnormal labyrinth layer in the placenta (Bader *et al.* 1998). The mice with a targeted disruption of the β3 integrin gene (Hodivala-Dilke *et al.* 1999) display fetal mortality among some of the homozygote offspring with defects that leads to a compact and poorly vascularized placenta (Table 3).

Adhesion molecules and the extracellular matrix have several important tasks during fetal development, in the guidance of migrating cells, in regulation of morphogenesis and differentiation and in rendering mechanical strength (Lin & Bissell 1993). The observed wide expression of type XIII collagen during mouse development, combined with the finding that the expression of mutant type XIII collagen results in fetal lethality caused by cardiovascular and placental defects that are highly similar to those displayed by mice with mutations in several classical cell adhesion molecules, increases the evidence that implicates type XIII collagen in cell adhesion, with functions that are essential for normal fetal development to proceed.

Table 3. Phenotype comparison of mice with targeted inactivation of some cell-adhesion molecules and the type XIII collagen COL2del transgenic mice

Molecule	Lethality	Defect	Reference
Plakoglobin	12.5 dpc-	Cardiovascular defect; abnormal desmosomes	Ruiz *et al.* 1996
Vinculin	10.5 dpc-	Cardiovascular defect; akinetic myocardium	Xu *et al.* 1998
α4 integrin	9.5-14.5 dpc	Placental and cardiovascular defects; lack of chorion and allantois fusion, detachment of epicardium	Yang *et al.* 1995
VCAM-1	9.5-14.5 dpc	Placental and cardiovascular defects; lack of chorion and allantois fusion, detachment of epicardium	Gurtner *et al.* 1995 Kwee *et al.* 1995
N-cadherin	10.5 dpc	Cardiovascular defect; dissociation of cardiomyocytes	Radice *et al.* 1997
αv integrin	9.5 –11.5 dpc (80% of fetuses)	Cardiovascular and placental defect; abnormal labyrinth layer, thin myocardium	Bader *et al.* 1998
β3 integrin	14-5-17.5 dpc (10% of fetuses)	Placental defect; abnormal labyrinth layer (25% of fetuses)	Hodivala-Dilke *et al.* 1999
α1(XIII)	10.5-13.5 dpc	Placental and cardiovascular defects; lack of chorion and allantois fusion, abnormal adherence junctions, reduced angiogenesis	Sund *et al.* II

The mice that were heterozygous for the COL2 deletion mutation were quite normal in appearance initially but began to display increased ratios of tumors with age, mainly T-cell lymphomas of the gastrointestinal tract and angiosarcomas of the liver and muscle (Paper III). Their wild-type littermates also displayed tumors with age, but the number

observed in these was clearly lower and the tumors were histologically different. The fact that most of the tumors observed in several transgenic lines were of two main types indicates direct involvement of mutant type XIII collagen in malignant transformation. Tumors only developed in the type XIII collagen transgenic mice at an older age and with moderate penetration, however, indicating that additional mutations are required for malignant transformation (Adams *et al.* 1999, Adams & Cory 1991). The expression of the mutant form of type XIII collagen somehow renders the organism more susceptible to T-cell lymphomas and angiosarcomas in particular. Since no intracellular ligands of type XIII collagen have yet been identified, it is difficult to speculate on the potential intracellular down-stream signalling events that take place as the mutant chain becomes located on the cell membrane. One possible mechanism is that the shortened COL2 domain changes the binding site of a ligand, with deleterious effects on the signalling cascade. The altered cascade could then either potentiate growth signals, inhibit growth suppression or affect cell adhesion, causing malignant transformation. It is also possible that the effects could be secondary to altered cell adhesion, as correct adhesion is of importance for normal control of cell growth.

Several transgenic strains overexpressing different oncogenes cause the development of T-cell lymphomas (Adams *et al.* 1999, Adams & Cory 1991, Seldin 1995). Interestingly, the null mutation for the tumor suppressor gene p53 results in a very similar phenotype to that observed for the type XIII collagen transgenic mice, which develop lymphomas and angiosarcomas in similar ratios (Donehower *et al.* 1992). Recent results obtained with mice overexpressing a mutant form of the intracellular enzyme PI3K suggest that this causes malignant transformation of the T-cell population (Borlado *et al.* 2000). The penetrance of the PI3K mutant mice phenotype was increased by mating into a p53 negative background, indicating that partial overlapping pathways may exist between these (Borlado *et al.* 2000). Another recently described transgenic line with a higher penetrance in the formation of T-cell lymphomas is Notch3, where overexpression of the intracellular domain of the receptor Notch3 creates a situation of ligand-independent signalling and malignant transformation (Bellavia *et al.* 2000). Although the possible function of type XIII collagen in these signalling pathways remains to be elucidated, the susceptibility of the present mutant mice to malignant transformation of the T-cell population and the occurrence of angiosarcoma implies that the molecule is most likely to be involved in the interpretation of signals from the surrounding extracellular matrix through binding of some of its ligands. As mutant type XIII collagen is expressed on the cell membrane, the balance of this function must be altered, leading to the effects seen in the transgenic mice.

Type XIII collagen is a large molecule spanning from the interior of the cell to the extracellular matrix, and its different domains can vary in their roles. We recently described the phenotype of a lack of the cytosolic and transmembrane domains of type XIII collagen in a transgenic mouse strain due to an N-terminal alteration. The mutant molecules are secreted, and can apparently take part in many of the functions of the ectodomain, but the mice display a progressive myopathy due to abnormal skeletal muscle-basement membrane interaction with an abnormal and occasionally ruptured basement membrane (Kvist *et al.* 2001). On the other hand, the deletion of a large portion of the COL2 domain is predicted to result in abnormal folding of most of the type XIII collagen ectodomain and lead to a more severe phenotype (II and III).

The results presented in this thesis increase the evidence for type XIII collagen as an important member of the group of proteins that convey information between the extracellular matrix and the cells. It is localized to several sites of cell-matrix and cell-cell adhesion and the functions of this intriguing molecule are now starting to emerge. It is widely expressed during fetal development and the developmental shifts in its expression patterns indicate that it functions in several developmental events. This is further underlined by the findings of fetal defects in mice that express a mutant form of this collagen. Finally, the development of tumors of certain cell populations in the type XIII collagen transgenics mice implies functions that extend beyond mere mechanical adhesion.

7 Future perspectives

The results presented in this thesis study add to the accumulating evidence that type XIII collagen is an important membrane protein in the cell. It is essential for normal fetal development and the it functions in the regulation of normal cell growth in the malignant transformation of certain mesenchymal cell populations.

Future studies using the COL2 deletion transgenic mice could point to additional subtle defects that have not yet been analyzed. The functional consequences of the defects in placental and cardiac development observed here should be further analyzed. No collagens have previously been found to be expressed by neurons, and therefore this unusual localization of type XIII collagen raises intriguing questions in the context of the development of the central and peripheral nervous systems in the normal situation and in the COL2 deletion transgenic mice. The high incidence of T-cell lymphomas in the COL2del transgenic mice combined with the observed expression of type XIII collagen in normal lymphatic tissues indicates possible functions for this collagen in cellular defense.

The increasing knowledge contributed by other type XIII collagen transgenic mouse lines with different mutations of the extracellular domain, together with the work done to characterize this molecule at the cellular and protein levels, will make the initially diffuse picture of this collagen clearer. Important questions that remain to be answered concern how the mutation of the COL2 domain of this membrane protein changes its ligand binding properties and what the subsequent cellular effects are. A prerequisite for understanding these questions is a knowledge of the folding and transport of type XIII collagen molecules containing one or more COL2 deletion α-chains. The intracellular ligands and the possible downstream signalling that might involve type XIII collagen also need to be addressed in future studies. The observed shedding of type XIII collagen related to cellular events during tissue remodeling and development is of importance. The overall role of this collagen and its mutations in a subset of human malignancies and in spontaneous abortions taking place in early human pregnancies, due to defects in either placental formation or cardiac function are also questions that will be addressed in future studies.

Evidence from two separate mouse strains, ColXIIIα1$^{n/n}$ and COL2del mice, indicate that type XIII collagen is involved in a variety of important cellular tasks that vary

between domains. It would be of great interest to see the consequences of a complete lack of type XIII collagen, through the generation of a mouse strain with inactivation of the gene, and more subtle mutations of the ectodomain can also give additional information on the function of this collagen.

References

Acton S, Resnick D, Freeman M, Ekkel Y, Ashkenas J & Krieger M (1993) The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands. J Biol Chem 268: 3530-3537.

Adams JM & Cory S (1991) Transgenic models of tumor development. Science 254: 1161-1167.

Adams JM, Harris AW, Strasser A, Ogilvy S & Cory S (1999) Transgenic models of lymphoid neoplasia and development of a pan- hematopoietic vector. Oncogene 18: 5268-5277.

Adamson ED & Ayers SE (1979) The localization and synthesis of some collagen types in developing mouse embryos. Cell 16: 953-965.

Aho S & Uitto J (1999) 180-kD bullous pemphigoid antigen/type XVII collagen: tissue-specific expression and molecular interactions with keratin 18. J Cell Biochem 72: 356-367.

Andrikopoulos K, Liu X, Keene DR, Jaenisch R & Ramirez F (1995) Targeted mutations in the col5a2 gene reveal a regulatory role for type V collagen during matrix assembly. Nat Genet 9: 31-36.

Andrikopoulos K, Suzuki HR, Solursh M & Ramirez F (1992) Localization of pro-alpha 2(V) collagen transcripts in the tissues of the developing mouse embryo. Dev Dyn 195: 113-120.

Angst BD, Khan LU, Severs NJ, Whitely K, Rothery S, Thompson RP, Magee AI & Gourdie RG (1997) Dissociated spatial patterning of gap junctions and cell adhesion junctions during postnatal differentiation of ventricular myocardium. Circ Res 80: 88-94.

Aplin JD (1991) Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. J Cell Sci 99 (Pt 4): 681-692.

Araki N, Higashi T, Mori T, Shibayama R, Kawabe Y, Kodama T, Takahashi K, Shichiri M & Horiuchi S (1995) Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction. Eur J Biochem 230: 408-415.

Ashkenas J, Penman M, Vasile E, Acton S, Freeman M & Krieger M (1993) Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. J Lipid Res 34: 983-1000.

Aszodi A, Pfeifer A, Wendel M, Hiripi L & Fässler R (1998) Mouse models for extracellular matrix diseases. J Mol Med 76: 238-252.

Bader BL, Rayburn H, Crowley D & Hynes RO (1998) Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. Cell 95: 507-519.

Bayes M, Hartung AJ, Ezer S, Pispa J, Thesleff I, Srivastava AK & Kere J (1998) The anhidrotic ectodermal dysplasia gene (EDA) undergoes alternative splicing and encodes ectodysplasin-A with deletion mutations in collagenous repeats. Hum Mol Genet 7: 1661-1669.

Bellavia D, Campese AF, Alesse E, Vacca A, Felli MP, Balestri A, Stoppacciaro A, Tiveron C, Tatangelo L, Giovarelli M, Gaetano C, Ruco L, Hoffman ES, Hayday AC, Lendahl U, Frati L, Gulino A & Screpanti I (2000) Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. EMBO J 19: 3337-3348.

Bierkamp C, Mclaughlin KJ, Schwarz H, Huber O & Kemler R (1996) Embryonic heart and skin defects in mice lacking plakoglobin. Dev Biol 180: 780-785.

Blomqvist C & Asko-Seljavaara S (1992) Pehmytkudossarkoomat. In: Holsti LR, Roberts PJ, & Teppo L (eds) Syöpätaudit 363-368. Kustannus Oy Duodecim, Vammala.

Bohme K, Li Y, Oh PS & Olsen BR (1995) Primary structure of the long and short splice variants of mouse collagen XII and their tissue-specific expression during embryonic development. Dev Dyn 204: 432-445.

Bonadio J, Saunders TL, Tsai E, Goldstein SA, Morris-Wiman J, Brinkley L, Dolan DF, Altschuler RA, Hawkins JE, Jr. & Bateman JF (1990) Transgenic mouse model of the mild dominant form of osteogenesis imperfecta. Proc Natl Acad Sci USA 87: 7145-7149.

Bonaldo P, Braghetta P, Zanetti M, Piccolo S, Volpin D & Bressan GM (1998) Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for bethlem myopathy. Hum Mol Genet 7: 2135-2140.

Borlado LR, Redondo C, Alvarez B, Jimenez C, Criado LM, Flores J, Marcos MA, Martinez A, Balomenos D & Carrera AC (2000) Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. FASEB J 14: 895-903.

Borradori L, Koch PJ, Niessen CM, Erkeland S, van Leusden MR & Sonnenberg A (1997) The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the beta4 integrin subunit. J Cell Biol 136: 1333-1347.

Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, Loos M, Pandolfi PP & Walport MJ (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet 19: 56-59.

Brewer GJ, Torricelli JR, Evege EK & Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35: 567-576.

Brodsky B & Shah NK (1995) Protein motifs. 8. The triple-helix motif in proteins. FASEB J 9: 1537-1546.

Brown JC & Timpl R (1995) The collagen superfamily. Int Arch Allergy Immunol 107: 484-490.

Brown KS, Cranley RE, Greene R, Kleinman HK & Pennypacker JP (1981) Disproportionate micromelia (Dmm): an incomplete dominant mouse dwarfism with abnormal cartilage matrix. J Embryol Exp Morphol 62: 165-182.

Buck CA, Edelman JM, Buck CE, Kennedy G & Baldwin HS (1996) Expression patterns of adhesion receptors in the developing mouse lung: functional implications. Cell Adhes Commun 4: 69-87.

Carmeliet P, Lampugnani MG, Moons L. Brevario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oostuyse B, Dewerchin M, Zanetti A, Angellio A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelman R, Lupu F, Herbert JM, Collen D & Dejana E (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 98: 147-157.

Carver W, Terracio L & Borg TK (1993) Expression and accumulation of interstitial collagen in the neonatal rat heart. Anat Rec 236: 511-520.

Cheah KS, Lau ET, Au PK & Tam PP (1991) Expression of the mouse alpha 1(II) collagen gene is not restricted to cartilage during development. Development 111: 945-953.

Chipman SD, Sweet HO, McBride DJ, Jr., Davisson MT, Marks SC, Jr., Shuldiner AR, Wenstrup RJ, Rowe DW & Shapiro JR (1993) Defective pro α2(I) collagen synthesis in a recessive mutation in mice: a model of human osteogenesis imperfecta. Proc Natl Acad Sci USA 90: 1701-1705.

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.

Christie RH, Freeman M & Hyman BT (1996) Expression of the macrophage scavenger receptor, a multifunctional lipoprotein receptor, in microglia associated with senile plaques in Alzheimer's disease. Am J Pathol 148: 399-403.

Cleaver O & Krieg PA (1999) Molecular mechanisms of vascular development. In: Harvey RP & Rosenthal N (eds) Heart Development. Academic Press, San Diego.

Cosgrove D, Meehan DT, Grunkemeyer JA, Kornak JM, Sayers R, Hunter WJ & Samuelson GC (1996) Collagen COL4A3 knockout: a mouse model for autosomal Alport syndrome. Genes Dev 10: 2981-2992.

Costell M, Gustafsson E, Aszodi A, Morgelin M, Bloch W, Hunziker E, Addicks K, Timpl R & Fässler R (1999) Perlecan maintains the integrity of cartilage and some basement membranes. J Cell Biol 147: 1109-1122.

Cross JC, Werb Z & Fisher SJ (1994) Implantation and the placenta: key pieces of the development puzzle. Science 266: 1508-1518.

De Arcangelis A & Georges-Labouesse E (2000) Integrin and ECM functions: roles in vertebrate development. Trends Genet 16: 389-395.

DeSimone DW (1994) Adhesion and matrix in vertebrate development. Curr Opin Cell Biol 6: 747-751.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS & Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356: 215-221.

Dunne DW, Resnick D, Greenberg J, Krieger M & Joiner KA (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc Natl Acad Sci USA 91: 1863-1867.

Dziadek M, Darling P, Bakker M, Overall M, Zhang RZ, Pan TC, Tillet E, Timpl R & Chu ML (1996) Deposition of collagen VI in the extracellular matrix during mouse embryogenesis correlates with expression of the alpha 3(VI) subunit gene. Exp Cell Res 226: 302-315.

Eggleton P, Reid KB & Tenner AJ (1998) C1q-how many functions? How many receptors? Trends Cell Biol 8: 428-431.

Eklund L, Piuhola J, Komulainen J, Sormunen R, Ongvarrasopone C, Fässler R, Muona A, Ilves M, Ruskoaho H, Takala TE & Pihlajaniemi T (2001) Lack of type XV collagen causes a skeletal myopathy and cardiovascular defects in mice. Proc Natl Acad Sci U S A 98: 1194-1199.

El Khoury J, Hickman SE, Thomas CA, Cao L, Silverstein SC & Loike JD (1996) Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. Nature 382: 716-719.

Elomaa O, Kangas M, Sahlberg C, Tuukkanen J, Sormunen R, Liakka A, Thesleff I, Kraal G & Tryggvason K (1995) Cloning of a novel bacterial-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell 80: 603-609.

Elomaa O, Sankala M, Pikkarainen T, Bergmann U, Tuuttila A, Raatikainen-Ahokas A, Sariola H & Tryggvason K (1998) Structure of the human macrophage MARCO receptor and characterization of its bacteria-binding region. J Biol Chem 273: 4530-4538.

Enenstein J, Waleh NS & Kramer RH (1992) Basic FGF and TGF-beta differentially modulate integrin expression of human microvascular endothelial cells. Exp Cell Res 203: 499-503.

Ezer S, Bayes M, Elomaa O, Schlessinger D & Kere J (1999) Ectodysplasin is a collagenous trimeric type II membrane protein with a tumor necrosis factor-like domain and co-localizes with cytoskeletal structures at lateral and apical surfaces of cells. Hum Mol Genet 8: 2079-2086.

Ezer S, Schlessinger D, Srivastava A & Kere J (1997) Anhidrotic ectodermal dysplasia (EDA) protein expressed in MCF-7 cells associates with cell membrane and induces rounding. Hum Mol Genet 6: 1581-1587.

Ferguson BM, Brockdorff N, Formstone E, Ngyuen T, Kronmiller JE & Zonana J (1997) Cloning of Tabby, the murine homolog of the human EDA gene: evidence for a membrane-associated protein with a short collagenous domain. Hum Mol Genet 6: 1589-1594.

Finnish Cancer Registry (1997) Cancer incidence in Finland 1995.

Franssila K (1992) Syövän synty, kasvu ja leviäminen. In: Holsti LR, Roberts PJ & Teppo L (eds) Syöpätaudit 9-20. Kustannus Oy Duodecim, Vammala.

Fraser I, Hughes D & Gordon S (1993) Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. Nature 364: 343-346.

Furuta Y, Ilic D, Kanazawa S, Takeda N, Yamamoto T & Aizawa S (1995) Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. Oncogene 11: 1989-1995.

Fässler R, Schnegelsberg PN, Dausman J, Shinya T, Muragaki Y, McCarthy MT, Olsen BR & Jaenisch R (1994) Mice lacking α1(IX) collagen develop noninflammatory degenerative joint disease. Proc Natl Acad Sci USA 91: 5070-5074.

Garofalo S, Metsäranta M, Ellard J, Smith C, Horton W, Vuorio E & de Crombrugghe B (1993) Assembly of cartilage collagen fibrils is disrupted by overexpression of normal type II collagen in transgenic mice. Proc Natl Acad Sci USA 90: 3825-3829.

Garofalo S, Vuorio E, Metsäranta M, Rosati R, Toman D, Vaughan J, Lozano G, Mayne R, Ellard J & Horton W (1991) Reduced amounts of cartilage collagen fibrils and growth plate anomalies in transgenic mice harboring a glycine-to-cysteine mutation in the mouse type II procollagen α1-chain gene. Proc Natl Acad Sci USA 88: 9648-9652.

George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H & Hynes RO (1993) Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development 119: 1079-1091.

Georges-Labouesse EN, George EL, Rayburn H & Hynes RO (1996) Mesodermal development in mouse embryos mutant for fibronectin. Dev Dyn 207: 145-156.

Gilbert SF (1997) Early vertebrate development: mesoderam and endoderm. In: Developmental Biology 5th edition: 341-388. Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, U.S.A.

Giudice GJ, Emegy DJ & Diaz LA (1992) Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. J Invest Dermatol 99: 243-250.

Giudice GJ, Squiquera HL, Elias PM & Diaz LA (1991) Identification of two collagen domains within the bullous pemphigoid autoantigen, BP180. J Clin Invest 87: 734-738.

Gough PJ, Greaves DR & Gordon S (1998) A naturally occurring isoform of the human macrophage scavenger receptor (SR-A) gene generated by alternative splicing blocks modified LDL uptake. J Lipid Res 39: 531-543.

Gress CJ & Jacenko O (2000) Growth plate compressions and altered hematopoiesis in collagen X null mice. J Cell Biol 149: 983-993.

Gumbiner BM (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84: 345-357.

Gurtner GC, Davis V, Li H, McCoy MJ, Sharpe A & Cybulsky MI (1995) Targeted disruption of the murine VCAM1 gene: essential role of VCAM-1 in chorioallantoic fusion and placentation. Genes Dev 9: 1-14.

Haegel H, Larue L, Ohsugi M, Fedorov L, Herrenknecht K & Kemler R (1995) Lack of β-catenin affects mouse development at gastrulation. Development 121: 3529-3537.

Hampton RY, Golenbock DT, Penman M, Krieger M & Raetz CR (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. Nature 352: 342-344.

Harbers K, Kuehn M, Delius H & Jaenisch R (1984) Insertion of retrovirus into the first intron of α1(I) collagen gene to embryonic lethal mutation in mice. Proc Natl Acad Sci USA 81: 1504-1508.

Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA & Bloomfield CD (1999) The World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues. Report of the Clinical Advisory Committee meeting, Airlie House, Virginia, November, 1997. Ann Oncol 10: 1419-1432.

Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, Wolf-Peeters C, Falini B & Gatter KC (1994) A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 84: 1361-1392.

Heinonen S, Männikkö M, Klement JF, Whitaker-Menezes D, Murphy GF & Uitto J (1999) Targeted inactivation of the type VII collagen gene (Col7a1) in mice results in severe blistering phenotype: a model for recessive dystrophic epidermolysis bullosa. J Cell Sci 112 (Pt 21): 3641-3648.

Hirako Y, Usukura J, Nishizawa Y & Owaribe K (1996) Demonstration of the molecular shape of BP180, a 180-kDa bullous pemphigoid antigen and its potential for trimer formation. J Biol Chem 271: 13739-13745.

Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S & Hynes RO (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103: 229-238.

Hogan B, Constantini F & Lacy E (1986) Manipulating the Mouse Embryo. Cold Spring Harbor Laboratories, Plainview, NY.

Hopkinson SB, Findlay K, deHart GW & Jones JC (1998) Interaction of BP180 (type XVII collagen) and alpha6 integrin is necessary for stabilization of hemidesmosome structure. J Invest Dermatol 111: 1015-1022.

Hopkinson SB & Jones JC (2000) The N terminus of the transmembrane protein BP180 interacts with the N- terminal domain of BP230, thereby mediating keratin cytoskeleton anchorage to the cell surface at the site of the hemidesmosome. Mol Biol Cell 11: 277-286.

Hopkinson SB, Riddelle KS & Jones JCR (1992) Cytoplasmic domain of the 180-kD bullous pemphigoid antigen, a hemidesmosomal component: molecular and cell biologic characterization. J Invest Dermatol 99: 264-270.

Hughes-Jones NC & Gardner B (1979) Reaction between the isolated globular sub-units of the complement component C1q and IgG-complexes. Mol Immunol 16: 697-701.

Hulmes DJS (1992) The collagen superfamily - diverse structures and assemblies. Essays Biochem 27: 49-67.

Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25.

Hynes RO (1996) Targeted mutations in cell adhesion genes: what have we learned from them? Dev Biol 180: 402-412.

Hägg P, Rehn M, Huhtala P, Väisänen T, Tamminen M & Pihlajaniemi T (1998) Type XIII collagen is identified as a plasma membrane protein. J Biol Chem 273: 15590-15597.

Hägg P, Väisänen T, Tuomisto A, Rehn M, Tu H, Huhtala P, Eskelinen S & Pihlajaniemi T (2001) Type XIII collagen: a novel cell adhesion component present in a range of cell-matrix adhesions and in the intercalated discs between cardiac muscle cells. Matrix Biol 19: 727-742.

Iruela-Arispe ML & Sage EH (1991) Expression of type VIII collagen during morphogenesis of the chicken and mouse heart. Dev Biol 144: 107-118.

Jacenko O, LuValle PA & Olsen BR (1993) Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage-to- bone transition. Nature 365: 56-61.

Jaenisch R, Harbers K, Schnieke A, Löhler J, Chumakov I, Jähner D, Grotkopp D & Hoffmann E (1983): Germline integration of Moloney murine leukemia virus at the *Mov13* locus leads to recessive lethal mutation and early embryonic death. Cell 32: 209-216.

Jandl JH (1987) Blood. Textbook of Hematology. Little, Brown and Company, Boston, MA.

Jiang H, Cooper B, Robey FA & Gewurz H (1992a) DNA binds and activates complement via residues 14-26 of the human C1q A chain. J Biol Chem 267: 25597-25601.

Jiang H, Robey FA & Gewurz H (1992b) Localization of sites through which C-reactive protein binds and activates complement to residues 14-26 and 76-92 of the human C1q A chain. J Exp Med 175: 1373-1379.

Juvonen M & Pihlajaniemi T (1992) Characterization of the spectrum of alternative splicing of α1(XIII) collagen transcripts in HT-1080 cells and calvarial tissue resulted in identification of two previously unidentified alternatively spliced sequences, one previously unidentified exon, and nine new mRNA variants. J Biol Chem 267: 24693-24699.

Juvonen M, Pihlajaniemi T & Autio-Harmainen H (1993) Location and alternative splicing of type XIII collagen RNA in the early human placenta. Lab Invest 69: 541-551.

Kapoor R, Sakai LY, Funk S, Roux E, Bornstein P & Sage EH (1988) Type VIII collagen has a restricted distribution in specialized extracellular matrices. J Cell Biol 107: 721-730.

Kaul M & Loos M (1993) C1q, the collagen-like subcomponent of the first component of complement C1, is a membrane protein of guinea pig macrophages. Eur J Immunol 23: 2166-2174.

Kaul M & Loos M (1995) Collagen-like complement component C1q is a membrane protein of human monocyte-derived macrophages that mediates endocytosis. J Immunol 155: 5795-5802.

Kere J, Srivastava AK, Montonen O, Zonana J, Thomas N, Ferguson B, Munoz F, Morgan D, Clarke A, Baybayan P, Chen EY, Ezer S, Saarialho-Kere U, de la Chapelle A & Schlessinger D (1996) X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. Nat Genet 13: 409-416.

Khetarpal U, Robertson NG, Yoo TJ & Morton CC (1994) Expression and localization of COL2A1 mRNA and type II collagen in human fetal cochlea. Hear Res 79: 59-73.

Khillan JS, Olsen AS, Kontusaari S, Sokolov B & Prockop DJ (1991) Transgenic mice that express a mini-gene version of the human gene for type I procollagen (COL1A1) develop a phenotype resembling a lethal form of osteogenesis imperfecta. J Biol Chem 266: 23373-23379.

Kivirikko KI (1993) Collagens and their abnormalities in a wide spectrum of diseases. Ann Med 25: 113-126.

Klein S, Giancotti FG, Presta M, Albelda SM, Buck CA & Rifkin DB (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. Mol Biol Cell 4: 973-982.

Knobel HR, Villiger W & Isliker H (1975) Chemical analysis and electron microscopy studies of human C1q prepared by different methods. Eur J Immunol 5: 78-82.

Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P & Krieger M (1990) Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. Nature 343: 531-535.

Kostin S, Hein S, Bauer EP & Schaper J (1999) Spatiotemporal development and distribution of intercellular junctions in adult rat cardiomyocytes in culture. Circ Res 85: 154-167.

Krengel S, Gotz W & Herken R (1996) Expression pattern of type II collagen mRNA during early vertebral development in the human embryo. Anat Embryol (Berl) 193: 43-51.

Krieger M & Herz J (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annu Rev Biochem 63: 601-637.

Krumdieck R, Hook M, Rosenberg LC & Volanakis JE (1992) The proteoglycan decorin binds C1q and inhibits the activity of the C1 complex. J Immunol 149: 3695-3701.

Kvist A-P, Latvanlehto A, Sund M, Eklund L, Väisänen T, Hägg PO, Sormunen R, Komulainen J, Fässler R & Pihlajaniemi T (2001) Lack of cytosolic and transmembrane domains of type XIII collagen results in progressive myopathy, Am J Pathol 159: 1581-1592.

Kvist AP, Latvanlehto A, Sund M, Horelli-Kuitunen N, Rehn M, Palotie A, Beier D & Pihlajaniemi T (1999) Complete exon-intron organization and chromosomal location of the gene for mouse type XIII collagen (col13a1) and comparison with its human homologue. Matrix Biol 18: 261-274.

Kwan KM, Pang MK, Zhou S, Cowan SK, Kong RY, Pfordte T, Olsen BR, Sillence DO, Tam PP & Cheah KS (1997) Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. J Cell Biol 136: 459-471.

Kwee L, Baldwin HS, Shen HM, Stewart CL, Buck C, Buck CA & Labow MA (1995) Defective development of the embryonic and extraembryonic circulatory systems in vascular cell adhesion molecule (VCAM-1) deficient mice. Development 121: 489-503.

Labib RS, Anhalt GJ, Patel HP, Mutasim DF & Diaz LA (1986) Molecular heterogeneity of bullous pemphigoid antigens as detected by immunoblotting. J Immunol 136: 1231-1235.

Lai CH & Chu ML (1996) Tissue distribution and developmental expression of type XVI collagen in the mouse. Tissue Cell 28: 155-164.

Larue L, Ohsugi M, Hirchenhain J & Kemler R (1994) E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc Natl Acad Sci USA 91: 8263-8267.

Letourneau PC, Condic ML & Snow DM (1994) Interactions of developing neurons with the extracellular matrix. J Neurosci 14: 915-928.

Li K, Sawamura D, Giudice GJ, Diaz LA, Mattei M-G, Chu ML & Uitto J (1991) Genomic organization of collagenous domains and chromosomal assignment of human 180-kDa bullous pemphigoid antigen-2, a novel collagen of stratified squamous epithelium. J Biol Chem 266: 24064-24069.

Li K, Tamai K, Tan EM & Uitto J (1993) Cloning of type XVII collagen. Complementary and genomic DNA sequences of mouse 180-kilodalton bullous pemphigoid antigen (BPAG2) predict an interrupted collagenous domain, a transmembrane segment, and unusual features in the 5'-end of the gene and the 3'-untranslated region of the mRNA. J Biol Chem 268: 8825-8834.

Li SW, Prockop DJ, Helminen H, Fässler R, Lapveteläinen T, Kiraly K, Peltarri A, Arokoski J, Lui H & Arita M (1995a) Transgenic mice with targeted inactivation of the Col2 α1 gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. Genes Dev 9: 2821-2830.

Li Y, Lacerda A, Warman ML, Beier DR, Yoshioka H, Ninomiya Y, Oxford JT, Morris NP, Andrikopoulos K, Ramirez F, Wardell BB, Lifferth GD, Teuscher C, Woodward SR, Taylor BA, Seegmiller RE & Olsen BR (1995b) A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. Cell 80: 423-430.

Lin CQ & Bissell MJ (1993) Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J 7: 737-743.

Lin Y, Zhang S, Rehn M, Itäranta P, Tuukkanen J, Heljasvaara R, Peltoketo H, Pihlajaniemi T & Vainio S (2001) Induced repatterning of type XVIII collagen expression in ureter bud from kidney to lung type: association with sonic hedgehog and ectopic surfactant protein C. Development 128: 1573-1585.

Liu CY, Olsen BR & Kao WW (1993) Developmental patterns of two α1(IX) collagen mRNA isoforms in mouse. Dev Dyn 198: 150-157.

Liu X, Wu H, Byrne M, Krane S & Jaenisch R (1997) Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. Proc Natl Acad Sci USA 94: 1852-1856.

Lohi J, Korhonen M, Leivo I, Kangas L, Tani T, Kalluri R, Miner JH, Lehto VP & Virtanen I (1997) Expression of type IV collagen alpha1(IV)-alpha6(IV) polypeptides in normal and developing human kidney and in renal cell carcinomas and oncocytomas. Int J Cancer 72: 43-49.

Lohler J, Timpl R & Jaenisch R (1984) Embryonic lethal mutation in mouse collagen I gene causes rupture of blood vessels and is associated with erythropoietic and mesenchymal cell death. Cell 38: 597-607.

Lu W, Phillips CL, Killen PD, Hlaing T, Harrison WR, Elder FFB, Miner JH, Overbeek PA & Meisler MH (1999): Insertional mutation of the collagen genes col4α3 and col4α4 in a mouse model of Alport syndrome. Genomics 61: 113-124.

Lui VC, Kong RY, Nicholls J, Cheung AN & Cheah KS (1995a) The mRNAs for the three chains of human collagen type XI are widely distributed but not necessarily co-expressed: implications for homotrimeric, heterotrimeric and heterotypic collagen molecules. Biochem J 311 (Pt 2): 511-516.

Lui VC, Ng LJ, Nicholls J, Tam PP & Cheah KS (1995b) Tissue-specific and differential expression of alternatively spliced alpha 1(II) collagen mRNAs in early human embryos. Dev Dyn 203: 198-211.

Luscinskas FW & Lawler J (1994) Integrins as dynamic regulators of vascular function. FASEB J 8: 929-938.

LuValle P, Daniels K, Hay ED & Olsen BR (1992) Type X collagen is transcriptionally activated and specifically localized during sternal cartilage maturation. Matrix 12: 404-413.

Marvulli D, Volpin D & Bressan GM (1996) Spatial and temporal changes of type VI collagen expression during mouse development. Dev Dyn 206: 447-454.

Mayer U, Saher G, Fässler R, Bornemann A, Echtermeyer F, von der M, Miosge N & Poschl E (1997) Absence of integrin α7 causes a novel form of muscular dystrophy. Nat Genet 17: 318-323.

Metsäranta M, Garofalo S, Decker G, Rintala M, de Crombrugghe B & Vuorio E (1992) Chondrodysplasia in transgenic mice harboring a 15-amino acid deletion in the triple helical domain of pro α1(II) collagen chain. J Cell Biol 118: 203-212.

Michelson PH, Tigue M & Jones JC (2000) Human bronchial epithelial cells secrete laminin 5, express hemidesmosomal proteins, and assemble hemidesmosomes. J Histochem Cytochem 48: 535-544.

Mikkola ML, Pispa J, Pekkanen M, Paulin L, Nieminen P, Kere J & Thesleff I (1999) Ectodysplasin, a protein required for epithelial morphogenesis, is a novel TNF homologue and promotes cell-matrix adhesion. Mech Dev 88: 133-146.

Miner JH, Cunningham J & Sanes JR (1998) Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. J Cell Biol 143: 1713-1723.

Miner JH & Sanes JR (1994) Collagen IV alpha 3, alpha 4, and alpha 5 chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches. J Cell Biol 127: 879-891.

Miner JH & Sanes JR (1996) Molecular and functional defects in kidneys of mice lacking collagen alpha 3(IV): implications for Alport syndrome. J Cell Biol 135: 1403-1413.

Montonen O, Ezer S, Saarialho-Kere UK, Herva R, Karjalainen-Lindsberg ML, Kaitila I, Schlessinger D, Srivastava AK, Thesleff I & Kere J (1998) The gene defective in anhidrotic ectodermal dysplasia is expressed in the developing epithelium, neuroectoderm, thymus, and bone. J Histochem Cytochem 46: 281-289.

Moorman AFM & Lamers WH (1999) Development of the conduction system of the vertebrate heart. In: Harvey M & Rosenthal N (eds) Heart Development 195-207. Academic Press, San Diego, CA.

Morgan BP & Walport MJ (1991) Complement deficiency and disease. Immunol Today 12: 301-306.

Morgan G, Vornanen M, Puitinen J, Naukkarinen A, Brincker H, Olsen J, Coeburgh JW, Vrints LW, Clayden D, McNally R, Jack A, Carli PM, Petrella T, Tomino R, D'Lollo S, Barchielli A & Cartwright R (1997) Changing trends in the incidence of non-Hodgkin's lymphoma in Europe. Biomed Study Group. Ann Oncol 8 Suppl 2: 49-54.

Muona A, Eklund L, Sormunen R, Väisänen T & Pihlajaniemi T (2001) Developmentally regulated expression of type XV collagen correlates with abnormalities in Col15a1-/- mice. In press. Matrix Biol

Müller U & Kupta R (1995) Molecular genetics of neuronal adhesion. Curr Opin Neurobiol 5:36-41.

Myllyharju J & Kivirikko KI (2001) Collagens and collagen-related diseases. Ann Med 33: 7-21.

Nakata K, Ono K, Miyazaki J, Olsen BR, Muragaki Y, Adachi E, Yamamura K & Kimura T (1993) Osteoarthritis associated with mild chondrodysplasia in transgenic mice expressing α1(IX) collagen chains with a central deletion. Proc Natl Acad Sci USA 90: 2870-2874.

Nerlich AG, Kirsch T, Wiest I, Betz P & von der MK (1992) Localization of collagen X in human fetal and juvenile articular cartilage and bone. Histochemistry 98: 275-281.

Niederreither K, D'Souza R, Metsäranta M, Eberspaecher H, Toman PD, Vuorio E & de Crombrugghe B (1995) Coordinate patterns of expression of type I and III collagens during mouse development. Matrix Biol 14: 705-713.

Niessen CM, Raaij-Helmer MH, Hulsman EH, van der NR, Jonkman MF & Sonnenberg A (1996) Deficiency of the integrin beta 4 subunit in junctional epidermolysis bullosa with pyloric atresia: consequences for hemidesmosome formation and adhesion properties. J Cell Sci 109 (Pt 7): 1695-1706.

Novak U & Kaye AH (2000) Extracellular matrix and the brain: components and function. J Clin Neurosci 7: 280-290.

Nykvist P, Tu H, Ivaska J, Käpylä J, Pihlajaniemi T & Heino J (2000) Distinct recognition of collagen subtypes by α1β1 and α2β1 integrins. α1β1 mediates cell adhesion to type XIII collagen. J Biol Chem 275: 8255-8261.

Oh SP, Griffith CM, Hay ED & Olsen BR (1993) Tissue-specific expression of type XII collagen during mouse embryonic development. Dev Dyn 196: 37-46.

Otter R, Bieger R, Kluin PM, Hermans J & Willemze R (1989) Primary gastrointestinal non-Hodgkins's lymphoma in a population-based registry. Br J Cancer 60: 745-750.

Pace JM, Li Y, Seegmiller RE, Teuscher C, Taylor BA & Olsen BR (1997) Disproportionate micromelia (Dmm) in mice caused by a mutation in the C-propeptide coding region of Col2a1. Dev Dyn 208: 25-33.

Palecanda A, Paulauskis J, Al Mutairi E, Imrich A, Qin G, Suzuki H, Kodama T, Tryggvason K, Koziel H & Kobzik L (1999) Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. J Exp Med 189: 1497-1506.

Pardanaud L, Yassine F & Dieterlen-Lievre F (1989) Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. Development 105: 473-485.

Paresce DM, Ghosh RN & Maxfield FR (1996) Microglial cells internalize aggregates of the Alzheimer's disease amyloid beta-protein via a scavenger receptor. Neuron 17: 553-565.

PE Applied Biosystems (1997) Relative quantification of gene expression. User Bulletin number 2, Abi Prism 7700 Sequence Detection System.

Peltonen S, Hentula M, Hägg P, Ylä-Outinen H, Tuukkanen J, Lakkakorpi J, Rehn M, Pihlajaniemi T & Peltonen J (1999) A novel component of epidermal cell-matrix and cell-cell contacts: transmembrane protein type XIII collagen. J Invest Dermatol 113: 635-642.

Peltonen S, Rehn M & Pihlajaniemi T (1997) Alternative splicing of mouse α1(XIII) collagen RNAs results in at least 17 different transcripts, predicting α1(XIII) collagen chains with length varying between 651 and 710 amino acid residues. DNA Cell Biol 16: 227-234.

Pereira R, Khillan JS, Helminen HJ, Hume EL & Prockop DJ (1993) Transgenic mice expressing a partially deleted gene for type I procollagen (COL1A1). A breeding line with a phenotype of spontaneous fractures and decreased bone collagen and mineral. J Clin Invest 91: 709-716.

Perälä M, Savontaus M, Metsäranta M & Vuorio E (1997) Developmental regulation of mRNA species for types II, IX and XI collagens during mouse embryogenesis. Biochem J 324 (Pt 1): 209-216.

Pihlajaniemi T, Myllylä R, Seyer J, Kurkinen M & Prockop DJ (1987) Partial characterization of a low molecular weight human collagen that undergoes alternative splicing. Proc Natl Acad Sci USA 84: 940-944.

Pihlajaniemi T & Rehn M (1995) Two new collagen subgroups: Membrane associated collagens and types XV and XVIII. Prog Nucleic Acid Res Mol Biol 50: 225-262.

Pihlajaniemi T & Tamminen M (1990) The α1 chain of type XIII collagen consists of three collagenous and four noncollagenous domains, and its primary transcript undergoes complex alternative splicing. J Biol Chem 265: 16922-16928.

Portier MM, Escurat M, Landon F, Djabali K & Bousquet O (1993) Peripherin and neurofilaments: expression and role during neural development. C R Acad Sci III 316: 1124-1140.

Prockop DJ & Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64: 403-434.

Pulkkinen L & Uitto J (1998) Hemidesmosomal variants of epidermolysis bullosa. Mutations in the alpha6beta4 integrin and the 180-kD bullous pemphigoid antigen/type XVII collagen genes. Exp Dermatol 7: 46-64.

Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M & Hynes RO (1997) Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181: 64-78.

Raulo E, Julkunen I, Merenmies J, Pihlaskari R & Rauvala H (1992) Secretion and biological activities of heparin-binding growth- associated molecule. Neurite outgrowth-promoting and mitogenic actions of the recombinant and tissue-derived protein. J Biol Chem 267: 11408-11416.

Rauvala H (1989) An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. EMBO J 8: 2933-2941.

Rauvala H, Merenmies J, Pihlaskari R, Korkolainen M, Huhtala ML & Panula P (1988) The adhesive and neurite-promoting molecule p30: analysis of the amino- terminal sequence and production of antipeptide antibodies that detect p30 at the surface of neuroblastoma cells and of brain neurons. J Cell Biol 107: 2293-2305.

Reichenberger E, Baur S, Sukotjo C, Olsen BR, Karimbux NY & Nishimura I (2000): Collagen XII mutation disrupts matrix structure of periodontal ligament and skin. J Dent Res 79: 1962-1968.

Resnick D, Chatterton JE, Schwartz K, Slayter H & Krieger M (1996) Structures of class A macrophage scavenger receptors. Electron microscopic study of flexible, multidomain, fibrous proteins and determination of the disulfide bond pattern of the scavenger receptor cysteine-rich domain. J Biol Chem 271: 26924-26930.

Riethmacher D, Brinkmann V & Birchmeier C (1995) A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. Proc Natl Acad Sci USA 92: 855-859.

Robbins SL, Kumar V & Cotran RS (1999a) Diseases of white cells, lymph nodes and the spleen. In: Robbins SL, Kumar V & Cotran RS (eds) Pathologic Basis of Disease 629-672. WB Saunders Company, Philadelphia, PA.

Robbins SL, Kumar V & Cotran RS (1999b) Neoplasia. In: Robbins SL, Kumar V & Cotran RS (eds) Pathologic Basis of Disease 241-303. WB Saunders Company, Philadelphia, PA.

Rohrer L, Freeman M, Kodama T, Penman M & Krieger M (1990) Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. Nature 343: 570-572.

Roman J & McDonald JA (1992) Expression of fibronectin, the integrin alpha 5, and alpha-smooth muscle actin in heart and lung development. Am J Respir Cell Mol Biol 6: 472-480.

Rosati R, Horan GS, Pinero GJ, Garofalo S, Keene DR, Horton WA, Vuorio E, de Crombrugghe B & Behringer RR (1994) Normal long bone growth and development in type X collagen-null mice. Nat Genet 8: 129-135.

Rossant J (1996) Mouse mutants and cardiac development: new molecular insights into cardiogenesis. Circ Res 78: 349-353.

Ruiz P, Brinkmann V, Ledermann B, Behrend M, Grund C, Thalhammer C, Vogel F, Birchmeier C, Gunthert U, Franke WW & Birchmeier W (1996) Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart. J Cell Biol 135: 215-225.

Ryynänen J, Sollberg S, Parente MG, Chung LC, Christiano AM & Uitto J (1992) Type VII collagen gene expression by cultured human cells and in fetal skin. Abundant mRNA and protein levels in epidermal keratinocytes. J Clin Invest 89: 163-168.

Saarela J (1998) Type XV and XVIII collagen. Structures of variant human a1(XVIII) chains, comparison of expression of type XV and XVIII collagen mRNA transcripts, and location of type XVIII protein in mature and developing human and mouse tissues. Acta Univ Oul D 449.

Sage H & Iruela-Arispe ML (1990) Type VIII collagen in murine development. Association with capillary formation in vitro. Ann N Y Acad Sci 580: 17-31.

Sandberg-Lall M, Hägg PO, Wahlström I & Pihlajaniemi T (2000) Type XIII collagen is widely expressed in the adult and developing human eye and accentuated in the ciliary muscle, the optic nerve and the neural retina. Exp Eye Res 70: 401-410.

Sandberg M, Tamminen M, Hirvonen H, Vuorio E & Pihlajaniemi T (1989) Expression of mRNAs coding for the α1 chain of type XIII collagen in human fetal tissues: comparison with expression of mRNAs for collagen types I, II, and III. J Cell Biol 109: 1371-1379.

Sandberg M & Vuorio E (1987) Localization of types I, II, and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. J Cell Biol 104: 1077-1084.

Sandberg MM, Hirvonen HE, Elima KJ & Vuorio EI (1993) Co-expression of collagens II and XI and alternative splicing of exon 2 of collagen II in several developing human tissues. Biochem J 294 (Pt 2): 595-602.

Sandell LJ, Morris N, Robbins JR & Goldring MB (1991) Alternatively spliced type II procollagen mRNAs define distinct populations of cells during vertebral development: differential expression of the amino-propeptide. J Cell Biol 114: 1307-1319.

Sandell LJ, Nalin AM & Reife RA (1994) Alternative splice form of type II procollagen mRNA (IIA) is predominant in skeletal precursors and non-cartilaginous tissues during early mouse development. Dev Dyn 199: 129-140.

Sariola H (1985) Interspecies chimeras: an experimental approach for studies on embryonic angiogenesis. Med Biol 63: 43-65.

Savontaus M, Ihanamäki T, Metsäranta M, Vuorio E & Sandberg-Lall M (1997) Localization of type II collagen mRNA isoforms in the developing eyes of normal and transgenic mice with a mutation in type II collagen gene. Investigative Ophthalmology & Visual Science 38: 930-942.

Seidman JG (1999) Manipulating the mouse genome. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K (eds) Current protocols in molcular biology 23.0.1-23.7.20. John Wiley and Sons.

Schacke H, Schumann H, Hammami-Hauasli N, Raghunath M & Brückner-Tuderman L (1998) Two forms of collagen XVII in keratinocytes. A full-length transmembrane protein and a soluble ectodomain. J Biol Chem 273: 25937-25943.

Schoen FJ (1999) Bloodvessels. In: Robbins SL, Kumar V & Cotran RS (eds) Pathologic Basis of Disease 467-516. WB Saunders Company, Philadelphia, PA.

Schoenfeld JR, Vasser M, Jhurani P, Ng P, Hunter JJ, Ross J, Jr., Chien KR & Lowe DG (1998) Distinct molecular phenotypes in murine cardiac muscle development, growth, and hypertrophy. J Mol Cell Cardiol 30: 2269-2280.

Seegmiller R, Fraser FC & Sheldon H (1971) A new chondrodystrophic mutant in mice. Electron microscopy of normal and abnormal chondrogenesis. J Cell Biol 48: 580-593.

Seldin DC (1995) New models of lymphoma in transgenic mice. Curr Opin Immunol 7: 665-673.

Shows TB, Tikka L, Byers MG, Eddy RL, Haley LL, Henry WM, Prockop DJ & Tryggvason K (1989) Assignment of the human collagen α1(XIII) chain gene (COL13A1) to the q22 region of chromosome 10. Genomics 5: 128-133.

Smolich JJ (1995) Ultrastructural and functional features of the developing mammalian heart: a brief overview. Reprod Fertil Dev 7: 451-461.

Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M & Edgar D (1999) Abscence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to gailure of endoderm differentiation. J Cell Biol 144: 151-160.

Snellman A, Keränen MR, Hägg PO, Lamberg A, Hiltunen JK, Kivirikko KI & Pihlajaniemi T (2000a) Type XIII collagen forms homotrimers with three triple helical collagenous domains and its association into disulfide-bonded trimers is enhanced by prolyl 4-hydroxylase. J Biol Chem 275: 8936-8944.

Snellman A, Tu H, Väisänen T, Kvist AP, Huhtala P & Pihlajaniemi T (2000b) A short sequence in the N-terminal region is required for the trimerization of type XIII collagen and is conserved in other collagenous transmembrane proteins. EMBO J 19: 5051-5059.

Srivastava AK, Pispa J, Hartung AJ, Du Y, Ezer S, Jenks T, Shimada T, Pekkanen M, Mikkola ML, Ko MS, Thesleff I, Kere J & Schlessinger D (1997) The Tabby phenotype is caused by mutation in a mouse homologue of the EDA gene that reveals novel mouse and human exons and encodes a protein (ectodysplasin-A) with collagenous domains. Proc Natl Acad Sci USA 94: 13069-13074.

Stacey A, Bateman J, Choi T, Mascara T, Cole W & Jaenisch R (1988) Perinatal lethal osteogenesis imperfecta in transgenic mice bearing an engineered mutant pro-α1(I) collagen gene. Nature 332: 131-136.

Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA & Damsky CH (1995) Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev 9: 1883-1895.

Sumiyoshi H, Inoguchi K, Khaleduzzaman M, Ninomiya Y & Yoshioka H (1997) Ubiquitous expression of the α1(XIX) collagen gene (Col19a1) during mouse embryogenesis becomes restricted to a few tissues in the adult organism. J Biol Chem 272: 17104-17111.

Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Noda T, Toyoda Y, Itakura H, Yazaki Y & Kodama T (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature 386: 292-296.

Takeichi M, Watabe M, Shibamoto S, Ito F, Oda H, Uemura T & Shimamura K (1993) Dynamic control of cell-cell adhesion for multicellular organization. C R Acad Sci III 316: 813-821.

Teereenhovi L & Sauvo M (1992) Lymfoomat. In: Holsti LR, Roberts PJ & Teppo L (eds) Syöpätaudit 415-430. Kustannus Oy Duodecim, Vammala.

Tenner AJ (1999) Membrane receptors for soluble defense collagens. Curr Opin Immunol 11: 34-41.

Thiel S & Reid KB (1989) Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. FEBS Lett 250: 78-84.

Tikka L, Elomaa O, Pihlajaniemi T & Tryggvason K (1991) Human α1(XIII) collagen gene. Multiple forms of the gene transcripts are generated through complex alternative splicing of several short exons. J Biol Chem 266: 17713-17719.

Tikka L, Pihlajaniemi T, Henttu P, Prockop DJ & Tryggvason K (1988) Gene structure for the α1 chain of a human short-chain collagen (type XIII) with alternatively spliced transcripts and translation termination codon at the 5' end of the last exon. Proc Natl Acad Sci USA 85: 7491-7495.

Tomaselli KJ (1991) Beta 1-integrin-mediated neuronal responses to extracellular matrix proteins. Ann N Y Acad Sci 633: 100-104.

Tomaselli KJ, Hall DE, Flier LA, Gehlsen KR, Turner DC, Carbonetto S & Reichardt LF (1990) A neuronal cell line (PC12) expresses two beta 1-class integrins-alpha 1 beta 1 and alpha 3 beta 1-that recognize different neurite outgrowth- promoting domains in laminin. Neuron 5: 651-662.

Tono-Oka S, Tanase S, Miike T & Tanaka H (1996) Transient expression of collagen type XIV during muscle development and its reappearance after denervation and degeneration. J Histochem Cytochem 44: 907-918.

van der Rest M & Garrone R (1991) Collagen family of proteins. FASEB J 5: 2814-2823.

Venstrom KA & Reichardt LF (1993) Extracellular matrix. 2: Role of extracellular matrix molecules and their receptors in the nervous system. FASEB J 7: 996-1003.

Vuorio E & de Crombrugghe B (1990) The family of collagen genes. Annu Rev Biochem 59: 837-872.

Wang T & Brown MJ (1999) mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. Anal Biochem 269: 198-201.

Williams RL, Courtneidge SA & Wagner EF (1988) Embryonic lethalities and endothelial tumors in chimeric mice expressing polyoma virus middle T oncogene. Cell 52: 121-131.

Wilson D (1983) The origin of the endothelium in the developing marginal vein of the chick wing-bud. Cell Differ 13: 63-67.

Winer J, Jung CK, Shackel I & Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270: 41-49.

Xu W, Baribault H & Adamson E (1998) Vinculin knockout results in heart and brain defects during embryonic development. Dev Suppl 125: 327-337.

Yamada Y, Doi T, Hamakubo T & Kodama T (1998) Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. Cell Mol Life Sci 54: 628-640.

Yang JT, Rayburn H & Hynes RO (1993) Embryonic mesodermal defects in α5 integrin-deficient mice. Development 119: 1093-1105.

Yang JT, Rayburn H & Hynes RO (1995) Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. Development 121: 549-560.

Yang TY, Chen SC, Leach MW, Manfra D, Homey B, Wiekowski M, Sullivan L, Jenh CH, Narula SK, Chensue SW & Lira SA (2000) Transgenic expression of the chemokine receptor encoded by human herpesvirus 8 induces an angioproliferative disease resembling Kaposi's sarcoma. J Exp Med 191: 445-454.

Yee SP, Mock D, Greer P, Maltby V, Rossant J, Bernstein A & Pawson T (1989) Lymphoid and mesenchymal tumors in transgenic mice expressing the v-fps protein-tyrosine kinase. Mol Cell Biol 9: 5491-5499.

Ying SC, Gewurz AT, Jiang H & Gewurz H (1993) Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14-26 and 76-92 of the A chain collagen-like region of C1q. J Immunol 150: 169-176.

Yoshioka H, Iyama K, Inoguchi K, Khaleduzzaman M, Ninomiya Y & Ramirez F (1995) Developmental pattern of expression of the mouse α1(XI) collagen gene (Col11a1). Dev Dyn 204: 41-47.

Young GA (1999) Lymphoma at uncommon sites. Hematol Oncol 17: 53-83.