OULU 2007
D 956

ACTA UNIVERSITATIS OULUENSIS

Juha Jäälinoja

THE STRUCTURE AND FUNCTION OF NORMAL AND MUTATED COLLAGEN IX

FACULTY OF MEDICINE,
DEPARTMENT OF MEDICAL BIOCHEMISTRY AND MOLECULAR BIOLOGY,
BIOCENTER OULU,
UNIVERSITY OF OULU

ACTA UNIVERSITATIS OULUENSIS
D Medica 956

JUHA JÄÄLINOJA

THE STRUCTURE AND FUNCTION OF NORMAL AND MUTATED COLLAGEN IX

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium F101 of the Department of Physiology (Aapistie 7), on December 21st, 2007, at 1 p.m.

OULUN YLIOPISTO, OULU 2007

Acta Univ. Oul. D 956, 2007

Supervised by
Professor Leena Ala-Kokko

Reviewed by
Professor Yrjö Konttinen
Docent Asta Pirskanen

ISBN 978-951-42-8653-7 (Paperback)
ISBN 978-951-42-8654-4 (PDF)
http://herkules.oulu.fi/isbn978951428654 4/
ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)
http://herkules.oulu.fi/issn03553221/

Cover design
Raimo Ahonen

OULU UNIVERSITY PRESS
OULU 2007

Jäälinoja, Juha, The structure and function of normal and mutated collagen IX
Faculty of Medicine, Department of Medical Biochemistry and Molecular Biology, University of Oulu, Biocenter Oulu, University of Oulu, P.O. Box 5000, FI-90014 University of Oulu, Finland
Acta Univ. Oul. D 956, 2007
Oulu, Finland

Abstract

Collagen IX belongs to the superfamily of collagenous proteins and is present on the surface of the heterotypic collagen fibrils that are predominantly composed of collagen II, and also collagen XI. The major sites of expression of collagen IX include the articular cartilage, intervertebral disc, inner ear and the vitreous body of the eye.

Previous reports have indicated that mutations in the genes encoding the three polypeptide chains of collagen IX may lead to intervertebral disc disease and multiple epiphyseal dysplasia, a chondrodysplasia characterized by early onset osteoarthritis. These observations and results from genetically modified mouse lines suggest that collagen IX is crucial in the maintenance of the long-term integrity of tissues. However, the structure-function relationship as well as detailed information concerning the functional roles of this protein has remained unclear.

Recombinant human collagen IX was obtained using an insect cell expression system. Besides full-length molecules, five truncated variants of collagen IX were produced to examine chain association and trimerization. Contrary to previous observations, it was shown that the COL1 and NC1 domains are not essential for trimerization. Instead, they seem to play an important role in the specificity of chain selection. The results also suggest that the N-terminal domains, NC3 or COL3, are required for complete folding and stabilization of collagen IX molecules, implicating cooperativity between different domains in the folding process.

Collagen IX was found to mediate cell adhesion and bind efficiently to collagen receptor integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$. The binding was found to represent a novel type of mechanism, and the binding site of the integrin I domain was located at the N-terminal end of the COL3 domain in collagen IX. The obtained results suggest that the FACITs may play an important role as mediators of cell adhesion to collagen fibrils.

Antibodies binding to human recombinant collagen IX were measured among 53 patients with seropositive rheumatoid arthritis (RA). These autoantibodies were significantly elevated among the RA patients when compared to the controls, suggesting that autoantibodies to collagen IX show diagnostic potential in early RA. However, no association was found between the antibody levels and outcome.

Keywords: collagen type IX, extracellular matrix, integrins, rheumatoid arthritis

Acknowledgements

This work was carried out at the Collagen Research Unit of the Department of Medical Biochemistry and Molecular Biology, University of Oulu, and Biocenter Oulu, during the years 1999-2007.

I wish to express my deepest gratitude to my supervisor, Professor Leena Ala-Kokko, for her encouragement, optimism and trust throughout these years. I would like to express my sincere thanks to Professors Kari Kivirikko, Taina Pihlajaniemi, Ilmo Hassinen and Johanna Myllyharju for providing excellent research facilities and creating a high-standard scientific environment as well as Professor Peppi Karppinen for leadership with a bright mind and tender heart.

I wish to acknowledge all my co-authors. Tero Pihlajamaa is warmly acknowledged for his mentorship and collaboration during the early steps of my scientific career. Joni Ylöstalo also deserves my thanks for his cooperation. More recently, I have had the privilege to work with Professor David Hulmes, largely recognized for his expertise in collagen fibrillogenesis, Docent Jarmo Käpylä, an expert in the field of integrins, and Professor Markku Hakala as well as Docent Markku Kauppi and colleagues, specialists in clinical medicine, especially in rheumatoid arthritis. Docent Sohvi Hörkkö deserves my deepest gratitude for her continuous encouragement and positive attitude as well as expertise regarding the ELISA work and immunology in general. In this regard I am also grateful to Professor Riitta Karttunen for her help in providing the controls for the study. Professor Yrjö Konttinen and Docent Asta Pirskainen are gratefully recognized for their valuable comments on this thesis and Sandra Hänninen, M.Sc., for the careful revision of the language.

Among the department personnel, I am especially grateful to Hongmin Tu who has always offered a helping hand when needed. Docent Minna Männikkö is warmly recognized not only for her support and good advice but also for open and relaxed conversations regarding any given subject. My warmest thanks belong also to Professor Johanna Myllyharju for her expert advice. I also want to thank Pertti Vuokila, Marja Leena Karjalainen, Marja-Leena Kivelä, Auli Kinnunen, Seppo Lähdesmäki and Risto Helminen for looking after the practical errands in the department.

I want to express my dearest thanks to Helena Satulehto for all her efforts in the lab; they have been instrumental in achieving this goal. Thanks also to the other technicians in our group, including Aira Harju and especially Minta Lumme, with whom I had many enjoyable conversations. Thanks to Eveliina

Jakkula and Olli-Pekka Kämäräinen for sharing their room and their thoughts. Thanks for many interesting conversations and companionship with Heini, Marja, Iita, Merja, and Mirka. Thanks also to other members of the group, both former and current, who are not mentioned by name.

I thank Anne Heikkinen, Eveliina Jakkula, Tero Pihlajaniemi and Jarmo Käpylä for their contribution to the tables and figures appearing in the text. I am also grateful to Marko Kervinen for his expertise in modifying figures into appropriate form.

Above all, I wish to thank my parents for their never-ending love and support. Finally, I am grateful to my beloved wife, Minna, for her love and understanding, and to our children – two little princesses who have melted my heart for good.

This thesis work was supported financially by the Finnish Centre of Excellence Programme (2000-2005) of the Academy of Finland, the Finnish Cultural Foundation (Northern Osthrobotnian Fund), the Tyyni Tani Foundation and the Maud Kuistila Foundation.

Oulu, September 7, 2007 Juha Jäälinoja

Abbreviations

BM	basement membrane
CCP	cyclic citrullinated protein
CIA	collagen-induced arthritis
CIX	collagen IX
COL	collagenous domain
COMP	cartilage oligomeric matrix protein
CRP	C-reactive protein
DMARD	disease-modifying anti-rheumatic drug
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FACIT	fibril associated collagen with interrupted triple helices
FcγR	Fc gamma receptor
GAG	glycosaminoglycan
IC	immune complex
IDD	intervertebral disc disease
Ig	immunoglobulin
IL	interleukin
MED	multiple epiphyseal dysplasia
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NC	noncollagenous domain
OA	osteoarthritis
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PPI	peptidyl proline cis/trans isomerase
RA	rheumatoid arthritis
rCIX	recombinant human collagen IX
RF	rheumatoid factor
SLRP	small leucine-rich repeat proteoglycans
TNFα	tumor necrosis factor alpha
vWFA	von Willebrand factor A-like domain
X or Y (in Gly-X-Y)	any amino acid

List of original articles

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

I Jäälinoja J, Ylöstalo J, Beckett W, Hulmes DJS & Ala-Kokko L (2007) Trimerization of collagen IX α chains does not require the presence of the COL1 and NC1 domains. Biochem J. In press.

II Käpylä J, Jäälinoja J, Tulla M, Ylöstalo J, Nissinen L, Viitasalo T, Vehviläinen P, Marjomäki V, Nykvist P, Säämänen AM, Farndale RW, Birk DE, Ala-Kokko L & Heino J (2004) The fibril-associated collagen IX provides a novel mechanism for cell adhesion to cartilaginous matrix. J Biol Chem 279: 51677-87.

III Jäälinoja J, Nissilä M, Kauppi M, Hakala M, Laiho, K, Karttunen R & Ala-Kokko L (2007) Serum antibodies against intact human collagen IX are elevated at the onset of RA but are not related to the development of erosions. Manuscript.

Contents

1 Introduction

The evolution of multicellular organisms has led to the formation of an extracellular matrix (ECM). This material provides structural support for the cells and also participates in many active processes in coordination with cells. These processes include development and morphogenesis, wound healing and tissue regeneration, inflammation, tumor growth and metastasis, for example. Specifically, cell survival, apoptosis, migration and proliferation as well as angiogenesis, formation and breakdown of matrix and release of growth factors and other components are regulated by the coordinated interplay between cells and the surrounding matrix. Thus, the ECM is far from an inert structural scaffold; rather it plays multiple important active roles in numerous physiological functions.

The main constituents of the ECM are proteoglycans, structural glycoproteins, such as laminin and fibronectin, and fibrils of elastin and collagen. The collagens are a family of structurally related proteins serving in a multitude of functional roles within the ECM of virtually all tissues. The typical feature of collagens is their triple-helical structure, which provides tissues with flexibility, rigidity and strength. Collagens are traditionally classified as structural proteins of the ECM, but they also have a wide variety of distinct functional motifs, and therefore participate in multiple other functions e.g. some of those described in the first paragraph. Moreover, there is a large variety of proteins that contain collagenous sequences and structures.

The formation of a structurally intact, correctly folded triple helix is a prerequisite for the proper function of collagen in tissues. The key questions in understanding collagen assembly are the mechanisms by which the individual α chains are able to discriminate between each other and associate in correct stoichiometry, and how this association leads to triple helix formation. One of the main aspects of this dissertation was to investigate these phenomena with the best known FACIT, collagen IX, present in cartilage ECM. Collagen IX was also analyzed for its ability to mediate cell adhesion via binding to specific cellular receptors, integrins. The binding was localized to the NC3 domain of collagen IX and was found to represent a novel mechanism of interaction. The third aspect of this thesis represents applied or clinical research. It was found that the sera of patients with recent onset rheumatoid arthritis (RA) contain elevated levels of autoantibodies towards full-length human collagen IX. The presence of these

autoantibodies already at the early stages of the disease may reflect their diagnostic potential in RA.

2 Review of the literature

2.1 Superfamily of collagens

Collagens constitute a protein family of extracellular matrix proteins that currently comprises 28 individual members with 42 different gene products. Collagens may assemble with themselves and with other collagen types, and they display affinity to variety of molecules through charged residues. Importantly, they act as ligands for cellular receptors including integrins. In addition to having a structural function, they play important roles in cell adhesion and migration during development and morphogenesis, growth, differentiation and wound healing. They are essential for the formation, homeostasis and regeneration of tissues and organs. They are also involved in numerous pathological processes. (Myllyharju and Kivirikko 2004.)

Collagens are numbered by roman numerals in the order of discovery. Traditionally they have been categorized as fibrillar or nonfibrillar collagens. When looking at their structure, supramolecular organization, and other features in more detail, collagens can be classified into the following groups: (i) fibril forming collagens, (ii) fibril-associated collagens with interrupted triple helices (FACITs), (iii) network-forming collagens, (iv) collagens forming beaded filaments, (v) collagens forming anchoring fibrils, (vi) basement membrane (BM) collagens, (vii) transmembrane collagens and (viii) multiplexins. (For reviews, see Gelse *et al.* 2003, Myllyharju & Kivirikko 2004 and Ricard-Blum & Ruggiero 2005). In addition, there is a heterogenous group of proteins that contain collagenous domains but have not been defined as collagens. These proteins include C1q of complement, adiponectin, acetylcholinesterase, ectodysplasin and several members of macrophage receptors, collectins and ficolins (Myllyharju & Kivirikko 2004). The collagen types represented in the different subgroups as well as their chain composition and major sites of expression are presented collectively in Table 1.

Table 1. Chain composition of collagen types, classification and major sites of expression.

Type	Constituent α chains	Structure/Group	Tissue distribution
I	α1(I), α2(I)	Fibrillar	Bone, tendon, ligament and skin
II	α1(II)	Fibrillar	Cartilage, intervertebral disc, and vitreous humor
III	α1(III)	Fibrillar	Co-expressed with collagen I in vasculature and skin
IV	α1(IV), α2(IV), α3(IV)	3D network	Most basement membranes
	α4(IV), α5(IV), α6(IV)		Glomerular and alveolar BM
V	α1(V),α2(V), α3(V)	Fibrillar	Co-expressed with collagen I in lung, cornea and bone
	α4(V)		Nervous system
VI	α1(VI), α2(VI), α3(VI)	Microfibril	Wide tissue distribution, not bone
VII	α1(VII)	Anchoring fibril	Squamous epithelium BM zone
VIII	α1(VIII), α2(VIII)	Hexagonal lattice	Many tissues, Descemet's membrane of cornea
IX	α1(IX), α2(IX), α3(IX)	FACIT	Associated with type II fibrils in cartilage and cornea
X	α1(X)	Hexagonal lattice	Hypertrophic cartilage
XI	α1(XI), α2(XI), α1(II)	Fibrillar	Co-expressed with collagen II
XII	α1(XII)	FACIT	Associated with type I fibrils in perichondrium, ligament, and tendon
XIII	α1(XIII)	Transmembrane	Many tissues at a low level
XIV	α1(XIV)	FACIT	Associated with type I fibrils in many tissues
XV	α1(XV)	Multiplexin	Many BM zones
XVI	α1(XVI)	FACIT	Associated with type II fibrils in hyaline cartilage and with microfibrils in skin
XVII	α1(XVII)	Transmembrane	Skin and intestinal epithelia
XVIII	α1(XVIII)	Multiplexin	Endothelial and epithelial BM zones
XIX	α1(XIX)	FACIT-like	Rare BM zones, in developing muscle
XX	α1(XX)	FACIT	Associated with type I fibrils in sternal cartilage, cornea, and tendon
XXI	α1(XXI)	FACIT	Associated with type I fibrils in vessel walls
XXII	α1(XXII)	FACIT-like	Associated with microfibrils at tissue junctions
XXIII	α1(XXIII)	Transmembrane	Heart, lung and brain, metastatic cells
XXIV	α1(XXIV)	Fibrillar	Co-expressed with collagen I in bone and cornea
XXV	α1(XXV)	Transmembrane	Neurons
XXVI	α1(XXVI)	FACIT-like	Testis and ovary
XXVII	α1(XXVII)	Fibrillar	Co-expressed with collagen II in cartilage and epithelia
XXVIII	α1(XXVIII)	Fibrillar	Peripheral nerves

Compiled according to the references quoted in the text.

The collagen subunits, termed as α helices, contain Gly-X-Y sequences characteristic of collagenous proteins. This repetitive amino acid triplet sequence

defines the boundaries of collagenous domains (COL) and allows the formation of the triple helix that is the characteristic structural feature of the collagen superfamily. Glycine residues, being the smallest amino acid, occupy the core of the α helix, thus enabling the tight packing of the left-handed helix. The X position is frequently occupied by proline and the Y position by hydroxyproline residues. The presence of 4-hydroxyproline residues is essential for the formation of hydrogen bonds that contribute to the stability of the triple helices, which are formed when the three individual α chains are brought into close proximity and supercoiled around a common axis in a right-handed manner. The constituent α chains can be identical or products of two to three different genes representing homotrimeric or heterotrimeric compositions, respectively. These monomeric collagen molecules are then assembled into supramolecular structures, for example networks or fibers. Collagen fibers are often known to consist of more than one collagen type. For example, collagen I fibrils often contain small amounts of collagens III, V and XII, whereas fibrils consisting primarily of collagen II also contain collagens IX and XI. Some collagens (V and XI) can also form hybrid molecules, e.g. have an α1(XI) and an α2(V) chain in the same molecule. Furthermore, additional heterogeneity within the superfamily is caused by alternative splicing of some transcripts or the use of alternative promoters in some genes. Thus, it is the relative composition and amounts of different collagen types that co-assemble into heterotypic fibrils that define the structure and organization of the matrix network and determine the biomechanical properties of the tissues.

The different collagen types are characterized by marked complexity and diversity in their structure, splicing, number and composition of non-collagenous domains and assembly. This complexity and the large number of different structures imply that the individual members of the collagen superfamily participate in numerous different biological functions. This is highlighted by the numerous diseases caused by mutations in the collagen coding genes. In addition, the varying roles of different collagens are reflected by their tissue- and time-specific expression, specific supramolecular structure and other characteristics. Furthermore, during the few last years more insight has been gained on the roles of noncollagenous domains of different collagen types. It has become evident that proteolytic fragments of these domains play regulatory functions during development, wound healing and angiogenesis. Most of these proteolytically generated fragments seem to exert an inhibitory effect on cell proliferation, angiogenesis and tumorigenesis. The best known of these fragments is the C-

terminal NC1 domain of collagen XVIII, endostatin (O'Reilly *et al.* 1997). The sister molecule to collagen XVIII, collagen XV, contains a homologous domain to endostatin called restin, in the NC10 domain of α1(XV) (Ramchandran *et al.* 1999). Other fragments with cryptic biological activity include the NC1 domains of α1(IV), α2(IV) and α3(IV) called arresten, canstatin and tumstatin, respectively. A lot of attention has been paid to these molecules because of their antitumor activity *in vitro* and *in vivo*. Thus, intense medical and clinical research has been aimed to develop cancer therapies based on these molecules (Colorado *et al.* 2000, Kamphaus *et al.* 2000, Maeshima *et al.* 2000). In parallel, our understanding of the distinct members of the collagen family has evolved from structural constituents to active organizers and regulators of matrix homeostasis as well as modulators of cell-matrix interactions (Ortega & Werb 2002).

More than 1300 disease-causing mutations have been described in 23 of the 42 human collagen genes (Myllyharju & Kivirikko 2004). Most of these mutations are single-base substitutions that convert the codon of the obligate glycine to that of a bulkier residue and either prevent the folding of the triple helix beyond this point or cause an interruption in the helix. Because the triple helix of most collagens is propagated from the C-terminus to the N-terminus, the closer the glycine substitution is to the C-terminal end, the more severe phenotype it usually produces (Kivirikko & Prockop 1995). However, this is not always the case. This may be explained by the fact that the triple helix appears to contain regions of high and low stability (Steplewski *et al*. 2004). Mutations in different regions, therefore, have different effects that take place at different levels of biosynthesis and organization of collagenous matrices. The levels include (i) formation of a single molecule, (ii) formation of homotypic fibrils, (iii) formation of heterotypic fibrils, and (iv) interaction with other molecules or cells (see DiLullo *et al.* 2002, Steplewski *et al.* 2005). In general, mutations that lead to the production of a structurally altered polypeptide chain that is still able to associate with other chains usually cause more severe phenotypes than mutations that prevent trimer formation and heterozygous null alleles. This is because the resulting triple helices contain abnormalities that interfere with the formation of supramolecular structures and/or lead to aberrant function (Kivirikko & Prockop 1995, Myllyharju & Kivirikko 2001). Other mutations involve codons in the X or Y position and cause substitutions or a translation stop codon. Most of the amino acid substitutions in the X and Y positions produce mild phenotypes or represent non-pathogenic polymorphisms. Further mutations may be represented by larger

reorganization of genetic material such as deletions, insertions, duplications or result in abnormal RNA splicing (Myllyharju & Kivirikko 2004).

The majority of the collagen mutations result in relatively rare inheritable diseases, such as osteogenesis imperfecta, Ehlers-Danlos syndrome, Alport syndrome, epidermolysis bullosa and various chondrodysplasias. These diseases display a considerable spectrum of severity from perinatal lethality to a very mild disease. The majority of these diseases show a dominant inheritance pattern, although examples of recessive inheritance are known as well. Diseases or phenotypes involving mutations in the genes encoding collagen IX α chains are described in detail in section 2.5.3.

2.1.1 Fibrillar collagens

Fibrillar collagens are the classical group of structural collagens and have been studied for decades. As their name implies, these collagens form fibrils, which primarily act as supporting elements in the extracellular matrix. They share similarities in structural elements, the most prominent being a continuous collagenous sequence of about 1000 amino acids. This sequence makes up the triple helical domain which forms a rod-like structure with a length of about 300 nm. All the exons encoding the triple-helical domain are of sizes that are multiples of 9 bp (e.g. 45, 54, 99, 108 and 162 bp), the most common size being 54 bp. Because of this, it has been suggested that the ancestral gene for the fibril-forming collagens has evolved by amplification of this 54-bp unit, possibly embedded in the intronic sequences (Yamada *et al.* 1980, see also Boot-Handford & Tuckwell 2003). Other similarities include the size and structure of the C-propeptide, which are highly conserved among the fibril-forming collagen types. The N- and C-propeptides are linked to the main triple-helical domain by short, noncollagenous sequences called telopeptides. The telopeptides act as sites for intermolecular crosslinking, which is important for the stabilization of the collagen fibers (Eyre *et al.* 1984). The conservation of the numbers and locations of the cysteine residues in the C- propeptides is especially high. The conserved features of the C-propeptides are believed to reflect their essential roles in initiating chain assembly and triple helix formation. The N-propeptides show more diversity in their sizes and structures. In some cases the N- propeptides are retained in the collagen molecules present in fibrils, although their removal is generally regarded as a prerequisite for proper fibrillogenesis. It has, therefore, been suggested that the N-propeptides may regulate the fibril diameter (Birk

2001, Chapman 1989, Hulmes *et al.* 1989, Linsenmayer *et al.* 1993) or the production of collagen by a feedback mechanism (Bateman & Lamande 1996). Collagen molecules are secreted in the extracellular space as precursors, called procollagens, retaining the C- and N-propeptides. The procollagen molecules are processed to mature collagen molecules by cleavage of the N- and C-propeptides by specific N- and C-proteinases. Cleavage of the C-propeptides reduces the solubility of the protein, and the collagen molecules are then capable of self-assembling into highly ordered, quarter-staggered, 67 nm banded fibrils typical of fibril-forming collagens (Kadler *et al.* 1987, Prockop & Hulmes 1994).

The group of fibrillar collagens includes the classical collagen types I, II and III together with the minor types V and XI and new members, namely collagens XXIV and XXVII. Collagen I, the first member of the collagen protein family to be characterized, is the most abundant collagen in the human body. It is found especially in bone, tendon, ligament and skin. It consists of two identical α1(I) chains and one α2(I) chain. Low levels of the [α1(I)]3 homotrimer are also found in various tissues (Kielty & Grant 2002). Collagen II is a homotrimer consisting of three identical α1(II) chains, being the major collagenous component of cartilage (Miller & Matukas 1969, Miller & Gay 1987). It is expressed mainly in cartilage, the vitreous of the eye, intervertebral disc and inner ear, but it can also be found in many non-chondrogenic tissues during development (Brewton & Mayne 1992). Collagen II is the most important structural component of cartilage, accounting for up to 90-95% of its total collagen content (Eyre 1991, Mendler *et al.* 1989). Collagen III is typically found together with collagen I in many connective tissues (Miller *et al.* 1971, Miller & Gay 1987). It is the major collagen in blood vessels, but it is also found in many tissues that require elastic properties such as skin, lung and a number of other tissues. It forms only homotrimeric molecules of three α1(III) chains. Collagens V and XI share a number of common characteristics. They are minor constituents in a number of tissues and associate with the much more abundant collagen I to form heterotypic fibers. They are located at the core of these fibers where they are thought to regulate the lateral packing and diameter of the heterotypic fibers (Fessler *et al.* 1986, Birk *et al.*1988 and Blaschke *et al.* 2000). At least three genetically distinct collagen V α chains, α1(V), α2(V) and α3(V), are currently known and they form homotrimers and heterotrimers with variable chain compositions (Mann *et al.* 1992, Fichard *et al.* 1995). An additional collagenous polypeptide, showing 82% sequence identity to human α3(V) collagen and expressed in neonatal and adult brain and in neonatal peripheral nerves, has been termed α4(V) (Chernousov *et al.*

2000). The expression pattern of collagen V generally follows that of collagen I. Collagen XI is a quantitatively minor fibril-forming collagen found in cartilage. Like collagen II, it is also expressed in the vitreous body of the eye and the nucleus pulposus of the intervertebral disc (Eyre *et al.* 1987, Mayne & Brewton 1993). Collagen XI is a heterotrimer of three α chains: α1(XI), α2(XI) and α3(XI). The α3(XI) chain is actually encoded by the collagen II gene COL2A1, except that the α3(XI) chain undergoes more extensive posttranslational glycosylation than α1(II) (Burgeson *et al.* 1982). Collagens XXIV (Koch *et al.* 2003) and XXVII (Boot-Handford & Tuckwell 2003b, Pace *et al.* 2003) are regarded as fibrillar collagens, even though they have interruptions in their collagenous sequences and are more homologous with each other than with the other fibrillar collagens. The expression of collagen XXIV is restricted to bone and cornea, whereas collagen XXVII is strongly expressed in cartilage containing heterotypic II/XI fibrils. A peculiar feature of human collagen XXVII is that it contains unexpected residues such as tryptophan and cysteine within the triple helical domain. Their significance is so far unclear.

2.1.2 FACIT collagens

The largest subgroup of non-fibrillar collagens are the FACITs. The members of the FACIT collagen subgroup are partially homologous multidomain molecules that possess common structural features. These collagens are characterized by (i) three or more triple helical domains interrupted by short noncollagenous sequences, (ii) the presence of two highly conserved cysteine residues separated by four amino acids at the NC1-COL1 junctions, (iii) the existence of two G–X–Y triplet imperfections within the COL2 domain and (iv) the presence of a large N-terminal domain containing a thrombospondin subdomain next to the collagenous region (Ricard-Blum & Ruggiero 2005). Unlike fibrillar collagens, they are not capable of forming fibrillar polymers alone but are colocalized with them, presumably acting as molecular bridges that anchor and organize the interstitial collagen fibrils within the extracellular matrix. As defined by these characteristics, these molecules are collectively named *f*ibril-*a*ssociated *c*ollagens with *i*nterrupted *t*riple helices, or FACIT collagens.

In contrast to the highly organized structure of the triple helix, noncollagenous domains are characterized by structural and functional diversity. There is considerable variety in the number of the collagenous domains as well as in the size and composition of the N-terminal domains. N-terminal domains of

some of the FACIT members contain different numbers of von Willebrand factor A-like domains (vWFA) that may or may not be accompanied by a variable number of fibronectin type III repeats (see Ricard-Blum and Ruggiero 2005). Furthermore, at least collagens XII, XIV and XIX undergo alternative splicing of the transcripts, making their study and understanding even more complex and challenging.

The members of the FACIT subgroup of collagens include collagens IX, XII, XIV, XVI (Myllyharju & Kivirikko 2004) and the more recently discovered collagens XX (Koch *et al.* 2001) and XXI (Fitzgerald & Bateman 2001, Tuckwell 2002). In addition, collagens XIX, XXII, and XXVI are included in this category because they share common features of their primary structure with FACIT collagens. However, there are features of these three collagens that are distinctive from the other FACIT members. For example, while collagens IX (Olsen 1997) and XVI associate with type II fibrils in cartilage (the latter aggregating also with microfibrils) (Kassner *et al.* 2003), collagens XIX and XXVI have not been shown to associate with fibrils (Sato *et al.* 2002). Instead, purified collagen XIX seems to self-aggregate into higher-order structures (Myers *et al.* 2003). In addition, collagen XXII does not show affinity to collagen fibril extracts either, but to extrafibrillar microfibrils (Koch *et al.* 2004). The expression patterns of collagens XII and XIV follow that of collagen I, and they participate in the formation of collagen I fibrils in dense connective tissues such as skin, tendon and periosteum (Gelse *et al.* 2003). Also, collagen XX is likely to be expressed in these tissues (Koch *et al.* 2001). Collagen XIX is mainly expressed in the epithelial basement membrane zones, where it plays an important role in muscle differentiation (Myers *et al.* 1997, Myers *et al.* 2003). Collagen XXI, in turn, is highly expressed during fetal development, being particularly abundant in the vascular wall (Chou & Li 2002). As judged by its unique expression, collagen XXII seems to represent a novel marker for the tissue junction (Koch *et al.* 2004). At present, the biological functions of almost all the FACIT collagens are surprisingly poorly understood. In general, the FACIT collagens associate with the collagen fibrils by their C-terminal collagenous domains, while the noncollagenous N-terminus points out to the extracellular space, allowing interactions with ECM proteins (Olsen 1997). However, based on their specific expression patterns and unique structural properties, it is very likely that the FACITs display more specific and subtle functions than the originally suggested structural role and the stabilization of extracellular matrices.

2.2 Biosynthesis of collagens

Biosynthesis of fibrillar collagens is usually regarded as a paradigm for collagen synthesis. It is a complex multistep process that requires multiple enzyme activities of which many are unique to collagens and collagen-like molecules. These activities include hydroxylation of proline and lysine residues by prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase (Kivirikko & Pihlajaniemi 1998), glycosylation of the hydroxylysine residues by galactosyltransferase and galactosylglucosyltransferase, cleavage of the N- and C-propeptides by the specific N- and C-proteinases, and oxidation of the lysine residues by lysyl oxidase through which crosslink formation is accomplished. Other enzymes participating in collagen biosynthesis are peptidyl proline cis-trans isomerase (PPI), Hsp47 that acts as a chaperone during collagen synthesis, and protein disulphide isomerase (PDI). PDI has three functions: (i) it catalyzes the formation of intra- and interchain disulfide bonds, (ii) it acts as a chaperone binding to nascent collagen chains, thus preventing their aggregation, and (iii) it is present in the enzyme tetramer prolyl 4-hydroxylase representing its ß subunit (Myllyharju & Kivirikko 2004).

Next, the main events of the biosynthesis of fibrillar collagens are briefly described. First, the intracellular events following the translation of the procollagen polypeptides in the ribosomes of the rough endoplasmic reticulum include cleavage of the signal peptide, hydroxylation of certain proline and lysine residues, and glycosylation of some hydroxylysine and asparagine residues. These hydroxylation steps start before the translation is complete and continue as posttranslational modifications until triple helix formation takes place (Kivirikko & Pihlajaniemi 1998). Further modifications are carried out by peptidyl proline cis-trans isomerase, Hsp47 and PDI. Then, association occurs at the C-terminus of α chains and initiates the nucleation and subsequent folding of the collagen triple helix. The process proceeds towards the N-terminus in a zipper-like manner. After intracellular processing, nascent triple-helical procollagen molecules are secreted from the cell into transport vesicles, followed by excision of the N- and C-propeptides by N- and C-proteinases. The cleavage of the C- propeptide, in particular, lowers the solubility of collagen and triggers the spontaneous self-assembly of the collagen fibrils into thicker fibers. Some recent data, however, suggest that processing of procollagens to collagens and collagen fibrillogenesis can also occur intracellularly or on the plasma membrane, thus providing a completely new perspective on the assembly of collagens (Canty *et al.* 2004,

Canty & Kadler 2005). Finally, the formation of intermolecular crosslinks provides the additional stability required for collagen to fulfill its structural functions (Prockop & Kivirikko 1995, Gelse *et al.* 2003). The assembly and fibril formation of the fibrillar and FACIT collagens are described in more detail in sections 2.4.1 and 2.4.2.

2.3 Collagen IX

2.3.1 Biosynthesis and degradation

The intracellular steps during biosynthesis of collagen IX follow mostly those of other collagens. When compared to the fibrillar collagens, there are marked differences in the secretion and assembly mechanisms. Collagen IX, like other FACITs, does not contain large C- or N-propeptides at its termini and is not secreted as a procollagen. Thus, collagen IX is not proteolytically processed prior to its deposition into the extracellular matrix. However, the molecule undergoes heavy posttranslational processing, similar to other collagens. A more detailed description of the assembly process of FACIT collagens has been provided in section 2.4.2.

Synthesis and degradation of the extracellular matrix and its constituents is a simultaneous continuous process taking place in both physiological and pathological states (see Matrisian 1992 or 2001 and Birkedal-Hansen *et al.* 1993). Matrix metalloproteinases (MMPs) are known to degrade a wide variety of ECM components and actively participate in the remodelling of the ECM. MMP-3 (also known as stromelysin-1) produced by chondrocytes and synovial fibroblasts is capable of degrading aggrecan, link protein and collagens II, IX, X and XI (Wu *et al.* 1991), and may therefore be able to remove collagen IX from the fibril surface. This would predispose the fibrils to attack by additional MMPs and other proteinases, thus compromising the integrity of the cartilage, seen for example in RA (Bonassar *et al.* 1995) and osteoarthritis (Okada *et al.* 1992). Also, MMP-9 (Bollen & Eyre 1993), MMP-2 (Brown *et al.* 1996), cathepsins B and L (Maciewicz & Wotton 1991) and neutrophil elastase (Gadher *et al.* 1988) are able to cleave collagen IX. However, it is worth noting that in most reports soluble collagen monomers have been utilized to study the proteolytic activities of these enzymes. In contrast to soluble monomers, the collagen in tissue is arranged as cross-linked fibrils that are poor substrates for proteolysis and are degraded

slowly if at all. As collagen IX lies on the surface of cartilage collagen fibrils, it is conceivable that collagen II is protected from proteolysis by collagenases until collagen IX is removed. Since none of the three known collagenases are able to degrade collagens IX or XI at their triple-helical domains, the heterotypic fibrils of cartilage seem to present a complex and challenging substrate for proteolysis (Eyre *et al.* 2002).

2.3.2 Tissue expression

In general, the expression pattern of collagen IX follows those of collagens II and XI. Collagen IX is present in all hyaline cartilages as a minor component, representing about 1-2% of adult and even 10 % of fetal cartilage collagen (Eyre 1991, 2002). A relatively constant ratio of 1:1:1 is usually exhibited in cartilage by the three different mRNAs (Perälä *et al.* 1997). Expression of the long variant of collagen IX is most abundant in mature tissues, while that of the short form predominates in the early stages of development (Hayashi *et al.* 1992, Swiderski & Solursh 1992) and in the vitreous of the eye (Warman *et al.* 1993). The presence of collagen IX in the ocular structures has also been demonstrated in chicken (Svoboda *et al.* 1988), bovine (Bishop *et al.* 1992 and 1994) and mouse (Liu *et al.* 1993) species.

Collagen IX is also found in the intervertebral disc, both in the innermost layer, the nucleus pulposus, where the short form is predominant, and in the outer layer, annulus fibrosus, where both forms are present (Newall & Ayad 1995). In addition, collagen IX is expressed as a long variant in the cartilaginous endplates between the discs and the vertebrae. Some, but not all of the synthesized collagen IX molecules are observed to carry a GAG side chain (Buckwalter 1995, Newall & Ayad 1995). Besides the eye and the intervertebral disc, collagen IX is present at the mRNA level also in other extracartilaginous tissues such as skin, brain, kidney and heart. However, the ratios of the α1(IX), α2(IX) and α3(IX) mRNAs seem to vary considerably in these tissues, implying that the mRNA may not actually be translated into protein (Perälä *et al.* 1997).

2.3.3 Structure of collagen IX

Gene structure

The human α1(IX), α2(IX) and α3(IX) chains are encoded by the COL9A1, COL9A2 and COL9A3 genes, located on chromosomes 6q12-q13, p32.3-p33 and 20q13.3, respectively (Kimura *et al.* 1989, Warman *et al.* 1993b, 1994, Brewton *et al.* 1995, Tiller *et al.* 1998). While the complete cDNA sequences for the human α1(IX), α2(IX) and α3(IX) chains have been characterized during the ninety's (Muragaki *et al.* 1990a, Perälä *et al.* 1993, Warman *et al.* 1994, Brewton *et al.* 1995, Pihlajamaa *et al.* 1998, Paassilta *et al.* 1999b), the first cDNA clones encoding α1(IX) were originally isolated from chicken already during the eighty's (Ninomiya & Olsen 1984). Later on, the complete cDNA structures for the chicken α1(IX) (Vasios *et al.* 1988, Nishimura *et al.* 1989) as well as for α2(IX) and α3(IX) were also defined (Brewton *et al.* 1992, Har-El *et al.* 1992).

There are some common features as well as differences between the three genes encoding collagen IX. For instance, they all seem to share a similar exon-intron structure. In addition, the coding regions of the genes display considerable similarity, suggesting that they have evolved by duplication of a common precursor gene (Ninomiya & Olsen 1984, Brewton *et al.* 1992, Har-El *et al.* 1992 and Brewton *et al.* 1995). Furthermore, additional similarity is presented by three short interruptions in the Gly-X-Y triplet motif, two in the COL1 and one in the COL3 domain of each chain. The three genes also display unique characteristics. Despite a similar exon-intron organization, the three genes are very different in size. The COL9A1 gene consists of 38 exons and is about 90 kb in length, while COL9A2 comprises 32 exons but is only 15 kb. The difference is largely explained by the divergent sizes of introns and the lack of sequences encoding the large NC4 in COL9A2 (exons 1-7 in COL9A1). The COL9A3 gene also contains 32 exons, but is 23 kb in length. Another distinguished feature is found in the exon encoding part of the NC3, producing NC3 domains of different sizes in all of the three genes (Table 2). Additional complexity is provided by the use of an alternative promoter of the COL9A1 gene (see next paragraph). Finally, besides the split codons for Gly that may be present at exon junctions, the genes of collagen IX (and other FACITs) present exon sizes other than multiples of 9 bp, in contrast to the fibrillar collagens that follow the 9 bp rule (Vuorio & Crombrugghe 1990).

Table 2. The individual domains and their size (amino acid residues) in human collagen IX.

Domain	α1(IX)	α2(IX)	α3(IX)
NC4	268[a]	26[a]	28[a]
COL3	137	137	137
NC3	12	17	15
COL2	339	339	339
NC2	30	30	31
COL1	115	115	112
NC1	20	25	22

[a] includes a signal peptide

Two transcripts of the COL9A1 gene have been identified. This results from the use of two different promoters, located about 20 kb apart, which are operated both in a tissue-specific and developmentally regulated manner. The upstream promoter encodes a large N-terminal domain of α1(IX) corresponding to exons 1-7. The resulting polypeptide consists of 245 residues and is predominantly expressed in cartilage (Vasios *et al.* 1988, Nishimura *et al.* 1989 and Muragaki *et al.* 1990a). The downstream promoter that gives rise to a shorter form of COL9A1 produces a transcript starting from alternative exon 1* that is directly spliced to sequences encoded by exon 8, thus resulting in the skipping of exon 7 (Nishimura *et al.* 1989, Muragaki *et al.* 1990a,b). The use of an alternative promoter leads to a transcript encoded by 32 exons, instead of the 38 exons included in the long form. As a result, the NC4 region of the human short α1(IX) variant contains only three amino acid residues. The short form of collagen IX is predominantly expressed in the eye (Svoboda *et al.* 1988) and, due to the absence of the large globular NC4 resulting in different surface properties, is probably involved in molecular interactions different from those of the long form (Nishimura *et al.* 1989).

Protein structure

First indications of the existence of collagen IX were discovered through isolation of disulphide-bonded collagenous fragments obtained from pepsin-treated mammalian cartilage and intervertebral disc (Shimokomaki *et al.* 1980, 1981, Ayad *et al.* 1981, 1982, Ricard-Blum *et al.* 1982). Additional evidence was demonstrated when these fragments (HMW and LMW or M1 and M2) were isolated from chicken sternal cartilage (Reese & Mayne 1981, Reese *et al.* 1982,

von der Mark *et al.* 1982), and finally, when isolation of the intact parental molecule was successfully completed (Bruckner *et al.* 1983, Noro *et al.* 1983, von der Mark *et al.* 1984).

Collagen IX is a heterotrimer consisting of three genetically distinct α chains, α1(IX), α2(IX) and α3(IX) (van der Rest & Mayne 1987). The overall structure of collagen IX molecules is stabilized by the bonding of α chains to each other by intramolecular disulfide bridges between cysteine residues located at the NC1/COL1 junction and NC3 domain of each chain. The molecules are divided into three collagenous domains, COL1-3, that are flanked by noncollagenous domains, NC1-4, numbered from the C-terminus (Figure 1). The collagenous domains show an equal number of residues, except for the COL1 domain of α3(IX) that is 3 residues shorter than the corresponding domains of α1(IX) and α2(IX). Instead, there is variance in the sizes of noncollagenous domains, especially at both termini of the molecule. The NC4 domain of α1(IX) is 245 residues long, while that of α2(IX) and α3(IX) is only 3 residues in length. However, a molecular form with equally-sized NC4 domains is also present in tissue-specific locations. The size difference between the two variants of collagen IX is explained by the use of an alternative promoter, resulting in short or long variants. Furthermore, the size of the NC3 domain is different in all three chains. This is actually necessary to provide the short arm of the molecule, comprised of COL3 and NC4, with the flexibility to bend off the fibril surface and allow interactions with other matrix molecules. In fact NC4, based on its calculated pI of 10.4, carries a strong positive charge at physiological pH, making it an excellent interaction partner candidate (Vasios *et al.* 1988). These interactions are described in section 2.5.1. Structurally, as shown by electron microscopy, the NC4 domain adopts a globular conformation, stabilized by four cysteine residues participating in intrachain disulfide bonding (Irwin *et al.* 1985, Mayne *et al.* 1985, Bruckner *et al.* 1988, Vaughan *et al.* 1988). The human NC4 domain also contains an attachment site for an N-linked oligosaccharide (Muragaki *et al.* 1990a, Warman *et al.* 1993a). Based on comparisons between recombinant full-length collagen IX and recombinant NC4 produced in *E. coli*, it seems that the folding of the NC4 domain follows an inherent pattern, independent of the presence of the collagen triple helix (Pihlajamaa *et al.* 2004). Circular dichroism analysis of the domain has revealed its exceptionally high thermal denaturation point of approximately 80 °C (Pihlajamaa *et al.* 2004). The thermal stability of the short arm, formed by the NC4 and COL3 domains, is thus markedly higher than that of the long arm, formed by the rest of the molecule (Bruckner *et al.* 1983, Miles *et*

al. 1988). This difference is due to the higher hydroxyproline content of the short arm (Reese *et al.* 1982) and the presence of disulfide bridges. The difference in the thermal stabilities seems to reflect the molecular arrangement of collagen IX, since the lower stability of the long arm is compensated by a stabilizing association on the cartilage collagen fibril surface (Kimura *et al.* 1989, Muragaki *et al.* 1990a,b, 1996, Perälä *et al.* 1993, 1997, Rokos *et al.* 1994, Warman *et al.* 1994, Brewton *et al.* 1995). The high stability and the preservation of the COL3 and NC4 domains during the course of evolution imply a specific role for these regions in cartilage development or function.

The domain structure of collagen IX represents a modular architecture in which unique structural or functional features can be envisaged or demonstrated for each domain. As described above and later in section 2.5.1, the NC4 domain is capable of interacting with other matrix components along with the COL3 domain, which also serves as an arm projecting NC4 into the extrafibrillar space (van der Rest & Mayne 1988, Vaughan *et al.* 1988). The NC3 domain, in turn, acts as a molecular "hinge", explaining the observed kinks in the molecule when visualized by electron microscopy (von der Mark *et al.* 1982, Reese *et al.* 1982, Duance *et al.* 1984). The COL2 domain of collagen IX contains the crosslinking site to collagen II (Eyre *et al.* 1987, van der Rest & Mayne 1988, Wu *et al.* 1992), while the COL1 and NC1 domains are essential for correct chain assembly (Mechling *et al.* 1996).

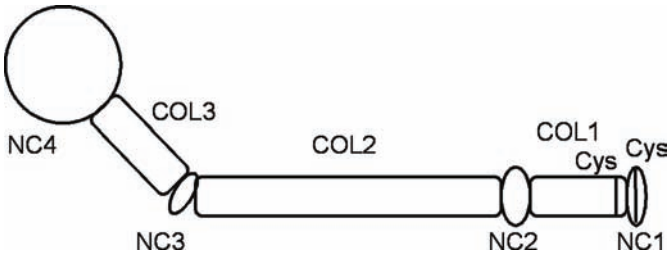

Fig. 1. Schematic domain structure of collagen IX. Cysteine residues at NC1 and the COL1 domain are indicated. In the short form expressed by the use of an alternative promoter, the globular NC4 domain is absent.

Collagen IX as a proteoglycan

The first observations of the proteoglycan form of collagen IX derive from chicken (Noro *et al.* 1983, Bruckner *et al.* 1985, Vaughan *et al.* 1985). Subsequently, this form of collagen IX has been demonstrated to exist also in humans and other mammals (Bruckner *et al.* 1988, Ayad *et al.* 1989, 1991, Arai *et al.* 1992, Bishop *et al.* 1992). The GAG side chain is known to be attached to a Ser residue within the NC3 domain of the α2(IX) chain (Huber *et al.* 1986, Konomi *et al.* 1986, McCormick *et al.* 1987, Huber *et al.* 1988). However, the presence of the GAG chain, representing either dermatan or chondroitin sulphate, is not a uniform feature of all collagen IX molecules, and the proportion of molecules containing the GAG substitution seems to vary depending on the developmental stage and origin of the tissue. While more than 80 % of collagen IX extracted from chicken embryonic sternal cartilage is in the proteoglycan form (Huber *et al*. 1988), it represents a minor component in human cartilage (Diab *et al*. 1996). It has also been proposed that the presence of the GAG chain would represent a transient phase and that it would be cleaved off when collagen IX molecules become decorated on the surface of maturing collagen II heterotypic fibrils (Diab *et al.* 1996). So far, distinct functions for the proteoglycan and non-proteoglycan forms of collagen IX have not been demonstrated.

2.4 Collagen chain assembly

Alpha-helical coiled coils are widely occurring protein oligomerization motifs found in collagens and collagen-like proteins including fibrillar collagens (I–III, V, XI, XXIV, and XXVII), FACITs (IX, XII, XIV, XIX, XX, and XXIII), transmembrane collagens (XIII, XVII, XXIII, and XXV) and MARCO, ectodysplasin, and scavenger receptors, the collectins, collagens XV and XVIII (the multiplexins), the emilins, the collagen-like tail subunit of acetylcholinesterase, and collagens VI, VII, and XXVI (McAlinden *et al.* 2003). Thus, almost all members of the collagen superfamily contain short, repeating heptad sequences typical of coiled coils. The amino acid sequence of the coiled-coil domain is characterized by up to four heptad repeats (*a-b-c-d-e-f-g*) in which hydrophobic amino acid residues occur at positions *a* and *d* (Kammerer 1997). They are located before, after, or between collagen-like regions as in the C-propeptides of fibrillar procollagens, in the N-terminal sequences of transmembrane collagens, and in the noncollagenous domains of FACITs. The

ability of the coiled coils to function as efficient tools in the oligomerization of polypeptide chains has been shown on multiple occasions. For example, the coiled coil region is both necessary and sufficient to drive the trimerization of the lung surfactant protein SP-D (Hoppe *et al.* 1994, Zhang *et al.* 2001). Moreover, when fused C-terminal to the N-propeptide region of procollagen IIA, the first two heptad repeats of SP-D were shown to be able to drive trimerization and folding (McAlinden 2002). Furthermore, coiled-coil oligomerization domains have also been shown to be essential for the trimerization and folding of the membrane-associated collagens XIII (Snellman *et al.* 2000, Latvanlehto *et al.* 2003) and XVII (Areida *et al.* 2001). However, it has been observed that not all of the heptad repeat-containing peptides form α-helical coiled coils independently (McAlinden *et al.* 2003). This suggests that trimerization is a cooperative process involving both coiled-coil and collagen-like regions. In summary, noncollagenous domains seem to be important for initial chain association and registration in order to ensure correct and sufficiently fast triple-helical folding. The widespread occurrence of the coiled-coil domains suggests a general role in the triple-helical assembly of collagens and collagen-like proteins. However, other structural features and mechanisms are also known to be involved in the assembly and trimerization of polypeptide chains. These include utilization of long noncollagenous domains such as NC1 of collagen IV or tightly packed ß-sandwich assembly domains, as with the members of the C1q family. (Kammerer 1997, McAlinden *et al.* 2003).

2.4.1 Fibrillar collagens

Fibrillar collagens are synthesized as large (~ 500 kDa) precursors, procollagens, which contain N- and C-terminal propeptide regions (Kadler *et al.* 1995). The C-propeptide domains that are highly conserved (Dion & Myers 1987) are approximately 245 amino acid residues long and together form a tri-lobed 3-D structure cruciform in shape (Bernocco *et al.* 2001). The importance of the C-propeptides in chain association has been demonstrated by both engineered (McLaughlin & Bulleid 1998, Lim *et al.* 1998) and naturally occurring mutations (Pace *et al.* 2001) that lead to abnormal procollagen assembly. Besides their participation in the assembly process of collagen, the C-propeptides also control collagen solubility during proteolytic processing and fibril formation and participate in feedback regulation of collagen biosynthesis (Bateman & Lamande 1996**)**. The main role of the procollagen C-propeptide domains, however, is to

direct chain association during intracellular assembly of procollagen molecules. They contain a specific discontinuous 15 amino acid sequence that has been observed to determine the type-specific assembly of procollagens (Lees *et al.* 1997). This sequence is, therefore, responsible for the initial recognition event and is necessary to ensure selective chain association. The molecular recognition of the component α chains as well as their correct stoichiometry is particularly important in cells producing more than one collagen type. For example, skin fibroblasts may express six genetically distinct, yet highly homologous collagen α chains that are able to assemble in a type specific manner into collagens I, III and V (McLaughlin & Bulleid 1998). The role of C-propeptides in the subsequent folding events, nucleation and the alignment of the chains, has been studied by Singh *et al.* (1990) as well as by Bulleid and colleagues (1997). They demonstrated that the transmembrane domain of the influenza virus haemagglutinin could functionally replace the C-propeptide domain of collagen III, thus negating roles other than initial association. Similarly, the telopeptide region as well as the formation of interchain disulfide bonds within the C-propeptide was shown to be unimportant in the helix nucleation; instead, the presence of hydroxylated Gly-X-Y triplets was essential for nucleation to occur (Bulleid *et al.* 1996). It is thus apparent that this triple-helical region is the site of nucleation, which takes place independently of the C-propeptides that play a crucial role in the early stages of procollagen assembly, being responsible for the initial interactions between the chains (for a review, see McLaughlin & Bulleid 1998). Nucleation and chain alignment, in turn, take place at the C-terminal region, after which triple helix formation is propagated towards the N-terminus in a zipper-like manner (Engel & Prockop 1991). Location-wise, this process may occur inside the cell, in specific saccular transport structures termed post-Golgi carriers, on the cell membrane, or in the extracellular space (see Canty & Kadler 2005). Since the above mentioned concepts are essential in understanding collagen chain assembly process and intimately related to the findings presented in the original article I, they are shortly described in the following table 3.

Table 3. Key concepts and their descriptions in the assembly of fibrillar collagens.

Concept	Description
Chain association	The initial event in chain assembly where collagen α chains are brought to close proximity allowing the C-propeptides to associate.
Chain selection	The collagen α chains discriminate between each other via specific interactions between amino acid side chains in order to assemble in a type-specific manner.
Nucleation	The collagen α chains at the C-terminal end of the triple helical domain condensate to form a "nucleus" to initiate the triple helix formation. The process requires the presence of at least two Pro-Pro-Gly triplets and adequate hydroxylation of proline residues but is not dependent on the formation of inter-chain disulfide bonds.
Triple helix formation / trimerization	Upon nucleation, the collagen α chains are aligned and triple helix formation is driven towards the N-terminus in a zipper-like manner. The process is completed by formation of inter-chain disulfide bonds that stabilize the structure. In certain conditions the associated α chains do not form a triple helix but a trimer that is not structurally stable.

Compiled according to McLaughlin & Bulleid (1998).

Collagen fibril assembly is a complex self-assembly process during which fibers spontaneously aggregate into ordered fibrillar structures *in vivo*. Yet, our understanding of the phenomenon is primarily based on *in vitro* experiments. The spontaneous folding of the triple helix is a slow and concentration dependent process in which cis-trans isomerations by PPI act as a rate-limiting step (Bächinger *et al.* 1980). The ability for self-assembly is encoded in the primary structure of the collagens, but other matrix molecules such as fibronectin, specific integrins and small leucine-rich repeat proteoglycans (SLRPs) such as lumican, decorin and fibromodulin have been demonstrated to affect collagen fibrillogenesis *in vitro* (see Canty & Kadler 2005). Although the mechanisms involved are not clear, they are known to function in limiting fibril diameter as well as regulating fibril fusion (Graham *et al.* 2000). The fibril formation is influenced by the propeptides of procollagen molecules and the key event controlling fibril formation is the cleavage of the C-propeptides. Thus, a major extracellular function of the C-propeptides is to prevent fibril formation. The function of the N-terminal propeptides in this process is not well understood and seems to differ between collagen types, implying that N-terminal processing of procollagens is a more complex event. The short non-helical telopeptides of the processed collagen monomers are involved in the covalent crosslinking of the collagen molecules catalyzed by lysyl oxidase, thus providing the tissues with

mechanical strength and resistance to force (Eyre *et al.* 1984). According to a recent report, telopeptides both at the N- and C-terminal regions are actively involved in mediating the fibril formation of fibrillar collagens (Steplewski *et al.* 2006).

After initial conversion from procollagen to collagen, the resulting fibrils organize in arrays of narrow fibrils of uniform diameter. The process of assembly involves hydrophobic and electrostatic interactions and is proposed to follow a liquid crystal-type ordering in solution prior to collagen processing and deposition as insoluble matrix (Hulmes 2002). The fibril-forming collagens assemble into prototype D-periodic, cross-striated fibrils consisting of an uninterrupted triple helix approximately 300 nm in length (Kadler *et al.* 1996). The fibrils are then able to grow by accretion, lateral fusion and by end-to-end fusion in the ECM (Birk *et al.* 1995, Graham *et al.* 2000), resulting in a variety of fibers with different diameters and lengths that form complex arrays typical to tissue type and location. For example in the cornea of the eye, the fibers are arranged as orthogonal lattices, whereas in tendon they are aligned parallel to each other forming bundles (Hulmes 2002). Thus, the suprastructural diversity reflects tissue-specific requirements and is achieved through the formation of macromolecular composites or alloys consisting of more than one collagen type (Hansen & Bruckner 2003). The collagen fibrils and related structures are able to provide the major biomechanical scaffold for cell attachment and anchorage of macromolecules, allowing the shape and form of tissues to be defined and maintained.

An interesting exception to the general principle concerning the direction of the trimerization of α chains is the group of transmembrane collagens. Because their N-terminal ends are anchored to the plasma membrane, it is feasible that the triple-helix formation would occur in an opposite N-to-C direction. In fact, this has been shown for collagens XIII (Snellman *et al.* 2000) and XVII (Areida *et al.* 2001). Furthermore, it has been shown experimentally by using designed collagen-like peptides linked to two different nucleation domains that collagen triple helix formation can be nucleated at either end of the molecule (Frank *et al.* 2003). These observations have changed the classical insight that triple helices may only (or preferentially) fold from the C-terminus.

2.4.2 FACIT collagens

As described above, the C-propeptides play a significant role in the initial association and in molecular recognition between α chains when fibrillar collagens are concerned. In the case of FACITs, however, C-propeptides are absent and replaced by markedly shorter non-triple-helical domains, NC1. This contrast in size is reflected by the number of amino acid residues in the NC1 domain, which for collagen IX is in the range of 20-25 (see Table 2) and approximately 75 for collagen XII. The NC1 domains of the FACITs do not display similarities in their sequences. Instead, common sequences are shared within the COL1 domain of FACITs. Furthermore, there is significant overall structural similarity at the COL1 domain with respect to size and the location of two imperfections in the triple helix. In addition, there are two conserved cysteine residues at the COL1-NC1 junction. These cysteines are involved in interchain disulfide bonding. When put together, these observations suggest that the COL1 domain or COL1-NC1 junction may serve a common function in all FACITs.

The role of the COL1 domain and its adjacent sequences has been studied in the chain assembly of collagen IX. Analysis of pepsin-resistant low molecular weight (LMW) fragments indicated that LMW fragments re-associated to form three different triple- helical forms, homotrimers of α1(IX) and α2(IX), and a heterotrimer of α1(IX)α2(IX)α3(IX) (Labourdette & van der Rest 1993). Analogous observations were obtained when insect cells were used as a host to express full-length collagen IX. In this system, collagen IX α chains assembled favorably into triple-helical α1(IX)α2(IX)α3(IX) heterotrimers, but also disulfide-bonded α1(IX) homotrimers were seen (Pihlajamaa *et al.* 1999). When chain assembly was investigated using synthetic peptides, it was found that peptides consisting of the C-terminus of the COL1 domain and the entire NC1 domain were able to form disulfide-bonded heterotrimers, but not triple helices. These observations suggest that the information for chain selection of collagen IX is located in the C-terminal part of COL1 and in the NC1 domain, but the triple helix formation is not the driving force for this interaction (Mechling *et al.* 1996).

Besides collagen IX, collagen XII has also been a target of research when studying the mechanisms of chain assembly and trimer formation of FACITs. These studies, carried out with a recombinant collagen XII minigene in HeLa and insect cells, suggested an important role for proline hydroxylation in stabilizing the COL1 domain to obtain disulfide-bonded trimers (Mazzorana *et al.* 1993, 1996). The data also support the notion of the NC1 and COL1 domains containing

information necessary for the chain association of FACITs. The role of this region was further analyzed by deleting most of the NC1 domain, except the first seven residues at the junction of the COL1 and NC1 domains. The results indicated that this deletion does not prevent the assembly of trimeric disulfide-bonded truncated collagen XII α chains (Mazzorana *et al.* 1995). The results suggest that if the NC1 domain is involved in the initial events during collagen XII chain assembly, this should take place via amino acids at the junction of NC1 and COL1. Based on these observations, the collagenous sequence was put at the center of interest. By using minicollagen XII in an insect cell expression system, Mazzorana & colleagues (2001) demonstrated that disulfide exchange between intra- and interchain bonding takes place, suggesting that the triple helix is formed first followed by interchain disulfide bonding in favorable redox conditions. The verification of these observations demonstrated that the formation of interchain disulfide bonds is not a prerequisite for the trimeric association and triple helical folding of collagen XII. The results also indicate that at least ten Gly-X-Y triplets in the C-terminal part of COL1 are required for the formation of a triple helix. Hence, it appears that the COL1/NC1 junction alone is not sufficient to drive association and that the role of the collagenous sequence and formation of the COL1 triple helix is critical for the trimeric assembly of collagen XII (Mazzorana *et al.* 2001).

2.5 Function of collagen IX

The functions of collagen IX have largely been proposed based on its structural properties and location on the surface of cartilage heterotypic fibrils. More recently, data confirming the suspected role of collagen IX interacting with other ECM partners, such as cartilage oligomeric matrix protein (COMP), heparin and matrilin-3 has been demonstrated (Pihlajamaa *et al*. 2004, Budde *et al*. 2005). In the end, a protein is able to fulfill its biological function only by interacting with other molecules or cells, and thus, it is the nature, quality and frequency of interaction that largely define the specific function(s) of a given protein. Valuable information of the potential roles of collagen IX has also been obtained from experimental animal models and from human disorders involving collagen IX.

2.5.1 Collagen IX as a cartilage component

Cartilage, representing a specialized form of connective tissue, consists of cartilage-specific cells (chondrocytes) and an abundant extracellular matrix. Cartilage can be divided into three major forms – elastic, fibrous and hyaline. Hyaline cartilage is the most prevalent and widespread type. Articular cartilage is a form of hyaline cartilage that covers the epiphyses of long bones at joint surfaces. It provides the joint with load-spreading and shock-absorbing properties and, together with the synovial fluid, enables frictionless movement of the joint. Cartilage formation is also essential for the development of long bones since cartilage serves as a model, being slowly replaced by bone. This process is known as endochondral ossification. Because cartilage is devoid of blood vessels and lymphatics, nutrition takes place by diffusion from adjacent tissues. Thus, the regeneration capacity of cartilage is poor (McCarty 1989, Buckwalter & Mankin 1998).

The primary constituents of the cartilaginous ECM are collagen II, proteoglycans and water. Proteoglycans are macromolecules that contain one or more GAG chains attached to a core protein, aggrecan. Large proteoglycan aggregates are formed when hyaluronic acid is bound by aggrecan and the structure is further stabilized by the binding of link protein. Because of the abundance of hydrophilic sulphated proteoglycans, cartilage is highly hydrated, providing the tissue with its osmotic properties and resistance to compression.

The swelling pressure caused by the presence of hydrated proteoglycan polymers is counterbalanced by a network of collagen fibrils. These fibrils, consisting primarily of collagens II, IX and XI, display typical D-periodic distribution (Vaughan *et al*. 1988). While the core of the fibril is formed by collagen XI, further growth in diameter is thought to occur by the deposition of collagen II, and finally the fibril becomes decorated by collagen IX (Eikenberry *et al*. 1992) (Figure 2). The structural integrity of the cartilage is known to depend on correct formation of these heterotypic fibrils, which relies on site-specific interactions between relatively small regions of interacting collagen molecules. Changes in these critical regions, for example single amino acid substitutions such as a Cys for Arg substitution at α1 -519 of collagen II (Fertala *et al.* 2001), may alter their structural and physicochemical characteristics and lead to aberrant interactions between collagens II and IX. Such changes in the affinity/interaction have been proposed to represent one of the steps in a cascade of changes affecting

the cartilaginous matrix and ultimately leading to its destabilization (Steplewski *et al.* 2005).

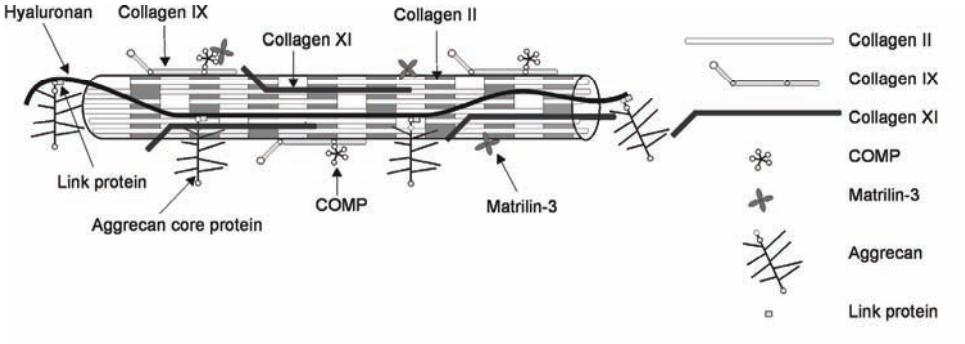

Fig. 2. Schematic presentation of cartilage ECM showing a heterotypic collagen fibril, consisting of collagens II, IX, and XI, and its association with some of the noncollagenous components of cartilage, including cartilage oligomeric matrix protein (COMP), aggrecan, hyaluronan, matrilin-3, and link protein. The figure has been originally published in Eveliina Jakkula's thesis, Acta Universitatis Ouluensis, D832.

The orientation of collagen IX molecules on the fibril is antiparallel in relation to collagen II molecules (Mendler *et al.* 1989, Wu *et al.* 1992). It has been shown that the critical regions for collagen IX binding on collagen II are located at the C-terminal part (D3 and D4 regions) of collagen II monomers and that collagen II telopeptides are unimportant for binding (Fertala *et al.* 2001). The interaction between collagens II and IX is stabilized by lysine-derived crosslinks at multiple locations. The crosslinking sites are located between the N-terminal region of the COL2 domain of each collagen IX α chain and the N-telopeptide of the α1(II) chain, and also between the central region of the COL2 domain within the α3(IX) chain and the C-telopeptide of the α1(II) chain (Eyre *et al.* 1987, van der Rest & Mayne 1988, Wu & Eyre 1989, Wu *et al.* 1992, Diab *et al.* 1996). Additional crosslinks are found between collagen IX molecules at the COL2 domain of either α1(IX) or α3(IX) chain and the NC1 domain of α3(IX) chain (Wu et *al.* 1992). A novel site at the NC1 domain of α1(IX) and Lys 930 of α1(II) has been discovered and a comprehensive model of cross-linking sites of collagen IX has been proposed (Eyre *et al.* 2004).

Several functions have been suggested for collagen IX, mainly based on its location on the surface of cartilage heterotypic fibrils. One of the proposed roles

is the stabilization of the collagen fibril network. This proposition is supported by the preferential location of collagen IX molecules at the intersections between two collagen fibrils (Müller-Glauser *et al.* 1986) and by the ability of two separate collagen IX molecules to interact independently of covalent crosslinks at the NC4 domains (Douglas *et al*. 1988). The surface position also implies a role as a modulator of the surface properties of collagen fibrils. The high positive charge of the NC4 domain (Vasios *et al*. 1988), in turn, suggests the capability of binding to adjacent proteoglycans or other ECM macromolecules. In fact, it has been demonstrated that a recombinant NC4 domain is able to bind heparin as well as cartilage matrix oligomeric protein (COMP) *in vitro* (Pihlajamaa *et al*. 2004). These observations support the idea of collagen IX modulating fibrillar surfaces and suggest a bridging mechanism by connecting or stabilizing macromolecular networks, a function also proposed for homologous collagens XII and XIV (Nishiyama *et al.* 1994, Akutsu *et al.* 1999). Furthermore, a role in regulating the fibril diameter has also been implicated for collagen IX. This is supported by the localization of collagen IX to the regions of cartilage where thin fibrils are the predominant type (Irwin *et al.* 1985, Müller-Glauser *et al.* 1986, Wotton *et al.* 1988, Hagg *et al.* 1998), and by the fact that the proportion of collagen IX decreases alongside with decreasing amounts of thin fibrils in maturing cartilage (Eyre *et al*. 1992). Another piece of evidence derives from experiments with *in vitro* fibrillogenesis. It is noteworthy that even though collagens II and XI may spontaneously initiate fibrillogenesis, they seemed incapable of generating correctly formed fibrils by themselves, and that a correct stoichiometry of 8:1:1 of collagens II, IX and XI was required for the formation and maintenance of proper thin fibrils (Vaughan *et al*. 1988, Eikenberry *et al*. 1992). However, collagen IX molecules have been observed on a few of the thicker collagen fibrils as well (Bruckner *et al.* 1988, Keene *et al.* 1995, Hagg *et al.* 1998).

Collagen fibrils in cartilage are known to vary in diameter and orientation depending on the location and developmental state. In contrast to the fibrils that are thicker and oriented perpendicular to the surface in the deeper zones of cartilage, fibrils at the surface tend to be thinner and run parallel to the surface. Most fibrils are 20 nm in diameter, but thicker fibrils have also been observed (Mendler et al. 1989). Moreover, the fibrils in embryonic cartilage are thinner in diameter as well as more randomly oriented when compared to adult cartilage (Horton 1993, Hagg *et al*. 1998). Fibril properties including diameter, spacing and morphology are known to be affected by fibril-associated components including the family members of small leusine-rich proteoglycans (SLRPs). These include

decorin, fibromodulin, lumican and biglycan, which are known to interact with collagen fibrils, regulating fibrillogenesis possibly by preventing lateral fusion of the fibrils (Hagg *et al*. 1998, Roughley & Lee 1994, Iozzo 1997). Other noncollagenous molecules known to associate with collagen are heparin (Munakata *et al.* 1999), COMP, a pentameric molecule capable of binding to collagens I, II and IX (Rosenberg *et al*. 1998, Thur *et al*. 2001, Holden *et al.* 2001), and matrilins 1 and 3 (see Deak *et al*. 1999). Matrilin-1 is able to bind to fibrillar collagens (Winterbottom *et al*. 1992), while matrilin-3 interacts directly with collagen IX (Budde *et al*. 2005). Besides types II, IX and XI, collagens III, VI, X, XII, XIII, XIV, XVI, XX and XXVII have also been detected in cartilage, but apart from collagen X they are not considered cartilage-specific as their main expression is in noncartilaginous tissues (Watt *et al*. 1992, Castagnola *et al*. 1992, Juvonen *et al*. 1992, Bruckner & van der Rest 1994, Young *et al*. 2000, Koch *et al*. 2001, Kassner *et al*. 2003, Pace *et al*. 2003, Boot-Handford *et al*. 2003).

2.5.2 Experimental mouse models

Genetically modified animal models provide a useful tool for studying the expression and function of collagens as well as other proteins. The functional significance of collagen IX has been studied by using different mouse models. A transgenic mouse line expressing a truncated α1(IX) chain with a large in-frame deletion of the complete NC3 domain and most of the COL2 and COL3 domains was generated (Nakata *et al.* 1993), exerting a dominant negative effect on the assembly of collagen IX molecules. The heterozygous offspring were normal at birth, but the homozygous mice developed mild osteochondrodysplasia with dwarfism and eye abnormalities. By the age of six months both heterozygous and homozygous mice developed degenerative changes in articular cartilage. The changes were progressive with age and resembled human OA. Histological specimens analyzed showed abnormally thin collagen fibrils. Furthermore, spinal changes including intervertebral disc degeneration and herniation were also detected (Kimura *et al.* 1996).

Mouse models totally lacking the α1(IX) chain have been created. In a recent report (Hu *et al.* 2006), age-dependent OA-like changes in the knee joints and temporomandibular joints, starting at the age of three months, were observed. Interestingly, the level of degraded collagen II was increased in the cartilage of these knock-out mice. Analogous results were reported previously, since the mice were normal at birth but, at the age of four months, developed a progressive

degenerative joint disease resembling human OA (Fässler *et al.* 1994). When studied in detail, it was discovered that the deficiency of the α1(IX) chain essentially leads to a functional knock-out of all three polypeptide chains of collagen IX, thus implicating a crucial role of the α1(IX) chains in the assembly process (Hagg *et al.* 1997). The cartilage collagen fibrils of the knock-out mice were apparently normal - a surprising finding, which is in contradiction to the proposed idea of collagen IX regulating fibril diameter. A slightly different function was, however, proposed based on experiments with immortalized chondrocytes derived from the same knock-out mice. The collagen fibrils produced were of normal diameter indicating that fibrillogenesis may occur without α(IX) chains. However, the fibrils behaved abnormally since they seemed to fuse laterally (Mallein-Gerin *et al.* 1995). This, together with the surface location of collagen IX, suggests a role in the prevention of lateral fusion and determining the correct spacing of parallel fibrils.

The normal skeletal morphogenesis and development of the α1(IX)-deficient mice suggests that collagen IX may not be essential for the development and formation of cartilage matrix. Instead, a structural function in maintaining the mechanical and biochemical stability in cartilage is implicated. This conclusion is supported by certain previous observations as well as the following two. First, mice overexpressing the NC4 domain of the α1(IX) chain developed age-related osteoarthritic lesions (Haimes *et al.* 1995). Secondly, when mice lacking the α1(IX) chain were backcrossed to the DBA/1 and B10.Q strains and immunologically challenged with collagen II, the CIX deficiency in both strains led to earlier and more severe arthritis when compared to controls (Carlsen *et al.* 2006). Taken together, these findings suggest that collagen IX may be unimportant in fibrillogenesis and the development of cartilage, but plays an important role in the maintenance of the long-term structural integrity of the tissue.

2.5.3 Human disorders involving collagen IX

Valuable information has also been obtained studying human disorders. Mutations in the genes encoding the three polypeptide chains of collagen IX are related to conditions including Stickler syndrome (Van Camp *et al.* 2006), multiple epiphyseal dysplasia (MED) (Chapman *et al.* 2003) and intervertebral disc disease (IDD) (Ala-Kokko 2002). As suggested by the mouse models, collagen IX might also be involved in the etiopathogenesis of primary OA, but so far this has

not been demonstrated in man (Jakkula *et al.* 2005a). The role of collagen IX in rheumatoid arthritis is discussed in section 2.6.

MED comprises a clinically and genetically heterogenous group of disorders that is characterized by delayed ossification and abnormalities in the epiphyses of long bones, short stature and early-onset degenerative OA of the large weight-bearing joints, primarily hips and/or knees. MED patients may also have spinal abnormalities. The phenotypic spectrum ranges from the mild Ribbing type to the more severe Fairbank type. Usually the symptoms include pain and stiffness in multiple joints, often leading to restricted joint motion. This may result in problems with gait.

To date, mutations in six different genes, *COMP*, *COL9A1*, *COL9A2*, *COL9A3*, *MATN3*, and *DTDST*, respectively, have been identified in MED patients. Mutations in all these genes, except *DTDST*, are dominantly inherited. It is known that the mutations in the known genes do not explain all MED cases (Jakkula *et al.* 2005b,c). When collagen IX is concerned, all mutations cluster in the splice-donor or acceptor site of exon 3 of *COL9A2* (Muragaki *et al.* 1996, Holden *et al.* 1999, Spayde *et al.* 2000, Fiedler *et al.* 2002) or *COL9A3* (Paassilta *et al.* 1999a, Bönnemann *et al.* 2000, Lohiniva *et al.* 2000, Nakashima *et al.* 2005). These mutations result in the skipping of exon 3 of *COL9A2* and *COL9A3* during RNA splicing. The resulting 36 bp deletion at the mRNA level gives rise to a 12-amino acid in-frame deletion in the α2(IX) or α3(IX) chains. Alternatively, mutations may occur in the splice-donor site of exon 8 of *COL9A1*, resulting in the skipping of exon 8 and/or 10 (Czarny-Ratajczak *et al.* 2001). The deletions in the *COL9A1* gene are predicted to cause an in-frame deletion of 25, 21 or 49 amino acids from the α1(IX) chain. All the deletions described are located in an equivalent region of the COL3 domain of collagen IX, suggesting an important function for this domain.

At least 22 different mutations in 25 probands have been described in the *COMP* gene, the first to be identified in MED (Briggs & Chapman 2002). The majority of the disease-causing *COMP* mutations cluster in exons 8-14, which encode the calcium-binding T3-repeats, or in exons 16-18, which encode the C-terminal globular domain (see Jakkula 2005a). The preponderance of these mutations represent point mutations leading to amino acid substitutions, small in-frame deletions or insertions. The exact mechanism by which COMP mutations cause MED is not clearly understood, but the incorporation of mutant COMP monomers into COMP pentamers is likely to cause a dominant-negative effect.

Thus, the mutations in COMP would represent a qualitative rather than quantitative defect (Chapman *et al.* 2003).

Matrilin-3 is the only member of the matrilin family known to be associated with MED (Jackson *et al.* 2004). All except one of the described nine mutations in the *MATN3* gene are missense mutations located in exon 2 of *MATN3,* affecting the β-sheet region of the vWFA domain (Chapman *et al.* 2001, Mostert *et al.* 2003, Jackson *et al.* 2004, Mabuchi *et al.* 2004). MATN3 is known to interact directly with the collagenous domains of collagen IX, and also indirectly with COMP serving as an adapter (Budde *et al.* 2005).

What is interesting in these mutations is the fact that collagen IX, COMP and matrilin-3 are known to interact with each other and it seems that a disruption to these interactions may represent a pathogenetic mechanism in MED (Holden *et al.* 2001, Chapman *et al.* 2003, Budde *et al.* 2005). The underlying disease mechanism has not yet been described in detail, but evidently, a large deletion in the COL3 domain of collagen IX may alter the conformation of the molecule with subsequent changes in its ability to interact with other ECM molecules (Chapman *et al.* 2003). In addition, it is known that mutations in the COMP and collagen IX genes may result in the accumulation of the corresponding proteins in the rough endoplasmic reticulum (ER) of chondrocytes, thus impeding their normal function (Maddox *et al.* 1997, Délot *et al.* 1998). This implies that the mutations may lead to misfolding of the protein that is subsequently recognized by the quality control system of the cell and results in the detrimental retention of the protein in the ER. This assumption is in agreement with the suspected dominant-negative effect, probably causing the entrapment of both the mutant and the wild-type protein. Similarly, when matrilin-3 is concerned, it is likely that it is not the overall loss but rather alterations in the structure/function that result in MED. However, the dilatation of the ER was not observed in MED patients with a mutation in the COL9A2 gene (van Mourik *et al.* 1998), implicating the existence of other disease mechanisms. Accordingly, certain collagen IX mutations have been reported to lead to decreased mRNA stability and incomplete splicing (Holden *et al.* 1999). The plurality of these diverse notions reveals that the cell and matrix pathology of MED is still inadequately comprehended and further research is necessary to understand the disease process and discover potential therapies.

IDD is a common musculoskeletal disease affecting the Finnish population with a prevalence of about 5 % (Heliövaara *et al.* 1987). IDD is characterized by intervertebral disc degeneration and herniation. These processes are often

accompanied by sciatica, pain felt in the lower back and radiating below knee level via the sciatic nerve. The symptoms are usually caused by disc herniation, i.e. extrusion of the inner disc layer, nucleus pulposus, through the outer layer, annulus fibrosus, causing mechanical compression or chemical irritation of the sciatic nerve root. Disc degeneration is an inevitable and common consequence of ageing, and it varies extensively in rate and extent between individuals and even in the same person over time (Buckwalter 1995). The degeneration impairs the structural integrity of the disc tissue predisposing to functional decline. The age-related degenerative changes include a decrease or loss of water and proteoglycans as well as collagen fibrils and viable cells within the matrix (Eyre 1979, Buckwalter 1995). The changes in the intervertebral discs are known to emerge as early as in the second decade of life (Nerlich *et al.* 1998).

Anthropometric, environmental and genetic factors are known to contribute to the etiology of IDD. Collectively, anthropometric and environmental factors include age, gender, occupation, hard physical work or a predisposition to vibration at work, cigarette smoking, physical trauma and body height. Their effects are evaluated as modest, however (Heliövaara 1989). Thus, genetic factors are suggested to play a role in IDD (Postacchini *et al.* 1988, Varlotta *et al.* 1991, Scapinelli *et al.* 1993, Battie *et al.* 1995, Matsui *et al.* 1998, Noponen-Hietala *et al.* 2005). The presence of collagen IX in the intervertebral disc (Newall & Ayad 1995) implicates mutations in the genes encoding collagen IX as possible contributors to the disease. Recently, it has been shown that sequence variations in the collagen IX and XI genes are associated with IDD (Noponen-Hietala *et al.* 2003). The observed changes in the intervertebral discs of α1(IX) transgenic mice (Nakata *et al.* 1993, Kimura *et al.* 1996) further strengthen this notion. Subsequently, two sequence variations that lead to incorporation of a tryptophan (Trp) residue into the α2(IX) or α3(IX) chain were detected by DNA analysis in a Finnish (Annunen *et al.* 1999, Paassilta *et al.* 2001) and Chinese population (Jim *et al.* 2005), and these variations were found to be associated with IDD. The molecular mechanisms underlying the disease process are not well understood. However, it seems that both the Trp2 and Trp3 allelic products are incorporated apparently normally into developing human cartilage (Matsui *et al.* 2002). This suggests that instead of misassembly of the matrix the pathological consequences of these sequence variations are likely to represent a long-term and indirect effect.

2.6 Integrins

Integrins constitute a major group of receptors playing important roles both in cell-cell adhesion and in recognition of extracellular matrix (ECM) components, including fibronectin, vitronectin, laminin and collagen. Structurally the integrins consist of one α and one β subunit forming heterodimeric receptors mediating dynamic linkages between extracellular milieu and the intracellular actin cytoskeleton. Integrins are expressed by all multicellular animals. Mammals are known to express 18 α and 8 β subunits that may combine to at least 24 different receptors (see Figure 3). During the last two decades, integrins have been, and still are, a target of intensive research. Studies with the genetic knock-out approach have indicated essential roles for almost all integrins and widespread functions in the maintenance of tissue integrity and modulation of cellular migration, differentiation and survival. While contributing to these processes in the physiological state, integrins are also implicated in disease processes such as infectious, inflammatory, traumatic and neoplastic conditions and have thus become acknowledged as potential therapeutic targets in a variety of conditions (see Humphries 2000 and Gullberg 2003).

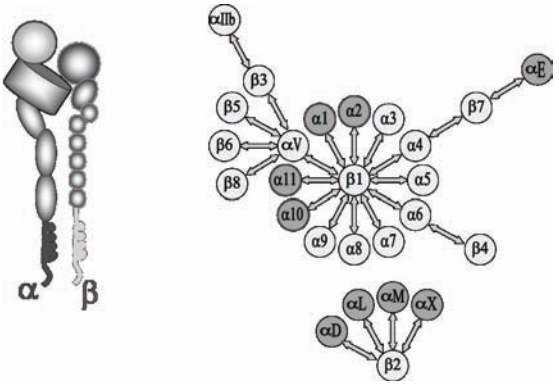

Fig. 3. The integrins form a family of cell surface receptors with 18 α and 8 β subunits expressed in mammals, and may combine to at least 24 different receptors. Schematic presentation of the overall structure of the integrin heterodimer is depicted on the left.

2.6.1 Structure and signaling properties

Integrins are αβ heterodimers consisting of one “head” with two “legs” that penetrate the plasma membrane with transmembrane helices and end in short cytoplasmic tails. The overall structure of integrins is shown on the left in Figure 3. The N-terminal head region is integral in ligand recognition. This region is different between the collagen binding integrins that contain an additional domain, the αI domain, which is crucial in collagen recognition, and the other integrins that recognize ECM molecules mostly via RGD sequences. The head of the α subunit is formed by a seven-bladed β-propeller domain and the inserted I domain, while the stalk or the leg region domains all adopt β-sandwich fold. The head region of the β subunit contains PSI, hybrid and an I-like domain (βI), which is the major ligand recognition site in integrins lacking αI domains (White *et al.* 2004). In the αI domain-containing integrins, the βI domain interacts with the α subunit head, affecting the integrin activation state and ligand affinity. Thus, the ligand specificities rely on both subunits of a given integrin heterodimer (Hynes 2002). The leg regions of the α and β subunits continue as single transmembrane helices that anchor the integrins to the lipid bilayer and end at the cytoplasmic domains that interact with each other and with cytoplasmic signaling components. The heterogeneity of the C-terminal sequences via multiple submembrane linker proteins enables the specific interactions with discrete cytoplasmic partners resulting in diverse cellular responses (Hynes 2002, White *et al.* 2004).

Many integrins are not constitutively active. The ECM adhesion and signaling properties of integrins are regulated by conformational changes between active and inactive forms. The active or high affinity conformer stands in an erect position on the plasma membrane, being exposed to interactions with the ECM while the inactive low affinity conformer adopts a bent position, being relatively inaccessible to ECM ligands. In this state, the cytoplasmic domains of α and β subunits are bound together, but, upon inside-out signaling, become separated. This leads to gross conformational rearrangements, resulting in an erect, open integrin conformation with high ligand affinity, with the head region exposed to the ECM. Outside-in signaling occurs when a ligand binds to an integrin with intermediate activity conformation resulting in the transition to an open active conformation and separation of the integrin cytoplasmic tails and activation of intracellular signal transduction pathways. Generally, this is thought to require specific high affinity binding by the ligand. (White *et al.* 2004.) The very recently published observation by Connors and colleagues shows that two synergistic

mechanisms, one related to the conformational state of the receptor regulated by amino acid Glu-336 and the other associated with receptor aggregation, affect the activation status of integrin α2ß1 (Connors *et al.* 2007).

2.6.2 Collagen-binding integrins α1ß1, α2ß1, α10ß1 and α11ß1

As described previously, the structure of collagen-binding integrins α1ß1, α2ß1, α10ß1 and α11ß1 is distinct from the other integrins. A significant role in collagen recognition is played by the αI domain. The structure of the α2I domain in the absence of ligand as well as bound to a collagen-like triple helix has been determined (Emsley *et al*. 1997, 2000, 2004). This, and the determination of crystal structures of several other integrin I domains, together with high sequence similarity between collagen-binding integrins has enabled the development of molecular models depicting the four α subunits aside their partner ß1 subunit (see White *et al.* 2004). These models provide information about the specific structural features possibly governing the recognition process between integrins and their ligands.

Tissue expression

The four collagen binding integrins display different expression patterns, although some overlapping may occur. Integrin α1β1 is mainly expressed on mesenchymal cell types including smooth muscle cells, endothelial cells, fibroblasts and chondrocytes. Integrin α2β1 is particularly abundant in epithelial cells and platelets, but it is expressed on endothelial cells, fibroblasts, chondrocytes, lymphocytes, mast cells, and neutrophilic granulocytes as well. The expression pattern of α10β1 and α11β1 integrins is mainly restricted to bone and cartilage (Heino 2000, White *et al.* 2004).

Collagen recognition

All the integrin I domains assume a “Rossman” fold composed of a central β sheet surrounded by seven α helices. The structure is homologous with the A domain of von Willebrand factors (vWFs), also capable of binding to collagen. Another characteristic feature of the I domains is the presence of an αC helix. This helix, as well as other regions, undergoes extensive reorganization upon ligand binding. It is suggested that the αC helix in the inactive conformation

prevents the integrin from interacting non-specifically with collagens (Gullberg & Lundgren-Åkerlund, 2002). It has also been proposed that the αC helix is guiding the collagen molecule to the right position and would therefore be necessary for binding (Käpylä *et al.* 2000, Heino 2000).

The ligand binding capability is dependent on the presence of a divalent cation, Mg^{2+} or Mn^{2+}, at the specific metal ion dependent adhesion site, MIDAS. The metal-coordinating residues within MIDAS are invariant among I domains, suggesting that integrins α1ß1, α2ß1, α10ß1 and α11ß1 engage collagen in a similar fashion. The importance of these three critical residues (two serines and a threonine) coordinating the MIDAS has been shown by mutagenesis that results in complete inhibition of ligand binding. Similarly, the presence of a glutamate residue in the collagen motif is an obligatory requirement for binding. The side chains surrounding the MIDAS motif, however, are more variable and provide additional ligand specificity, eg. preference with a certain collagen type (Emsley 2000, Gullberg 2003). This preference or selectivity in collagen binding is illustrated in numerous reports (Nykvist *et al.* 2000, Tulla *et al.* 2001, Zhang *et al.* 2003). Generally, α1ß1 prefers basement membrane collagen IV or collagen VI over fibril-forming collagens, contrary to the α2ß1 that displays the opposite preference (Kern *et al.* 1993, Tulla *et al.* 2001). Likewise, α1ß1, unlike α2ß1, is able to bind collagen XIII (Nykvist *et al.* 2000). The binding pattern of α10ß1 resembles that of α1ß1 (Tulla *et al.* 2001), while that of α11ß1 shows similarities with α2ß1 (Zhang *et al.* 2003). The key residues that determine the specificity of binding have been discovered by mutagenesis. These are D219 (aspartic acid) in the α2I domain and R218 (arginine) in the α1I domain. Switching these two residues with each other resulted in the reversal of the characteristic collagen type preference (Tulla *et al.* 2001) indicating that very subtle structural differences may explain the functional specifics of collagen binding integrins.

The recognition of fibrillar collagens by integrins α1ß1 and α2ß1 involves a specific, six-residue sequence GFOGER where O represents a hydroxyproline (Knight *et al.* 1998, 2000). Related sequences like GLOGER and GASGER have also been identified as potential binding sites (Xu *et al.* 2000), but GFOGER seems to be the sequence with the highest affinity with the ability to interact with the α2I domain even in the absence of activation (Siljander *et al.* 2004). The recognition of collagen IV by α1ß1 integrin is different from the previous case and involves specific R and D residues present on different collagen α chains (Eble *et al.* 1993, Golbik *et al.* 2000). This, together with other findings (Ivaska *et al.* 1999), suggests that collagen binding occurs via multiple α chains and requires

a certain three dimensional structure. The differences between integrins in their collagen type specificity also suggest that the underlying binding mechanisms may be different. This has been exemplified by a mutagenesis approach, demonstrating that collagens I and IV were indeed recognized by different mechanisms (Käpylä *et al.* 2000).

Signaling and cellular responses

Cell culture studies *in vitro* have demonstrated that cellular adhesion and signaling is different in a two-dimensional monolayer when compared to a three-dimensional matrix (Gullberg 2003, Heino 2000). This underlines the importance of the surrounding matrix and suggests that the function of the collagen receptors is dependent on the organization of the collagenous matrix. Signaling mediated by α1ß1 is known to inhibit collagen synthesis, while signaling through α2ß1 results in up-regulation of collagen synthesis. Analogously, α1ß1-mediated signaling stimulates cell proliferation, while signaling through α2ß1 may have an inhibitory effect on proliferation (see White *et al.* 2004). α2ß1 is also known to be important in mediating contraction. Both receptors, however, are involved in the induction of MMPs, especially collagenases 1 and 3. These cellular responses are largely mediated by the activation of the p38 pathway in the case of α2ß1, and, in the case of α1ß1, activation of mitogen-activated protein kinases, MAPKs through Shc and Ras (Heino 2000). Taken together, these results suggest that integrin α1ß1 is mainly associated with cell proliferation, while α2ß1 is more involved with matrix remodeling. The observations also indicate that certain signaling events take place only inside the three-dimensional collagen matrix, but not in monolayer cultures, suggesting that the architecture of the matrix as well as the shape of cells may modify signaling. Furthermore, integrin-mediated binding avidities and cellular effects are different with monomeric vs. fibrillar collagen (Jokinen *et al.* 2004).

Despite our increasing amount of knowledge concerning integrins, the biological significance of cell-collagen interactions is still far from clear. Both α1ß1 and α2ß1 are able to mediate cell adhesion and cell migration and participate in matrix remodeling during development and adulthood, in physiological and pathological states. The α1 knock-out mouse line demonstrates that integrin α1ß1 is not an absolute requirement for normal development, but points to roles in the regulation of angiogenesis, inflammation, fracture healing and fibrosis. Likewise, α2ß1-deficient mice are unaffected in terms of

development and viability, but exhibit disturbances in mammary gland branching morphogenesis and platelet adhesion (White *et al.* 2004). Loss of α10ß1 causes disturbances of chondrocyte shape, arrangement and proliferation, resulting in growth retardation of the long bones, demonstrating a specific role in growth plate morphogenesis and function (Bengtsson *et al.* 2005). Mouse lines deficient in α11ß1, in turn, display defects in dental development, causing malnutrition (Popova *et al.* 2007). For a comprehensive understanding of collagen recognition in mammals, the reader is advised to see the recent reviews by Heino (2007) and by Leitinger & Hohenester (2007).

2.7 Rheumatoid arthritis

RA is a systemic autoimmune disease with a prevalence of about 1 % (Feldmann *et al.* 1996b). It is more frequent in women, suggesting a role for sex hormones. The disease is most commonly manifested between the ages of 40-70 years. In its early phases RA primarily affects peripheral joints, where active inflammatory synovitis may result in cartilage break down, bone erosion and, as a result of joint deformity and loss of function, severe disability. The clinical course of RA is highly variable and often unpredictable. Acute disease phases with pain, swelling and inflammation of the joints are interspersed by periods with less prominent symptoms. Despite the fact that RA is primarily focused at joints, the systemic nature of the disease, with manifestations in multiple organs, may be demonstrated in the later stages of RA, or in severe cases, in which mortality is increased. The etiology of RA is so far unknown, but it has been postulated that both environmental and genetic factors play a role in the etiopathogenesis. Of the genetic factors, major histocompatibility complex (MHC) class II genes, in particular those encoding the β-chain of the DR1 and DR4 molecules, have been identified as major contributors to disease predisposition (Gregersen *et al.* 1987).

The diagnosis of RA is mainly based on clinical characteristics. At the moment, no single objective and specific diagnostic test exists, and diagnosis is usually achieved by combining information regarding clinical symptoms, radiographic imaging and laboratory markers, such as erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP), that are useful indicators of disease activity (Banks *et al.* 1998). Generally, the ACR 1987 revised criteria for the classification of RA (Arnett *et al.* 1988) have been used in the diagnosis of RA (Table 4). These criteria include only one serological parameter, rheumatoid factor (RF). The presence of IgM RF has traditionally been regarded as the most

important marker for RA. Based on its presence or absence, RA has been divided into two categories, seropositive or seronegative, respectively. This categorization may be meaningful since the presence of RF and its serum levels seem to predict a more aggressive and destructive course of disease (Richardson & Emery 1996, Swedler *et al.* 1997, Pai *et al.* 1998), while RF-negative RA generally presents with clearly less severity. The emergence of RF may precede the onset of RA by several years (Rantapää-Dahlqvist *et al.* 2003), but the problem with RF in the diagnostics of RA is its lack of specificity. Indeed, RF can be detected in up to 70-80% of RA patients but it is also detected in other rheumatic diseases, infectious diseases, and even in 3-5% of apparently healthy individuals (Aho *et al.* 1994, see also Vallbracht & Helmke 2005). In addition to RF, other serological markers such as antibodies to collagen II, antiperinuclear factor, antikeratin antibody and antibodies against cyclic citrullinated protein (CCP) may be utilized in the diagnostics of RA. Of these, only CCP has been accepted for clinical use.

Table 4. The revised classification criteria for RA by the American College of Rheumatology (ACR) (Arnett et al. 1988).

Criteria
1. Morning stiffness for at least one hour.
2. Soft tissue swelling of 3 or more joint areas observed by a physician.
3. Swelling of wrist, MCP or PIP areas.
4. Symmetric swelling.
5. Presence of rheumatoid nodules observed by a physician.
6. Presence of rheumatoid factor (RF).
7. Radiographic changes (erosions and/or osteopenia) in the wrist, MCP or PIP areas.

Four out of seven criteria must be fulfilled. The symptoms defined by the criteria 1-4 must have been present for at least six weeks.

The main objective in the treatment of RA is to induce complete remission and thus prevent joint destruction and immobility. When remission is not achieved, treatment is aimed at alleviating pain, controlling disease activity, slowing down the progression of tissue damage and maximizing the quality of life. Further objects of management include the maintenance of functional capacity as well as capacity for employment (Möttönen *et al.* 1999). An early diagnosis of RA is of vital importance since the outcome and the therapeutic effect depends largely on the timing of initiation of therapy (see O`Dell 2002, Visser *et al.* 2002, Kary *et al.* 2004). Therefore, diagnosis should be attempted and therapy initiated as early as

possible (Nell *et al.* 2004). New biomarkers might provide additional specificity and reliability for this purpose.

Disease-modifying anti-rheumatic drugs (DMARDs) are the most commonly used group of drugs to treat RA. Recent reports (O'Dell 2002, Kary *et al.* 2004, Nell *et al.* 2004) indicate that combination therapy with multiple DMARDs in early RA is able to effectively slow down the progression of RA. For example, aggressive initial treatment of early RA with a combination of DMARDs limited peripheral joint damage for at least 5 years (Korpela *et al.* 2004) and reduced occupational disability (Puolakka *et al.* 2004).

Besides DMARDs, RA is treated with corticosteroids or immunomodulatory drugs. Because of limited management potential and possibly significant side effects, the use of corticosteroids is primarily focused on local injections or low-dose oral treatment. Recently, new biological drugs have become available and introduced into clinical use. These drugs are targeted to molecules and cells important in the immunopathogenesis of RA and indicate that blocking single cytokines may produce profound effects in inflammatory diseases, also those other than RA (see Edwards 2005). Since the discovery of the fact that blockade of tumour necrosis factor alpha (TNFα) is able to reduce all other pro-inflammatory cytokines found in rheumatic joints (Feldmann *et al.* 1996a), it has risen to center stage among cytokine-targeted therapies. The efficacy of TNFα blockers is significantly increased by co-administration of methotrexate, and this therapy has been demonstrated to considerably retard or even restore the radiographically observed destruction of joints in RA (see Feldmann *et al.* 2005, Navarro-Sarabia *et al.* 2005).

2.7.1 Cartilage collagens and autoimmunity in RA

In many ways, cartilage is found at the center of disease activity in RA. For example, the synovitis in RA is known to remit within a particular joint after the removal of cartilage at arthroplasty (Konttinen *et al.* 2001), while the rest of the affected joints remain inflamed. In addition, cartilage collagens have shown an ability to elicit autoimmune-mediated arthritis both in rodents and primates. The pathogenic potential of the resulting autoantibodies is shown by their ability to passively transfer arthritis into susceptible recipients. The detection of antibodies against cartilage collagens in humans with RA or other inflammatory arthritic diseases also suggests a common role of collagen autoimmunity in these diseases.

Thus, it appears that cartilage-specific collagens are involved in the RA disease process as they serve as targets for autoimmunity.

2.7.2 Antibodies against collagens II and XI

The presence of collagen II (CII) antibodies in the sera of human subjects suffering from RA was noted as early as 1976 (Andriopoulos *et al.* 1976b), and high frequencies of these antibodies are detected in rheumatoid synovial fluid (Andriopoulos *et al.* 1976a, Clague & Moore 1984) and cartilage specimens (Jasin 1985, Terato *et al.* 1990). In accordance with these observations, IgG-producing B cells specific to CII are present in the rheumatoid synovium (Tarkowski *et al.* 1989) and synovial fluid (Rönnelid *et al.* 1994, Rudolphi *et al.* 1997, He *et al.* 2001), suggesting the presence of a local antigen-driven immune process. Subsequently, antibodies to CII have been detected in RA sera at multiple occasions (Morgan *et al.* 1987, 1988, 1989, Charrière *et al.* 1988, Collins *et al.* 1988, Rowley *et al.* 1988, Terato *et al.* 1990, 1996). While some reports have demonstrated that the antibody response to CII may correlate with disease progression (Cook *et al.* 1996) and inflammatory activity (Kim *et al.* 2000) in RA, some reports indicate that anti-CII antibodies tend to appear at a high incidence during the early phases of the disease, suggesting a possible value in diagnostic use (Fujii *et al.* 1992, Cook *et al.* 1994). Interestingly, Cook and colleagues found that the frequency of antibodies to the CB10 fragment of CII in cases of well-established RA was significantly increased, from 24% to 88%, when compared to the frequency using an intact CII molecule. This finding demonstrates that the CB10 fragment is a markedly more sensitive substrate than the intact CII and, together with a high specificity of 94%, implies clinical utilization (Cook *et al.* 2004). It has also been observed that autoimmunity to CII differs in fine specificity between distinct disease entities (Burkhardt *et al.* 2002). Interesting new data has also been presented regarding the role of citrullination in the autoimmunity to CII. Autoantibodies to citrullinated CII were detected in 78.5% of serum samples from 130 RA patients, while autoantibodies to native noncitrullinated CII were detected in only 14.6% of sera (Yoshida *et al.* 2006). Furthermore, autoantibodies recognizing the citrullinated telopeptide of CII were detected in RA sera (Koivula *et al.* 2005) and shown to predict the development of seropositive RA (Koivula *et al.* 2007). As a whole, these results encourage the introduction of CII antibody tests in clinical use.

Proinflammatory cytokines are important in the inflammatory expression of RA. Recently, the role of CII-reactive T cells in the stimulation of fibroblast-like synoviocytes to produce proinflammatory cytokines was investigated (Kim *et al.* 2004). The results demonstrated that high reactivity to CII was frequently observed in RA patients and that enhanced T cell responses to CII were strongly correlated with increased production of proinflammatory cytokines and chemokines crucial in the inflammatory response in RA. Parallel results were recently obtained, showing that immune complexes (ICs) containing anti-CII antibodies from arthritis patients induced the production of TNFα, interleukin-1 beta (IL-1β) and IL-8 (Mullazehi *et al.* 2006). The induced cytokine response was abolished after monocyte depletion or significantly reduced after blocking the activating Fc gamma receptor (FcγRIIa). FcγRs are leukocytic receptors present on macrophages, neutrophils and B cells and their expression is highly elevated in RA synovium (see Magnusson *et al.* 2007). Crosslinking of FcγRs by IgG-ICs is known to activate cellular effector functions such as antibody-dependent cellular toxicity and the production of inflammatory cytokines (Hogarth 2002). The expression of FcγRs has also been noted to correlate with the expression of TNFα and MMP-1 (Blom *et al.* 2003), providing a link both to inflammatory and degradative expression of RA. The role of ICs containing anti-CII has subsequently been shown in a clinical setting, as patients with very high anti-CII levels seem to represent a distinct phenotype characterized by significantly elevated cytokine profiles as well as elevated CRP and ESR at the baseline (Mullazehi *et al.* 2007). Thus, it is possible that anti-CII-containing ICs mediate an early acute phase response in RA. Taken together, these reports suggest that autoimmunity to CII may play an integral role not only in the amplification and perpetuation but also in the initiation of the inflammatory process in RA.

Research carried out with animal models is also supportive of the relevance of collagen autoimmunity as the susceptible strains of rodents and non-human primates immunized with collagens II or XI are known to develop autoreactive antibodies resulting in a collagen-induced arthritis (CIA) that bears immunological, pathological and phenotypic similarities to RA (Morgan *et al.* 1983, Holmdahl 1990a, 2002, Boissier *et al.* 1990, Cremer *et al.* 1992, 1994, 1995, 1998, Myers *et al.* 1997, Brand *et al.* 2003). Interestingly, when compared to CII collagen XI induces a different form of erosive and chronic relapsing polyarthritis in rats, thus providing a slightly different model of CIA (Lu *et al.* 2002). The pathogenic potential of the resulting antibodies has been demonstrated in experiments in which purified human or murine anti-CII antibodies were

shown to transfer arthritis passively to naïve recipients and result in their deposition in the joint, producing an acute arthritis with cartilage destruction (Stuart *et al.* 1982, 1983, Wooley *et al.* 1984, Loutis *et al.* 1988, Kerwar & Oronsky 1988, Holmdahl 1990b, Terato *et al.* 1992). Moreover, collagens II and XI are shown to induce tolerance and suppress arthritis when given as intravenous, oral or nasal tolerogens (Yoshino *et al.* 1995, Myers *et al.* 1998, Lu & Holmdahl 1999). Based on promising results with animal models of RA, oral CII has also been attempted as a treatment for human RA. The results of these studies (Trentham *et al.* 1993, Sieper *et al.* 1996, Barnett *et al.* 1998) show a trend toward clinical improvement but fail to reach statistical significance.

As in human RA, anti-CII-induced proinflammatory cytokines, such as TNFα and IL-1β, are abundantly expressed in the arthritic joints of mice with CIA (Williams 2004, 2007). While certain reports indicate that induction of CIA is dependent on FcγR status and related to the expression of these cytokines (Kleinau *et al.* 2000, Nandakumar *et al.* 2003) or the involvement of the MHC class II genes (Fugger *et al.* 1996, Rosloniec *et al.* 1997, 1998), other findings strongly support the role of the classical complement activation pathway, mediated by antibodies to CII, in the development CIA (Watson *et al.* 1987, Terato *et al.* 1992, Wang *et al.* 1995, Hietala *et al.* 2004).

The divergent pathways of adaptive immunity, represented by T and B cells, both play an important role in the pathogenesis of CIA, but their relative significance in priming the immune system and, on the other hand, contributing to joint destruction remain partly unclear. However, it is known that while the B cell response in terms of production of anti-CII antibodies is critical to the development of the disease, the role of T cells in CIA is more complex. Firstly, T cells are known to provide help to B cells in the production of antibodies directed to CII and they also participate and regulate the production of proinflammatory cytokines. Secondly, T cells may play a modulatory role in joint inflammation by activating other cells, e.g. synovial macrophages (see Holmdahl 2002). However, Zhang and coworkers showed by depleting the CII-reactive T cells that they play an instrumental role in the development of CIA and that the anti-CII antibodies strongly contributed to the development of CIA (Zhang *et al.* 2002). Most importantly, a direct role of CII antibodies has been shown by an experiment, where mice deficient for T or B cells were susceptible to collagen antibody-induced arthritis, demonstrating that CII-specific antibodies are able to induce arthritis even in the absence of an adaptive immune system (Nandakumar *et al.* 2004). Further support of the relevance of CII autoimmunity is demonstrated by

reports showing *in vitro* evidence that CII-C1 antibodies, recognizing the C1 conformational epitope of CII, impair cartilage formation in cultured chondrocytes and strongly inhibit the self-assembly of collagen fibrils from CII in solution (Amirahmadi *et al.* 2004, Gray *et al.* 2004). Furthermore, besides CII-C1, M2139, another antibody arthritogenic in CIA, was able to affect chondrocyte morphology and matrix synthesis, causing disorganization of CII fibrils in the ECM without the influence of cells of the immune system or their secreted products (Amirahmadi *et al.* 2005). These two antibodies were also able to induce loss of proteoglycan and CII in the ECM, interfering with the structural integrity of the pre-existing cartilage (Crombie *et al.* 2005) and providing evidence that these antibodies may contribute directly to cartilage destruction. Interestingly, the C1 epitope targeted by these antibodies is also recognized by human IgG antibodies in RA sera (Burkhardt *et al.* 2002). Thus, it appears that antibodies produced in RA are directed to the same conformational epitopes on CII as in CIA (Holmdahl 2002, Nandakumar *et al.* 2005). Collectively, the data suggest that CIA provides a meaningful tool to study RA pathogenesis and validate therapeutic targets.

2.7.3 Antibodies against collagen IX

The ability of CIX to induce arthritis has been investigated primarily in rodent models. Rats immunized with CIX developed a good T cell response to CIX, high levels of complement fixing antibodies to CIX, and deposits of antibody in the cartilage (Cremer *et al.* 1998). Intraperitoneal injection of lipopolysaccharide to stimulate the production of proinflammatory cytokines was ineffective, but intra-articular injection with CIX produced transient synovitis. Based on these observations CIX is a strong immunogen in rats, but surprisingly, incapable of inducing arthritis. Parallel results were obtained by using recombinant human collagen IX (rCIX) to induce murine CIA (Myers *et al.* 2002). When B10 congenic mice were immunized with rCIX, they developed a substantial antibody response to rCIX, but again, no erosive arthritis. The results of these two studies contradict those obtained by Boissier *et al.* (1990), which demonstrated a mild arthritis in DBA/1 mice after immunization with CIX. CIX has also been shown to be effective in the treatment of pristane-induced arthritis in rats, demonstrating potency in preventing and ameliorating established arthritis in rats (Lu & Holmdahl 1999). Furthermore, it has been shown that two mouse lines, lacking CIX as a result of transgenic disruption of the COL9A1 gene, developed an

earlier and more severe form of arthritis (Carlsen *et al.* 2006). The authors hypothesized that the deficiency of CIX may not only destabilize the cartilage but might also allow more prominent exposure of cartilage epitopes to antibody binding. By quantifying the binding of the CIIC1 antibody, targeted to CII, in the cartilage of CIX-deficient and control mice, the authors demonstrated a larger and denser staining in the CIX-deficient mice, thus confirming the hypothesis. Collectively, the data suggest that CIX is capable of inducing autoimmunity but not arthritis in the applied models. For a more comprehensive understanding, the reader is advised to see reviews by Diab (1993) and Cremer et al. (1998).

A rabbit model of RA has also been studied. Kojima and coworkers (2001) demonstrated that the proteoglycan aggregan and the NC4 domain of CIX were reduced in femoral and tibial cartilage, concomitant with the induction of inflammatory arthritis, reflecting rapid and extensive ECM degradation caused by joint inflammation. The amount of denatured CII in cartilage was also increased, but only after CIX and aggregan were first degraded and lost from the cartilage. The loss of CII in cartilage could be detected by measuring collagenase-generated peptides in synovial fluid, but interestingly, the changes were not reflected in serum.

The literature regarding the role of autoimmunity to CIX in RA is sparse. Morgan and colleagues have produced two reports, one describing the incidence of CIX autoimmunity in a cross-sectional setting (Morgan *et al.* 1987) and the other following the anticollagen antibody patterns of RA patients in a longitudinal study (Morgan *et al.* 1989). The former report indicated that the incidence of sera containing autoantibodies to CIX was found low (1.8-3.9% to native antigen vs. 3.6-11.8% to denatured antigen, depending on CII antibody positivity), while the latter demonstrated that the patients showed a selective and varying immune response to collagens, with a strong correlation between CRP and collagen autoimmunity in a subgroup of patients. Antibodies to CIX and other collagens were also examined in patients with rheumatic diseases, including RA, OA, osteoporosis and Paget's disease (Charrière *et al.* 1988). In this study, antibodies to CIX were found at a high frequency (44%) in RA patients only, contradicting to results by Morgan *et al.* (1987, 1989). The presence of collagen autoimmunity was associated with shorter disease duration or lower severity. No association was discovered between the presence of antibodies to CIX and CII. Besides these reports, only one study concerning the role of CIX in RA has been published, demonstrating that immunoreactive peptides of CIX are present in the synovial fluids of patients with arthritic diseases (RA or OA) (Wotton *et al.* 1999).

Interestingly, these peptides were found to contain significant amounts of non-collagenous material. The accessibility of CIX on the surfaces of cartilage heterotypic fibrils to autoantibodies and degradative enzymes leads the authors to hypothesize that fragments of CIX may be among the first to be released into the synovial fluid during joint inflammation (Wotton *et al.* 1999).

2.7.4 Markers of cartilage metabolism in RA

Along with antibodies targeted to cartilage collagens, other markers reflecting cartilage or bone matrix turnover have been evaluated as diagnostic or prognostic aids in RA. These include the C-terminal telopeptide of collagen I (Åman 1999, 2000), N-terminal propeptides of collagens I and III (Hakala et al. 1995), pyridinoline and deoxypyridinoline crosslinks of collagens I and II (Müller *et al.* 2003), a helical peptide derived from CII termed HELIX-II (Charni *et al.* 2005) and C-terminal crosslinking telopeptides of collagens I and II, termed CTX-I and CTX-II, respectively (Garnero *et al.* 2002, Landewé *et al.* 2006). The results demonstrate that markers of collagen metabolism may have clinical relevance in the diagnostics and treatment of RA, as increased levels of HELIX-II as well as CTX-I and CTX-II independently predict an increased risk of disease progression (Garnero *et al.* 2002, Charni *et al.* 2005). HELIX-II is also specific for CII degradation and is capable of distinguishing RA patients from healthy controls. Of particular interest are the results showing that arthritis instantaneously causes damage to collagens II, and to a lesser extent, collagen I in patients with early RA (Landewé *et al.* 2006). The results demonstrate that clinically perceptible arthritis is responsible for immediate damage to cartilage, thus stressing the importance of early diagnosis and effective intervention.

3 Outlines of the present study

When this thesis project was started, the genes encoding the three polypeptide chains of collagen IX, COL9A1, COL9A2 and COL9A3, had recently been characterized and the baculovirus system for the production of recombinant collagen IX set up. The heterotrimeric protein had been successfully produced in insect cells and characterized. In other words, the basic infrastructure for studying the biological function of collagen IX had been laid down. Thus, it was a logical continuation of the previous work to further investigate both the structural and functional role of collagen IX as a constituent of the extracellular matrix as well as its role in disease.

The assembly process of fibrillar collagens, the prototype in the collagen superfamily, is currently relatively well understood. The corresponding process with the FACITs is different and poorly understood. Understanding this process would be important since the formation of the correctly folded triple helix is a prerequisite for the proper function of collagen in tissues. Existing reports point to a significant role of the COL1-NC1 junction and adequate hydroxylation in the assembly of FACITs. However, only two reports have been published with the focus on collagen IX, leaving many questions, including the role of individual domains or the direction of trimer formation, unanswered.

Integrins make up a large family of heterodimeric transmembrane proteins that may act as receptors for various ECM components, including collagens. Molecular interactions between integrins and several collagen types including fibrillar collagens I, II and III, transmembrane collagen XIII, microfibril-forming collagen VI and basement membrane collagen IV have been described, but no data has been available concerning the interaction with the FACITs.

Cartilage collagens are implicated in the pathogenesis of RA. While representing the bulk of collagenous material in the cartilage, collagen II has been intensively studied. For example, human or murine anti-CII antibodies were shown to be able to transfer arthritis passively to susceptible recipients. Furthermore, antibodies against collagen II have been detected in the serum, synovium and cartilage of RA patients. Antibodies to collagen II are also known to correlate with inflammatory activity and disease progression in RA. However, very little is known about the possible role of collagen IX in RA, although it decorates the surfaces of collagen II. Three reports, all dated in the mid-eighties, have been published with regard to antibodies to collagen IX in RA. All of them were carried out with animal-derived tissue-extracted collagen, which is not ideal

for antibody screening. Our method of choice is to use recombinantly produced human collagen as an antigen. This approach offers many practical advantages when compared to tissue-extracted antigen.

The following specific aims were set for this doctoral project:

1. to investigate the chain assembly process and trimer formation of collagen IX by using truncated protein variants, with the emphasis of determining specific roles of individual domains and critical regions for chain selection,
2. to analyze the molecular interactions between collagen IX and collagen-binding integrins α1ß1, α2ß1, α10ß1 and α11ß1, to identify the location(s) of interaction, and to establish whether collagen IX can mediate cell adhesion,
3. to determine the relative levels of autoantibodies towards full-length human collagen IX in the sera of patients with recent onset rheumatoid arthritis, to asses the usefulness of these antibodies as a diagnostic marker, and to investigate whether the antibody levels associate with disease activity or outcome.

4 Materials and methods

The materials and methods used in this thesis are summarized in Table 5 and in Figure 4 below. Detailed descriptions of the experimental procedures, including the references, are presented in the original articles I – III. The subjects, clinical variables and the statistical methods used in the original article III are shortly described below. For detailed descriptions see the articles.

Table 5. Methods used in the original publications.

Method	Original publication
Cloning techniques	I
Recombinant protein expression in insect cells	I, II, III
Recombinant protein expression in *E. coli*	II
Protein purification	I, II, III
Gel filtration	I, II, III
Ion exchange chromatography	I, II, III
Protein analysis	I, II, III
SDS-PAGE & Western Blotting	I, II, III
Amino acid analysis	I, II, III
Circular dichroism spectroscopy	I
Chemical cross-linking	I
Solid-phase binding assays	II
Cell spreading experiments	II
Rotary shadowing and transmission electron microscopy	II
Detection of anti-CIX antibodies by ELISA	III

The subjects consisted of 53 seropositive RA patients and 30 healthy controls. The RA patients included 36 females and 17 males, with a mean age of 50 years, and fulfilled the classification criteria of the ACR for RA. The duration of their symptoms at the time of the initial diagnosis was less than 12 months (median duration 6 months) and the patients had received no previous anti-rheumatic medication. The presence of rCIX antibodies and markers of disease activity were analyzed at the moment of initial diagnosis and after 3, 6, 12 and 24 months of follow-up.

The following measures of disease activity were monitored: erythrocyte sedimentation rate (ESR), C-reactive protein, thrombocyte count, duration of morning stiffness, number of swollen joints, number of tender joints, patient's global assessment on the visual analogue scale (VAS) and physical function as

measured by the health assessment questionnaire (HAQ). The radiographic progression was evaluated by the number of erosive joints in hands and feet.

The results were expressed as means or medians, with standard deviations (SD) or ranges and 95 per cent confidence intervals (95% CI). Statistical comparisons between the groups were made using a bootstrap-type t-test. Correlation coefficients were calculated by the Spearman method.

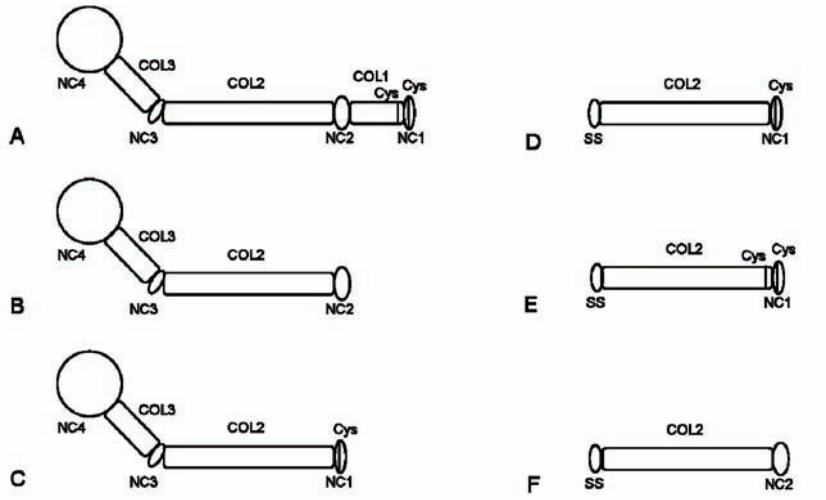

Fig. 4. Schematic representation of the expressed collagen IX variants. Full-length collagen IX (A) consists of three collagenous domains (COL1-3) and four noncollagenous domains (NC1-4), named from the C-terminus of the molecule. Two cysteines (Cys) located in the COL1/NC1 junction are conserved in FACITs. NC4-NC2 collagen IX (B) consists of NC4, COL3, NC3, COL2, and NC2 domains, while NC4-COL2/NC1 collagen IX (C) consists of NC4, COL3, NC3, COL2 and NC1 domains. In COL2/NC1 collagen IX (D) the α1(IX) chain signal sequence (SS) is attached to the COL2 domain followed by the NC1 domain, which contains one of the conserved cysteines. COL2/C/NC1 collagen IX (E) is comprised of SS and COL2 and NC1 domains with an engineered cysteine residue at the C-terminus of the COL2 domain in addition to the conserved cysteine in the NC1 domain. COL2/NC2 collagen IX (F) consists of SS and COL2 and NC2 domains.

5 Results

5.1 Collagen IX α chains can trimerize in the absence of NC1 and COL1 domains

Chain association of collagen IX was studied by expressing full-length CIX as well as five different variants (the “long” variants NC4-COL2, NC4-COL2/NC1 and the “short” variants COL2-NC1, COL2-NC2 and COL2/C/NC1; Figures 1 and 2 in I) in an insect cell expression system and analyzing the ability of the chains to assemble into trimeric composition by SDS-PAGE and immunoblotting. First, the variants were amplified by PCR (primers described in Table 1 in I) and cloned into baculovirus expression vectors. These vectors were then co-transfected in insect cells with the nuclear polyhedrosis virus. The resulting viral pools were plaque purified, amplified and tested for expression on adherent insect cell cultures. Best viruses were selected for subsequent co-expression with the virus encoding human prolyl 4-hydroxylase. Conditioned media from the adherent insect cell cultures was collected after 72h of expression and analyzed by SDS-PAGE followed by Western blotting and detection with the monoclonal antibody 95D1A.

When collagen IX α chains were expressed in diverse compositions together with prolyl 4-hydroxylase, both full-length and NC4-NC2 chains assembled mainly into monomeric and dimeric molecules (Figures 3 and 4 in I). However, also small amounts of homotrimeric α1 FL molecules, but not α2 and α3, could be detected for full length chains (Figure 3B). Trimers of α1 and α3 were seen for the NC4-NC2 chains (Figure 4B), indicating an increased tendency to form homotrimers. When full-length chains were co-expressed in pairs (α1/α2, α1/α3 or α2/α3), trimers were only observed when the α1 chain was present (Figure 3B). When all three chains were co-expressed, a new trimer band was observed corresponding to the heterotrimeric molecule consisting of all three chains (Figure 3B). In contrast, when different NC4-NC2 chains were co-expressed (Figure 4B), there was no indication of a new heterotrimeric band, although heterotrimers were observed with the pairwise combinations. The obtained results indicate that collagen IX NC4-NC2 α chains lacking NC1 and COL1 may assemble at the NC2 or NC3 domain and form disulfide-bonded trimers. Thus, the COL1 and NC1 domains are not essential for trimerization. Instead, based on the

obtained results, they seem to play an important role in the specificity of chain selection.

To verify the triple helicity of the NC4-NC2 variant, it was produced in insect cell suspension cultures in large amounts and purified. Collagen IX was precipitated from the conditioned medium (after 72 h) with 26% saturation of ammonium sulphate, and subjected to gel filtration and cation exchange chromatography. Purified proteins were analyzed by SDS-PAGE (Figure 6 in I), dialyzed against 50 mM acetic acid and subjected to circular dichroism analysis at +5°C. Far UV spectra were studied by performing wavelength scans from 250 to 190 nm. Typical collagen triple helical spectra with maximum ellipticity around 221 nm and minimum around 198 nm (Piez & Sherman 1970) were observed both for full-length and for NC4-NC2 collagen IX (Figure 7 in I). The results suggest that the trimeric molecules seen on the gels under non-reducing conditions (Fig. 6D in I) were indeed triple helical and not only disulfide bonded.

5.2 Structural information provided by N-terminal regions is necessary to ensure triple helical folding of collagen IX

Along with the NC4-NC2 construct, another variant, in which NC2 was replaced by NC1, was constructed for comparison (Figures 1 and 2 in I). When the conditioned medium from adherent insect cell cultures was analyzed, it was found that all three chains of NC4-COL2/NC1 were able to assemble into homotrimers (Figure 5B in I), but a heterotrimeric composition typical of full-length collagen IX was not observed. Again, this is indicative of poorer chain selection specificity. Since trimerization could take place at an artificial COL2/NC1 junction, the results also imply that collagenous and noncollagenous domains may act in different combinations and that the information for association may not be junction-specific.

To further investigate the roles of different domains in the folding of collagen IX molecules, three different variants were constructed in which the N-terminal domains NC4, COL3 and NC3 were deleted. The short variants termed COL2/NC2, COL2/NC1 and COL2/C/NC1 contained a long collagenous domain to ensure triple helical folding (see Nokelainen *et al.* 1998). COL2/C/NC1 was constructed to mimick the two cysteines found at the COL1/NC1 junction of CIX, since the corresponding cysteine residues in collagen XII have been shown to be important in chain association. To ensure secretion of the resulting proteins, a cloned signal sequence of the α1(IX) chain (9A1 SS) was added to the 5’-end of

the constructs. When chain association of COL2/NC1 and COL2/C/NC1 was analyzed, monomers of the expected sizes were found, as demonstrated in Figure 8A in I. Under non-reducing conditions (Figure 8B in I), both monomeric and dimeric compositions were detected, but not trimers. Similar results were obtained also for the COL2/NC2 variant (data not shown).

The fact that the long variants (NC4-NC2 and NC4-COL2/NC1) were able to trimerize, but the short ones (COL2/NC2, COL2/NC1 and COL2/C/NC1) were not, was surprising. Therefore, the conditioned media from the expressions with the short variants was subjected to covalent crosslinking using BS3 (McAlinden *et al.* 2003) to stabilize any dimers or trimers present. When analyzed by SDS-PAGE and Western blotting, only monomeric and dimeric molecules, but not trimers, were again detected. This indicates that the COL2/NC2 region alone appears unable to trimerize, even after substituting NC2 for NC1 and introducing a cysteine at the end of COL2. Collectively, the data suggest that in the absence of NC1 and COL1, the N-terminal domains NC3 or COL3 are required for complete folding and stabilization of collagen IX molecules.

5.3 Collagen IX mediates cell adhesion via integrin receptors

First, the potential cellular receptors for collagen IX were mapped by analyzing the expression patterns of collagen receptor integrins α1ß1, α2ß2, α10ß1 and α11ß1 by RT-PCR in human fetal tissues. All four integrin mRNAs could be detected (Figure 1 in II). α1 was predominantly expressed in the growth plate, meniscus and eye, α2 in skin, bone, growth plate and eye, and α10 in bone and growth plate. Integrin α11 presented with a broad expression pattern covering all the analyzed tissues. The expression of collagen IX was also tested in the same samples by analyzing the presence or absence of COL9A1. The highest levels of expression were found in cartilage and eye, while low levels were detected in bone. Based on these results, all the integrins studied may have a potential to act as receptors for collagen IX.

The expression of the four collagen receptor integrins was further studied in human chondrosarcoma cell lines (HTB-99) and human primary fetal chondrocytes. Both cell types were found to express all the analyzed integrin mRNAs (Figure 2 in II). The presence of the corresponding protein was confirmed by metabolic labeling and immunoprecipitation (Figure 2 in II). The results suggest that all four collagen receptor integrins may be concomitantly expressed in a single cartilage-derived cell.

The adhesive properties of human collagen IX were next studied by testing the ability of HTB-99 and CHO cells to attach and spread on collagen (Figure 3 in II). CHO cells, contrary to HTB-99, do not express collagen-binding integrins on their surfaces and do not spread on collagen (Nykvist *et al.* 2000). Thus, previously created CHO cell lines expressing either α1ß1 or α2ß1 as a sole collagen receptor, and a newly created cell line expressing α10ß1, were utilized. First, it was found that the spreading of HTB-99 cells on collagen IX was comparable to spreading on collagens I and IV. CHO-α1ß1 cells spread efficiently on collagen IV as well as on collagen IX, while their spreading on collagen I was poor (Figure 3 in II). CHO-α2ß1 spread fastest on collagen I, and collagen IX was almost as good a ligand as collagen I. Spreading on collagen IV was slower. The ability of CHO-α10ß1 cells to spread was tested on multiple collagen types (Figure 4C in II). It turned out that CHO-α10ß1 cells are able to spread efficiently on all collagen types tested. Collectively, the results indicated that collagen IX may function as a cell adhesion protein and that α1ß1, α2ß1, α10ß1 and possibly also α11ß1 integrins act as receptors for collagen IX. Interestingly, collagen IX was among the best collagenous ligands for every integrin tested.

5.4 Recombinant αI domains of α1ß1, α2ß1, α10ß1 and α11ß1 integrins bind to collagen IX with high affinity

To investigate the binding mechanism and kinetics, all the described integrin αI domains were first expressed as recombinant proteins in *E. coli* and then tested in a solid-phase binding assay. All the tested αI domains bound effectively to collagen IX in a metal-dependent fashion. Since the number of binding sites could not be deduced from the data, the binding affinities were estimated by using a simple Michaelis-Menten type equation and assuming that the possible multiple binding sites were identical. Approximated K_d values obtained were 30 ± 5 nM for α1I, 55 ± 13 nM for α2I, 294 ± 25 nM for α10I, and 53 ± 5 nM for α11I (Figure 5 in II). When these results were compared to previous reports with a similar experimental setting (Nykvist *et al.* 2000, Tulla *et al.* 2001), it was observed that collagen IX, unlike other collagen types tested so far, is effectively recognized by all integrin αI domains. This makes collagen IX unique. Collagen IX is also the first FACIT demonstrated to bind to integrins.

5.5 Collagen IX is recognized by the integrin α2I domain with a novel mechanism

Based on pre-existing knowledge, the α2I domain was selected to study further the interaction between collagen IX and integrins. It has been shown by crystallography that amino acids Asp-219 and His-258 are known to bind to the GFOGER motif within collagen I (Emsley *et al.* 2000). To study the similarities and differences in the recognition of different collagens, two mutations (Asp-219 to Arg and His-258 to Val) were introduced to the above-mentioned critical amino acids. Subsequently, the consequences of the mutations were studied by binding assays (Figure 7 in II). Both mutations decreased the binding to collagens I and IV, but the preference of D219R-α2I and H258V-α2I to collagens I and IV was the opposite (Figure 7 A-B in II). Similarly, when GFOGER and related peptides were tested, both mutations resulted in decreased binding (Figure 7C in II). When the binding of the mutant α2I domains to collagen IX was tested, the mutation D219R had no effect on the binding, whereas mutation H258V caused a considerable decrease in the affinity to collagen IX (Figure 7D in II). As there is no GFOGER sequence to be found at the site of interaction (Figure 9 in II) and the effects of mutations on binding of collagen IX to α2I were clearly different from those on binding to collagen I, these data suggest that the recognition of collagen IX by α2I takes place by a novel, previously undescribed mechanism. Thus, additional integrin recognition sequences in collagen remain to be discovered.

The binding mechanism of the α1I domain to collagen I is considered to be similar to the α2I domain (Xu *et al.* 2000). Also, imaging of the α1I-collagen IX complex by rotary shadowing electron microscopy (Figures 8D-E in II) suggested that the binding site is the same as for α2I (Figure 6 in II). However, integrin I domains are known to display a preference towards certain types of collagen, for example α1I prefers basement membrane collagen IV or collagen VI over fibril-forming collagens, while α2I displays an opposite preference (Kern *et al.* 1993, Tulla *et al.* 2001). This preference is achieved through key residues that mediate selective binding to collagens. In the α1I domain this residue is Arg-218 and in α2I, Asp-219 (Tulla *et al.* 2001). Based on this, the effect of the R218D mutation on the binding of α1I to collagens I, IV, and IX was studied (Figures 8A-C in II). The binding of R218D-α1I to collagen I was not significantly affected, but the binding to collagens IV and IX was markedly reduced. Thus, the residue Arg-218 seems to play an essential role in the recognition of collagen IX. The importance

of this residue in recognition has been previously demonstrated also for collagen I (Eble *et al.* 1993).

5.6 Integrin αI domain has one binding site in the COL3 domain of collagen IX

To define the sites of interaction, both the α2I- and α1I-collagen IX complexes were visualized by rotary-shadowing and electron microscopy (Figures 6 and 8D-E in II). In the electron micrographs one clear binding site could be observed close to the amino terminus of collagen IX. The binding site is located in the triple-helical COL3 domain near the NC3 domain and the area where the kink characteristic to collagen IX is situated. To rule out the possible participation of NC3 to binding, linear peptides of α1(IX), α2(IX) and α3(IX) were also tested. None of the tested peptides showed any effect on binding. What makes the observed binding site interesting is the fact that this area is devoid of known integrin binding motifs like GFOGER or GLOGER (Figure 9 in II). The determination of the exact binding site will thus be a task for future research. However, this may pose a considerable challenge since recognition is likely to take place via two or three different α chains (Eble *et al.* 1993, Golbik *et al.* 2000).

5.7 Serum IgG autoantibodies against collagen IX are elevated in early RA

The early diagnosis and treatment of RA may be difficult because of the variable and often unpredictable course of the disease. New information and new markers regarding the pathogenetic process involved in RA are therefore needed to improve the diagnostics and predictability of RA (Feldmann *et al.* 1996b). The purpose of this study was to measure the presence of autoantibodies binding to intact human recombinant collagen IX (rCIX) and assess their usefulness as a diagnostic marker and an indicator of disease activity in rheumatoid factor (RF)-positive RA.

First, rCIX was produced as described previously (Pihlajamaa *et al.* 1999). The structural properties and the amino acid content of the purified rCIX were analyzed and found to correspond to those of native tissue extracted CIX. Then, before actual experiments, the specificity of the antibody binding to rCIX was determined in a competition ELISA. A serum dilution curve of RA patient IgG

binding to rCIX was first carried out (Figure 2A in III). Then the ability of RA patient IgG to bind to rCIX in the presence or absence of soluble rCIX as a competitor was measured. Figure 2B (in III) demonstrates that prior incubation with rCIX resulted in an over 90% reduction in IgG binding to rCIX when 10 or 30 µg of rCIX was added as a competitor, while incubation with non-specific ovalbumin showed no effect, indicating high specificity of IgG binding to rCIX. Subsequently, a conventional ELISA assay was used to detect autoantibodies in RA sera binding to rCIX. The results were expressed as arbitrary OD units. The measurements indicated that the baseline serum IgG antibody binding to native rCIX was significantly higher in the RA patients (0.361 ± 0.124, mean ± SD) than in the controls (0.203 ± 0.117, mean ± SD) (95% CI 0.102 – 0.212: $p<0.001$) (Figure 1A in III). Likewise, the IgG binding to denatured rCIX was significantly higher in the RA patients (0.926 ± 0.230, mean ± SD) than in the controls (0.541 ± 0.241, mean ± SD) (95%CI 0.278 – 0.491: $p<0.001$) (Figure 1B in III). There was no measurable IgM or IgA autoantibody binding to native or denatured rCIX in RA or control samples, when measured in a subset of samples (data not shown). The IgG antibody binding to the native and denatured collagen was associated with each other ($p < 0.001$).

5.8 Collagen IX autoantibodies are not associated with disease activity or outcome in RA

To assess if the IgG antibody levels to rCIX among the RA patients were related to the disease activity, the patients were followed for 24 months. The median count of joints with erosion was 0 (IQR: 0, 1) at baseline and the increase during the two-year follow-up was 3 (95% CI: 1 to 5). The IgG antibody levels to rCIX among all the RA patients remained highly elevated throughout the 24 months follow-up and remained notably higher with denatured than with native antigen (Figure 3 in III). However, the autoantibody levels to rCIX did not correlate with outcome defined by radiological progression during the two-year follow-up as judged by the number of erosive joints in the hands and feet. Also, no relationship between the antibody levels to rCIX and the disease activity could be demonstrated during the follow-up when the disease activity was measured by ESR, CRP, thrombocyte count, duration of morning stiffness, number of swollen or tender joints, VAS or HAQ.

6 Discussion

Besides scientific use, collagens may have numerous medical, pharmaceutical and even industrial applications. To be eligible in these applications, the biochemical properties of collagen as well as its interactions with other proteins need to be known. Furthermore, the protein should be available in sufficient amounts and, if aimed to be used in a human context, free from host-derived pathogens or immunogens. The use of recombinant techniques has provided a feasible tool to produce collagen with the ability to fulfill most, if not all of the above mentioned requirements. For example, simultaneous co-expression of collagen polypeptide chains with recombinant prolyl 4-hydroxylase has enabled the production of fully hydroxylated, stable collagens in insect cells (Lamberg *et al.* 1996, Myllyharju *et al.* 1997, Nokelainen *et al.* 1998).

In this thesis project, collagen IX has been recombinantly expressed in insect cells using the baculovirus expression system. This expression system is relatively simple to use, suited for the simultaneous expression of multiple genes or large proteins, and known to provide many of the appropriate posttranslational modifications (Kidd & Emery 1993, Miller 1993, Patterson *et al.* 1995). However, the expression of rCIX has been a challenging task. This is due to both the complexity of the collagen biosynthetic pathway and to the multiplicity of the expressed genes. Because of the heterotrimeric nature of collagen IX, three different genes need to be expressed simultaneously in equimolar amounts. Furthermore, to achieve adequate stability and species-specific posttranslational modifications, human prolyl 4-hydroxylase α and β subunits were co-expressed with collagen IX polypeptide chains. This results in the simultaneous expression of five different genes. Because of the transient nature of the expression, the system is also subject to fluctuation.

6.1 Chain association is a multi-step process with functional interplay between collagenous and non-collagenous domains

Formation of a correctly folded triple helix is a fundamental requirement for the proper function of collagen in tissues. Formation of an intact triple helix, in turn, is dependent on a complex sequence of events encompassing association of the appropriate α chains (three or more), nucleation and propagation of the triple helix, and stabilization of the structure by interchain disulfide bonds (see Prockop & Kivirikko 1995, Gelse *et al.* 2003). The current knowledge regarding the chain

assembly and triple helix formation with fibrillar collagens is relatively good, but highly limited with the FACITs (see sections 2.4.1 and 2.4.2).

Previous reports have demonstrated that the information for chain association and nucleation is likely to reside in the COL1/NC1 junction of FACIT collagens (Labourdette & van der Rest 1993, Mechling *et al.* 1996, Mazzorana *et al.* 1993, 1995, 1996, 2001). However, prior to this thesis work, it was not known whether the information in the COL1/NC1 junction is specific, or whether noncollagenous and collagenous domains may function independently, or in different combinations. Likewise, it was unclear whether FACITs can associate and form triple helices starting at noncollagenous domains other than NC1.

In the present work, collagen IX chain association was studied by expressing five different deletion variants along with the full-length α chains (see Figures 1 and 2 in I) and by analyzing their ability to trimerize. First, full-length collagen IX molecules accompanied by the long variants (starting at the NC4 domain and ending at the NC2 or NC1 domain), were investigated. When analyzed by SDS-PAGE and immunoblotting, each chain of the three variants could be detected whenever the corresponding virus was used in the expression. Also, the chains were of expected sizes. Full-length collagen IX appeared as three distinct bands under reducing conditions and as a single heterotrimeric band under non-reducing conditions, as previously reported (Pihlajamaa *et al.* 1999). However, this heterotrimeric composition was not detected with the NC4-NC2 and NC4-COL2/NC1 variants. Instead, these variants presented with new homotrimeric and aberrant heterotrimeric (eg. $\alpha1_2\alpha2$ and $\alpha2_2\alpha3$) compositions, suggesting that the specificity of chain selection is reduced in the absence of NC1 and COL1 domains. The results are in agreement with previous observations (Mazzorana *et al.* 1993, 1995), supporting the role of the NC1-COL1 junction in the chain selection of collagen IX. Furthermore, it was found that collagen IX NC4-NC2 α chains (i.e. devoid of both COL1 and NC1 domains) were able to trimerize in insect cells, as demonstrated by SDS-PAGE and Western blotting under non-reducing conditions. Since trimerization occured in the absence of NC1 and COL1 domains, other domains, probably NC2 or NC3, must contain the ability to direct and drive the association of collagen IX α chains. This ability has been previously suggested only for the NC1 domain. Moreover, when the NC2 domain in these variants was replaced by NC1 (to gain NC4-COL2/NC1 variant), the α chains were, again, found to trimerize. Thus, the results demonstrate that collagen IX chain association may take place at an artificial COL2/NC1 junction. This observation suggests that i) noncollagenous and collagenous domains may act in

different combinations, and ii) association of α chains may not require the authentic order of these domains. Moreover, noncollagenous domains seem to be able to fold autonomously, as has been shown for the NC4 domain of collagen IX (Pihlajamaa *et al.* 2004). An alternative explanation for the obtained results could be that association takes place at the NC3 domain and trimerization proceeds in the N to C direction, as is the case with collagen XIII, which displays a high overall structural similarity to collagen IX (Snellman *et al.* 2000). Nevertheless, it is likely that chain selection plays an essential role in defining the correct specificity and stoichiometry of α chains thus enabling the ordered organization of the ECM and, ultimately, structural integrity of the tissue.

The triple helicity of the NC4-NC2 heterotrimeric variant was confirmed by circular dichroism analysis. The circular dichroism spectra for NC4-NC2 suggested that the trimeric molecules seen on the gels under non-reducing conditions were triple-helical and not only disulfide-bonded. These results are consistent with the previous observation indicating that a naturally occurring mutation leading to deletion of one Gly-X-Y repeat in the COL1 domain of α3(IX), with normal α1(IX) and α2(IX) chains, did not affect trimerization and triple helix formation (Paassilta *et al.* 1999).

The results described above further suggest that noncollagenous domains may act independently as association domains regardless of the adjacent collagenous domains, provided that the requirements of sufficient length and hydroxylation of the collagenous region are met. Since the degree of prolyl hydroxylation has a profound effect on the trimerization (Mazzorana *et al.* 1993, 1996) and stability of collagen (Bella *et al.* 1994), the levels of unhydroxylated and hydroxylated proline residues were determined by amino acid analysis in the produced collagen IX molecules. Since they were found to represent approximately 80% of the theoretical maximum they were considered adequate. Thus, it can be argued that prolyl hydroxylation is not a factor that may reduce the reliability of the obtained results.

6.2 Cooperativity is implicated in the folding of collagen IX molecules

In order to obtain results unaffected by other domains, COL2-NC1, COL2/C/NC1 and COL2-NC2 variants were expressed in insect cells. COL2 was chosen as the basic collagenous unit among the constructs because it was expected to provide adequate length to ensure triple-helical folding (Nokelainen *et al.* 1998). When

chain compositions of all the short variants were analyzed, monomers and dimers were detected, but trimers were completely absent. Hence, it appears that while COL1/NC1 fragments do have the ability to trimerize (Labourdette & van der Rest 1993, Mechling *et al.* 1996), COL2/NC1 and COL2/NC2 do not. In light of the results obtained with the long variants, this observation was unexpected. Therefore, the results were verified by using chemical crosslinking to indicate and stabilize the presence of trimers. However, only monomeric or dimeric molecules, but not trimers, were detected. Thus, it appears that both NC1 and NC2 domains are unable to drive trimerization of the COL2 and NC2- or NC1-containing variants. Similar results were obtained even when the final residue in the COL2 domain was substituted by cysteine, thereby mimicking the two conserved cysteines separated by four amino acids found at the COL1/NC1 junction. This observation is rather surprising, since a coiled region, with a shown potency to induce trimerization in several collagens and collagen-like proteins, has been identified in the NC2 domains of FACITs (McAlinden *et al.* 2003). Furthermore, the results by Paassilta *et al.* (1999) suggest that the NC2 domain of collagen IX may actively participate in association by correcting chain alignment.

Taken together, our results demonstrate that the long variants are able to trimerize but not the corresponding short chain variants. The inability of the short variants to assemble into trimeric molecules may be attributed to the absence of NC3 and COL3 domains, which may reflect a cooperative process in the assembly and folding of collagen IX molecules. These results are in line with a previous finding indicating that the COL1/NC1 junction alone is not sufficient to drive association of collagen XII α chains and that the role of the collagenous sequence and formation of the COL1 triple helix is critical in the trimeric assembly, suggesting cooperativity between non-collagenous and collagenous domains (Mazzorana *et al.* 2001).

As a conclusion, the observed results indicate that NC1 is not essential for association, which may take place at NC2 or NC3. Since COL2/NC1 or COL2/C/NC1 variants were not able to trimerize, the NC1 domain does not contain all the information necessary for association and trimerization of collagen IX. However, based on our results and previous reports, NC1, together with the COL1 domain, seem to play an important role in governing the specificity of the chain association. Furthermore, association and complete stabilization of collagen IX α chains seems to require the presence of N-terminal COL3 and NC3 domains, suggesting cooperativity between different regions in the folding of

collagen IX molecules. Whether the obtained results may be generalized to concern also other FACITS will be a question of future research.

6.3 Collagen IX mediates cell adhesion and cell signaling through integrin receptors

Cell adhesion is fundamental for all multicellular animals. The integrins form a conserved protein family that operates both in cell adhesion and in intercellular communication. They consist of two evolutionarily unrelated subunits (α and β) that heterodimerize to form a functional receptor (Hughes 2001, Hynes 2002). Nine out of the 18 human α subunits contain an I domain encompassing the metal ion-dependent adhesion site (MIDAS), which forms the ligand-binding site of the corresponding receptors (Michishita *et al.* 1993).

Nearly thirty collagen types form fibrils and networks in the ECM. The cellular response to collagenous matrix may be dependent on several factors. First, different collagen receptors regulate distinct signaling pathways. Second, collagen types may be differentially recognized by the collagen receptors. Third, the organization of the collagenous matrix may be critical. More information about integrin function and integrin–collagen interaction is needed to address the structural requirements for integrin signaling. This aspect was studied by using the best known member of the FACIT family, collagen IX, and the collagen receptor integrins α1ß1, α2ß2, α10ß1 and α11 ß1, as an example.

Several lines of evidence were collected to show that the four collagen receptor integrins may interact with collagen IX. First, the expression pattern of all four integrins was analyzed at the same time in the same samples of human fetal tissues. The results indicate that the four receptors are co-expressed with collagen IX at the mRNA level in fetal cartilage. The findings are in line with previous observations (Camper *et al.* 2001, Tiger *et al.* 2001). Next, the ability of cartilage-derived cells and transfected CHO cells to attach and spread on collagen IX was studied. The results showed that all cells could efficiently spread on collagen IX. What was exceptional, though, was the fact that it was among the best ligands for every αI domain or integrin tested. In addition, recombinant αI domains were produced as fusion proteins and analyzed for their capacity to bind collagen IX in solid-phase binding assays. All αI domains were found to bind efficiently to collagen IX in a metal-dependent fashion. Approximated binding affinities of 30 nM for α1I, 55 nM for α2I, 294 nM for α10I, and 53 nM for α11I were derived by using a simple Michaelis-Menten type equation. These results,

when compared to previous reports utilizing other collagen types in a similar experimental setting (Tulla *et al.* 2001, Zhang *et al.* 2003), indicate that collagen IX is one of the best collagenous ligands for every integrin αI domain. This characteristic is unique to collagen IX among collagens and suggests that collagen IX has evolved to mediate the high affinity binding of all collagen receptor integrins to cartilaginous matrix. This observation also suggests that the FACITs may have an important function as mediators of cell adhesion to collagen fibrils.

The location of the integrin I domain binding site in collagen IX was next determined. Rotary shadowing and electron microscopy revealed only one binding site for both the α1I domain and α2I domain in collagen IX. This binding site seems to be at the same location for both αI domains, and it resides in the triple-helical COL3 region very close to the NC3 domain. As the COL3 domain protrudes into the perifibrillar space, the binding site may be easily accessible to cell surface receptors. However, this site is also in the close vicinity of the GAG binding site present in NC3, and it is therefore possible that the GAG chain may act as a negative regulator of cell adhesion. In addition, COMP, shown to interact with NC domains in collagen IX (Holden *et al.* 2001), may also compete with collagen receptors.

The recognition of fibril-forming collagens by integrins takes place primarily via GFOGER (or related) sequences, which form high affinity binding sites (Emsley *et al.* 2000, Knight *et al.* 2000, Siljander *et al.* 2004). GFOGER is also known to act as a recognition site in BM collagen IV (Knight *et al.* 2000). However, the observation that Chinese hamster ovary cells expressing a double mutant α2 D219N/D292N integrin showed significantly reduced cell spreading on collagen I, but had no effect on collagen IV, suggested that the two collagen types are recognized by different mechanisms (Käpylä *et al.* 2000). Indeed, another type of binding motif is known in collagen IV. This motif is composed of one R and two D residues in different α chains (Eble *et al.* 1993, Golbik *et al.* 2000, Saccà *et al.* 2003). The fact that the discovered integrin binding site in collagen IX does not contain GFOGER or any similar motif suggests that collagen receptors binding to FACITs may represent yet another binding mechanism. This was tested experimentally by introducing mutations in the amino acid residues D219 and H258 known to be important in the recognition of collagen I by the α2I domain (Käpylä *et al.* 2000, Smith *et al.* 2000). Similarly, R218 in α1I was mutated, and the effect of mutation on collagen binding was analyzed. Mutated I domains D219R-α2I and H258V-α2I displayed different behaviors when binding to collagen I or to collagen IX. Both mutations decreased the binding of the α2I

domain to collagen I as well as to the GFOGER peptide. However, while binding to collagen IX was not affected by the D219R mutation, the H258V mutation caused a marked decrease in the affinity to collagen IX. This data, together with the noted absence of GFOGER-related sequences in the integrin binding region in collagen IX, demonstrates that binding of the α2I domain to collagen IX represents a novel mechanism of collagen recognition by integrins. As the effect of the R218D mutation in α1I domain on binding to collagen I was minimal, and while binding to collagens IV and IX was nearly prevented, it seems that the residue R218 in the α1I domain is very important for collagen IX binding. This agrees with the previous observation and indicates that arginine residue(s) are essential for the binding of both collagens IV (Eble *et al.* 1993) and IX. Despite possible similarities, however, there are certain observations that suggest that binding of these collagens to α1I domain cannot be identical. These include (i) differences noted in the binding behaviour between collagen IV and collagen IX (Figure 7B and D in II), (ii) lack of Arg, Asp, Asp motif in collagen IX at or near the discovered binding region, and (iii) the fact that collagen IX was exceptional in its ability to effectively bind to all integrin I domains studied. It was interesting to note that the D219 residue in the α2I domain, which corresponds to R218 in the α1I domain, was found insignificant in the same context.

Previously, it has been shown that integrins, α2β1 in particular, are able to bind directly to collagen fibrils (Jokinen *et al.* 2004). The present study shows that cell attachment to collagen fibrils may take place in a collagen IX-mediated manner and suggests a more general role for FACITs as mediators of cell-ECM interactions. This would be only natural as the FACITs decorate the surfaces of collagen fibrils and are better available for cell surface receptors than the collagens at the fibril core. Furthermore, the results indicate that the binding of collagen IX most likely takes place at or close to the metal ion-dependent adhesion site in α1I and α2I domains. In the absence of unique binding sequences in any of the collagen IX α chains, the binding is proposed to involve residues in multiple polypeptide chains. Interestingly, the vWFA3 domain that is structurally closely related to integrin αI domains, binds collagen with a different mechanism: the binding is metal independent and occurs on a different face of the domain when compared to the MIDAS (Nishida *et al.* 2003). The existence of two different modes of recognition may open up perspectives into integrin evolution as it is speculated that vWFA domains represent an ancient collagen binding mechanism, shared by the first αI domain integrins (Tulla *et al.* 2007). According to this hypothesis, MIDAS-mediated and divalent cation-dependent binding to the

GFOGER sequence in collagens has evolved more recently. This proposition is not without experimental support, as the αI domain of urochordate *Ciona intestinalis* is able to specifically and strongly bind to collagen IX (but not to other collagen types investigated), despite the absence of the structural features required for binding to the GFOGER motif in the collagen triple helix (Tulla *et al.* 2007). This observation suggests that the GFOGER-dependent binding mechanism of collagen receptor integrins seems to have arisen in the vertebrate lineage only. Thus, it is possible to speculate that the generation of this subgroup of integrins is associated with the evolution of cartilage and bone. The presence of several unique collagen types in cartilage also points to the importance of chondrocyte collagen receptors.

In conclusion, the present results show that collagen IX, a FACIT prototype, acts as a cell adhesion protein and is recognized by the four known collagen receptor integrins in a previously undescribed, GFOGER-independent mechanism. When compared against other collagen types, collagen IX seems unique in its ability to interact efficiently with all collagen receptor integrins or the respective αI domains. The determination of the three-dimensional integrin binding motif in collagen IX and the analysis of structural changes on integrin receptors induced by collagen IX binding provide interesting challenges for future research.

6.4 New markers are needed to aid in the diagnostics of early RA

The diagnostics of early RA poses a challenge to clinicians as there is no distinctive feature in the early phases of the disease. In the absence of reliable and specific diagnostic tools, the diagnosis of RA is usually based on the patient's clinical symptoms. Also, the classification criteria of the ACR are taken into account. The use of these criteria as a diagnostic aid is, however, problematic. First, they were originally created to serve classification but not diagnostic aims, and second, they are not adequate to distinguish early stages of RA from other forms of joint inflammation. The earlier a diagnosis is attempted, the more atypical symptoms are present with the patients. Yet, early diagnosis is of crucial importance since the outcome and the therapeutic effect seem to depend less on the pharmacological capacity of a given medication than on the timing of the initiation of therapy (see Quinn 2001, Möttönen *et al.* 2002, O'dell 2002, Visser *et al.* 2002, Kary *et al.* 2004). Indeed, it is recognized that diagnosis should be achieved in RA and therapy initiated as early as possible. As the alterations in

cartilage matrix turnover take place early in the disease process, long before any significant damage can be detected radiographically, it is important to find sensitive, non-invasively detectable biochemical markers of cartilage matrix homeostasis. These new biomarkers would aid, either alone or in conjunction with the existing markers such as RF and CCP, in achieving the diagnosis of early RA. Current study was started to investigate the role of collagen IX autoantibodies in RA and their potential usefulness as a diagnostic or prognostic aid.

Cartilage is an exceptional tissue as it does not contain capillary network or lymph ducts. Thus, it is regarded immunologically privileged. There are several lines of evidence to indicate that cartilage specific collagens may play a role in autoimmune-mediated arthritis (Morgan *et al.* 1983, Wooley *et al.* 1984, Tarkowski *et al.* 1989, Holmdahl *et al.* 1990, Cook *et al.* 1996, Rudolphi *et al.* 1997, Cremer *et al.* 1998, Kim *et al.* 2000, Burkhardt *et al.* 2002, Myers *et al.* 2002, Carlsen *et al.* 2006). One of the first critical steps in the disease process is the breakdown of the collagenous network that is essential for the maintenance and integrity of the tissue. This process is either accompanied or preceded by the formation of collagen-related autoantibodies, which may perpetuate the disease. It is not known, however, whether these antibodies initiate the inflammatory process in the joint or whether they are formed during cartilage breakdown, being unrelated to the initiation of arthritis. Experiments with certain antibodies binding to collagen II (Amirahmadi *et al.* 2004, 2005, Gray *et al.* 2004, Crombie *et al.* 2005) suggest that they may interfere with the structural integrity of cartilage and precede or possibly initiate the inflammatory expression of disease, thus representing an important component of pathogenesis. Despite some controversy concerning the role of collagen antibodies in the pathogenesis of RA, these observations favor the view that collagen-related autoimmunity occurs during the early initiating events in the development of RA. Nevertheless, independent of our understanding of the disease mechanism, autoantibodies binding to collagen or degradation products of collagen may reflect the biochemical status of the joint and thus serve as diagnostic or prognostic tools.

6.5 Antibodies to rCIX may have diagnostic value in RA

In this study, 53 patients with recent-onset RF-seropositive RA were analyzed for the presence of rCIX antibodies of the IgG type at the moment of initial diagnosis and after 3, 6, 12 and 24 months of follow-up. The RA sera were accompanied by

30 controls. Associations were looked for between patients' antibody titers, the development of erosions in the hands and feet, and various clinical and laboratory markers. Our data shows that serum levels of IgG binding to rCIX were significantly higher in the RA patient sera compared to the controls. The difference was two-fold in magnitude and was not affected by age. These autoantibodies were observed already at the early stages of the disease, which may reflect their diagnostic potential in RA.

To evaluate if the IgG antibody levels to rCIX among the RA patients were related to the disease activity, the patients were followed for 24 months. The levels of rCIX antibodies remained elevated, showing a very slight decrease towards the end of the follow-up. This kind of attenuation of collagen antibodies over time is frequently observed in RA (Pereira *et al.* 1985, Cook *et al.* 1996). In the control population very low or low levels of rCIX antibodies were commonly detected but few sera displayed high reactivity. This might be due to unknown joint pathology, since no exclusion criteria could be applied to the controls at study entry. Even though this setting may overestimate the autoimmunity to rCIX among the controls, the diagnostic usefulness of these antibodies requires further examination with a large patient set and a control group with a well documented history and confirmed absence of inflammatory arthritis. Moreover, as also healthy individuals may exhibit rCIX autoantibodies in their sera, the difference between the patients and healthy individuals is quantitative, not qualitative. Distinguishing between these groups would require careful calibration of threshold antibody level, thus narrowing the potential of rCIX antibody test in clinical use. Furthermore, one limitation of the current study is that it does not provide information regarding patients with RF-seronegative RA. Finally, it is worth noting that the presence of rheumatoid factors may cause interference in various immunological assays.

The fact that no correlation was observed between rCIX antibody levels, laboratory or clinical parameters, and radiological progression is in line with the report by Charrière *et al.* (1988) and indicates that rCIX may not be a suitable marker to reflect disease activity, the response to treatment or the outcome in RA. However, the measurement of rCIX antibodies might be useful in the diagnosis of early RA. This assumption is supported by a number of reports. When various collagen antibodies were studied in the serum of patients with arthritic diseases, only antibodies to collagen IX were observed at high frequencies in the RA patients (Charrière *et al.* 1988), although low frequencies of RA patients with collagen IX antibodies have also been demonstrated elsewhere (Morgan *et al.*

1987 & 1989). In addition, the frequencies of RA patients with CII antibody positivity may be as high as 60-75% in the early stages of the disease (Pereira *et al.* 1985, Fujii *et al.* 1992, Cook *et al.* 1994) but fall to 24-30% later, as shown by a cross-sectional approach in cases of well-established RA (Cook *et al.* 1996). Another interesting observation is that antibodies against CII precede the appearance of rheumatoid factors in most patients (O'Dell 2002). Moreover, it would be of interest to examine, whether antibodies to rCIX display differences in epitope specificity depending on the underlying disease, as has been demonstrated for CII (Burkhardt *et al.* 2002). High values of sensitivity (88%) and specificity (94%) in the detection of RA have also been found when a fragment, termed CB10, was used as a substrate in ELISA (Cook *et al.* 2004). These reports illustrate that epitope-specific antibodies are produced in RA and suggest their exploitation in clinical use. Thus, it is quite possible that both common and unique epitopes exist in cartilage collagens, and antibodies to these different epitopes may have different roles in RA. Keeping the focus on CIX, the results by Carlsen *et al.* (2006) suggest that CIX might hold a gatekeeper role permitting, or in its absence allowing, the antibody attack against the bulk of collagen fibril, CII. In addition, the results by both Kojima *et al.* (2001) and Wotton *et al.* (1999) also support the relevance of CIX autoimmunity in the early initiating phase of arthritis. If rCIX antibodies were to prove suitable as a predictive marker for early RA, this would be a valuable asset for RA diagnostics, especially in cases where the diagnosis is not clear on the basis of laboratory and clinical findings. It is not currently known, however, whether the rCIX antibodies are associated with RA specifically or with chronic arthritis in general. These aspects need clarification by further studies, preferably conducted in a prospective research setting and with large patient and control groups. The potential diagnostic or prognostic value of rCIX autoantibody test in RA could be evaluated by comparing its performance and ability to distinguish RA in a group of rheumatic or other autoimmune diseases. Alternatively, samples and data in existing cohort studies could be exploited to investigate whether the emergence of rCIX autoantibodies predates the onset of RA (see Aho *et al.* 1985 and Rantapää-Dahlqvist *et al.* 2003). Still, it is likely that the measurement of these antibodies would only be beneficial in a limited number of cases where RF and anti-CCP antibodies are negative and the clinical symptoms too unspecific to enable diagnosis of early RA.

To our knowledge there are no previous studies published on autoantibodies binding to intact full-length collagen IX in humans. We utilized recombinant human-like collagen as an antigen instead of pepsin-digested tissue-derived

material. With this approach we aimed to have an antigen that resembles that encountered *in vivo*, being intact and containing the non-helical regions that may function as natural epitopes. The fact that CIX antibodies were significantly elevated among the RA patients in this study contrasts to certain previous reports (Morgan *et al.* 1987, 1989) and suggests that the noncollagenous regions in collagen IX show a potential as new RA epitopes. However, this proposition is without solid experimental support since tissue-extracted CIX was not available for comparison. The recombinant material is also devoid of impurities that can cause cross-reactivity, such as other cartilage collagens that might be present in tissue-extracted collagen preparations. However, when a protein is recombinantly expressed, there are many perspectives that need to be considered. One of these aspects is the degree and quality of posttranslational modifications, which seem to affect recognition of self-antigens. Increasing data support the notion that posttranslational modifications of self proteins may play a crucial role in the development of autoimmunity (see Kuhn *et al.* 2006). Furthermore, it has been shown that posttranslational modifications may affect collagen-related autoimmunity, for example the degree of glycosylation is known to affect T cell recognition of CII in a mouse model (Myers *et al.* 1998, von Delwig *et al.* 2006). Likewise, it has been shown that the potency of the immune response against CII correlates with the extent of lysine hydroxylation and glycosylation (Myers *et al.* 2004). The degree of these modifications affects the immune response to collagen II and plays a role in the induction of arthritis. These observations relate to our findings, since the baculovirus expression system used here is able to generate moderate but not adequate levels of these modifications. Thus, the antibody levels measured here might be higher if an antigen with the degree of modifications matching those of tissue-derived collagen was available.

Another interesting aspect is the relevance of citrullination and citrullinated antibodies as determinants of autoimmunity in RA. In an experimental setting, antibodies against citrullinated proteins enhance tissue injury in autoimmune arthritis, demonstrating that these antibodies are centrally involved in its pathogenesis (Kuhn *et al.* 2006). When evaluated in a clinical setting, the anti-CCP antibodies are highly specific to RA, appear before the occurrence of clinical symptoms, predicting the development of RA in patients with undifferentiated arthritis (van Gaalen *et al.* 2004) and in apparently healthy individuals (Rantapää-Dahlqvist *et al.* 2003), and provide prognostic information as anti-CCP positivity is associated with less favorable disease course and more severe radiological damage (Kroot *et al.* 2000, Rönnelid *et al.* 2005). These antibodies rise primarily

against filaggrin, which is not present in rheumatoid synovium. Thus, other proteins naturally occurring in joint and synovial tissue may function as targets for autoimmunity. Recently, collagens I and II have been identified as such targets in RA (Koivula *et al.* 2005, 2007, Suzuki *et al.* 2005, Yoshida *et al.* 2006). This raises the question, whether collagen IX might also represent a substrate for citrullination and if so, would the antibodies against citrullinated collagen IX constitute a novel marker that is intimately linked to RA pathophysiology?

7 Conclusions

The development of novel technologies has opened new avenues in the field of molecular biology. For example, production of recombinant proteins has allowed their use in research and in other applications, including the pharmaceutical industry. This has also allowed the investigation of molecular mechanisms underlying various disease conditions. In this thesis project CIX, the availability of which is otherwise greatly limited, was produced as a recombinant protein in order to better understand its structural properties and folding as well as its functional characteristics, particularly with regard to recognition of CIX by cellular receptors and with autoimmunity in RA.

Since the formation of the correctly folded triple helix is a functional prerequisite of collagen molecules in tissue, the chain assembly and folding of CIX was investigated. The results indicated that the NC1 and COL1 domains are not necessary for the association of CIX α chains and that complete folding and stabilization of CIX molecules in the absence of these domains requires cooperativity between C-terminal and N-terminal domains. However, the results also confirmed the role of NC1-COL1 domains for the specificity of chain association. Collectively, the obtained results increase our understanding of the molecular assembly of CIX, and FACITs in general.

Data from both the genetically modified mouse lines and from the human chondrodysplasia phenotype, MED, imply that CIX is relevant for the long-term stability and integrity of articular cartilage. It has been shown that proteins may work as complex networks interacting with several partners. The importance and delicacy of these interactions is exemplified in MED, in which mutations in any of the interacting molecules (CIX, COMP or MATN3) may result in disease. The effects of these mutations are exerted on multiple levels from the fine structure of the parent molecule to the macroscopic matrix, ultimately defining the resulting phenotype.

In the current study, the function of CIX, regarding its interaction with the cellular receptor integrins, was further elucidated. It was found that the recombinant αI domains of all the investigated integrins effectively bound to CIX. The recognition of CIX by the α2I domain was shown to represent a novel mechanism of binding. The binding site of αI was located in the COL3 domain of CIX. The results also provide the first demonstration of integrins recognizing FACIT collagens. The finding suggests that FACITs may have an important function as mediators of cell adhesion to collagen fibrils.

Autoimmunity to cartilage collagens is implicated in RA, and recent investigations clearly demonstrate that newly aroused interest in collagen autoimmunity in the pathogenesis of RA is warranted. In the present work, autoantibodies binding to recombinant human CIX in RA sera were measured and their usefulness as a diagnostic or prognostic marker was evaluated. Importantly, for the first time, the antigen used in the assay represented an intact human molecule with the noncollagenous domains preserved. The results showed that the levels of autoantibodies recognizing rCIX were significantly higher in RA sera when compared to the controls. However, also healthy controls had these antibodies. The emergence of these antibodies already at the early stages of the disease may reflect their potential as a possible diagnostic aid in RA. Moreover, the presence of these antibodies in high frequency among RA patients contradicts previous reports and suggests that the noncollagenous regions in CIX show a potential as new RA epitopes. Currently, autoimmunity to CIX is also investigated in other rheumatic diseases and in Meniere's disease.

In the future, recombinant technology with the possibility to utilize various CIX constructs described in this thesis manuscript will allow more precise determination of CIX epitopes in RA. Another interesting prospect is to investigate the role of citrullination as a determinant of autoimmunity to CIX in RA.

References

Aho K, Palusuo T & Kurki P (1994) Marker antibodies of rheumatoid arthritis: diagnostic and pathogenetic implications. Semin.Arthritis Rheum. 23(6): 379-387.

Aho K, Palosuo T, Raunio V, Puska P, Aromaa A & Salonen JT (1985) When does rheumatoid disease start? Arthritis Rheum. 28(5): 485-489.

Akutsu N, Milbury CM, Burgeson RE & Nishiyama T (1999) Effect of type XII or XIV collagen NC-3 domain on the human dermal fibroblast migration into reconstituted collagen gel. Exp.Dermatol. 8(1): 17-21.

Ala-Kokko L (2002) Genetic risk factors for lumbar disc disease. Ann.Med. 34(1): 42-47.

Aman S, Paimela L, Leirisalo-Repo M, Risteli J, Kautiainen H, Helve T & Hakala M (2000) Prediction of disease progression in early rheumatoid arthritis by ICTP, RF and CRP. A comparative 3-year follow-up study. Rheumatology (Oxford) 39(9): 1009-1013.

Aman S, Risteli J, Luukkainen R, Risteli L, Kauppi M, Nieminen P & Hakala M (1999) The value of synovial fluid analysis in the assessment of knee joint destruction in arthritis in a three year follow up study. Ann.Rheum.Dis. 58(9): 559-562.

Amirahmadi SF, Pho MH, Gray RE, Crombie DE, Whittingham SF, Zuasti BB, Van Damme MP & Rowley MJ (2004) An arthritogenic monoclonal antibody to type II collagen, CII-C1, impairs cartilage formation by cultured chondrocytes. Immunol.Cell Biol. 82(4): 427-434.

Amirahmadi SF, Whittingham S, Crombie DE, Nandakumar KS, Holmdahl R, Mackay IR, van Damme MP & Rowley MJ (2005) Arthritogenic anti-type II collagen antibodies are pathogenic for cartilage-derived chondrocytes independent of inflammatory cells. Arthritis Rheum. 52(6): 1897-1906.

Andriopoulos NA, Mestecky J, Miller EJ & Bennett JC (1976a) Antibodies to human native and denatured collagens in synovial fluids of patients with rheumatoid arthritis. Clin.Immunol.Immunopathol. 6(2): 209-212.

Andriopoulos NA, Mestecky J, Miller EJ & Bradley EL (1976b) Antibodies to native and denatured collagens in sera of patients with rheumatoid arthritis. Arthritis Rheum. 19(3): 613-617.

Annunen S, Paassilta P, Lohiniva J, Perala M, Pihlajamaa T, Karppinen J, Tervonen O, Kroger H, Lahde S, Vanharanta H, Ryhanen L, Goring HH, Ott J, Prockop DJ & Ala-Kokko L (1999) An allele of COL9A2 associated with intervertebral disc disease. Science 285(5426): 409-412.

Arai M, Yada T, Suzuki S & Kimata K (1992) Isolation and characterization of type IX collagen-proteoglycan from the Swarm rat chondrosarcoma. Biochim.Biophys.Acta 1117(1): 60-70.

Areida SK, Reinhardt DP, Muller PK, Fietzek PP, Kowitz J, Marinkovich MP & Notbohm H (2001) Properties of the collagen type XVII ectodomain. Evidence for n- to c-terminal triple helix folding. J.Biol.Chem. 276(2): 1594-1601.

Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH & Luthra HS (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 31(3): 315-324.

Ayad S, Abedin MZ, Grundy SM & Weiss JB (1981) Isolation and characterisation of an unusual collagen from hyaline cartilage and intervertebral disc. FEBS Lett. 123(2): 195-199.

Ayad S, Abedin MZ, Weiss JB & Grundy SM (1982) Characterisation of another short-chain disulphide-bonded collagen from cartilage, vitreous and intervertebral disc. FEBS Lett. 139(2): 300-304.

Ayad S, Marriott A, Brierley VH & Grant ME (1991) Mammalian cartilage synthesizes both proteoglycan and non-proteoglycan forms of type IX collagen. Biochem.J. 278 (Pt 2)(Pt 2): 441-445.

Ayad S, Marriott A, Morgan K & Grant ME (1989) Bovine cartilage types VI and IX collagens. Characterization of their forms in vivo. Biochem.J. 262(3): 753-761.

Bachinger HP, Bruckner P, Timpl R, Prockop DJ & Engel J (1980) Folding mechanism of the triple helix in type-III collagen and type-III pN-collagen. Role of disulfide bridges and peptide bond isomerization. Eur.J.Biochem. 106(2): 619-632.

Banks RF, Whicher JT, Thompson D & Bird HA (1998) Acute phase response. In: Maddison PJ, Isenberg DA, Woo P & Glass DN (eds) Oxford Textbook of Rheumatology, 2nd Edn. Oxford University Press, Oxford, 623–631.

Barnett ML, Kremer JM, St Clair EW, Clegg DO, Furst D, Weisman M, Fletcher MJ, Chasan-Taber S, Finger E, Morales A, Le CH & Trentham DE (1998) Treatment of rheumatoid arthritis with oral type II collagen. Results of a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum. 41(2): 290-297.

Bateman JF, Lamande SR, Ramshaw JAM (1996) Collagen Superfamily. In: Comper WD (ed.), Extracellular Matrix, Harwood Academic Publishers, Amsterdam, 22– 67.

Battie MC, Videman T, Gibbons LE, Fisher LD, Manninen H & Gill K (1995) 1995 Volvo Award in clinical sciences. Determinants of lumbar disc degeneration. A study relating lifetime exposures and magnetic resonance imaging findings in identical twins. Spine 20(24): 2601-2612.

Bella J, Eaton M, Brodsky B & Berman HM (1994) Crystal and molecular structure of a collagen-like peptide at 1.9 A resolution. Science 266(5182): 75-81.

Bengtsson T, Aszodi A, Nicolae C, Hunziker EB, Lundgren-Akerlund E & Fassler R (2005) Loss of alpha10beta1 integrin expression leads to moderate dysfunction of growth plate chondrocytes. J.Cell.Sci. 118(Pt 5): 929-936.

Bernocco S, Finet S, Ebel C, Eichenberger D, Mazzorana M, Farjanel J & Hulmes DJ (2001) Biophysical characterization of the C-propeptide trimer from human procollagen III reveals a tri-lobed structure. J.Biol.Chem. 276(52): 48930-48936.

Birk DE (2001) Type V collagen: heterotypic type I/V collagen interactions in the regulation of fibril assembly. Micron 32(3): 223-237.

Birk DE, Fitch JM, Babiarz JP & Linsenmayer TF (1988) Collagen type I and type V are present in the same fibril in the avian corneal stroma. J.Cell Biol. 106(3): 999-1008.

Birk DE, Nurminskaya MV & Zycband EI (1995) Collagen fibrillogenesis in situ: fibril segments undergo post-depositional modifications resulting in linear and lateral growth during matrix development. Dev.Dyn. 202(3): 229-243.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A & Engler JA (1993) Matrix metalloproteinases: a review. Crit.Rev.Oral Biol.Med. 4(2): 197-250.

Bishop P, McLeod D & Ayad S (1992) Extraction and characterisation of the intact form of bovine vitreous type IX collagen. Biochem.Biophys.Res.Commun. 185(1): 392-397.

Bishop PN, Crossman MV, McLeod D & Ayad S (1994) Extraction and characterization of the tissue forms of collagen types II and IX from bovine vitreous. Biochem.J. 299 (Pt 2)(Pt 2): 497-505.

Blaschke UK, Eikenberry EF, Hulmes DJ, Galla HJ & Bruckner P (2000) Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. J.Biol.Chem. 275(14): 10370-10378.

Blom AB, Radstake TR, Holthuysen AE, Sloetjes AW, Pesman GJ, Sweep FG, van de Loo FA, Joosten LA, Barrera P, van Lent PL & van den Berg WB (2003) Increased expression of Fcgamma receptors II and III on macrophages of rheumatoid arthritis patients results in higher production of tumor necrosis factor alpha and matrix metalloproteinase. Arthritis Rheum. 48(4): 1002-1014.

Boissier MC, Chiocchia G, Ronziere MC, Herbage D & Fournier C (1990) Arthritogenicity of minor cartilage collagens (types IX and XI) in mice. Arthritis Rheum. 33(1): 1-8.

Bollen AM & Eyre DR (1993) Direct extraction of gelatinases from rat bone. Connect.Tissue Res. 29(3): 223-230.

Bonassar LJ, Frank EH, Murray JC, Paguio CG, Moore VL, Lark MW, Sandy JD, Wu JJ, Eyre DR & Grodzinsky AJ (1995) Changes in cartilage composition and physical properties due to stromelysin degradation. Arthritis Rheum. 38(2): 173-183.

Bonnemann CG, Cox GF, Shapiro F, Wu JJ, Feener CA, Thompson TG, Anthony DC, Eyre DR, Darras BT & Kunkel LM (2000) A mutation in the alpha 3 chain of type IX collagen causes autosomal dominant multiple epiphyseal dysplasia with mild myopathy. Proc.Natl.Acad.Sci.U.S.A. 97(3): 1212-1217.

Boot-Handford RP & Tuckwell DS (2003) Fibrillar collagen: the key to vertebrate evolution? A tale of molecular incest. Bioessays 25(2): 142-151.

Boot-Handford RP, Tuckwell DS, Plumb DA, Rock CF & Poulsom R (2003) A novel and highly conserved collagen (pro(alpha)1(XXVII)) with a unique expression pattern and unusual molecular characteristics establishes a new clade within the vertebrate fibrillar collagen family. J.Biol.Chem. 278(33): 31067-31077.

Brand DD, Kang AH & Rosloniec EF (2003) Immunopathogenesis of collagen arthritis. Springer Semin.Immunopathol. 25(1): 3-18.

Brewton RG & Mayne R (1992) Mammalian vitreous humor contains networks of hyaluronan molecules: electron microscopic analysis using the hyaluronan-binding region (G1) of aggrecan and link protein. Exp.Cell Res. 198(2): 237-249.

Brewton RG, Ouspenskaia MV, van der Rest M & Mayne R (1992) Cloning of the chicken alpha 3(IX) collagen chain completes the primary structure of type IX collagen. Eur.J.Biochem. 205(2): 443-449.

Brewton RG, Wood BM, Ren ZX, Gong Y, Tiller GE, Warman ML, Lee B, Horton WA, Olsen BR & Baker JR (1995) Molecular cloning of the alpha 3 chain of human type IX collagen: linkage of the gene COL9A3 to chromosome 20q13.3. Genomics 30(2): 329-336.

Briggs MD & Chapman KL (2002) Pseudoachondroplasia and multiple epiphyseal dysplasia: mutation review, molecular interactions, and genotype to phenotype correlations. Hum.Mutat. 19(5): 465-478.

Brown DJ, Bishop P, Hamdi H & Kenney MC (1996) Cleavage of structural components of mammalian vitreous by endogenous matrix metalloproteinase-2. Curr.Eye Res. 15(4): 439-445.

Bruckner P, Mayne R & Tuderman L (1983) p-HMW-collagen, a minor collagen obtained from chick embryo cartilage without proteolytic treatment of the tissue. Eur.J.Biochem. 136(2): 333-339.

Bruckner P, Mendler M, Steinmann B, Huber S & Winterhalter KH (1988) The structure of human collagen type IX and its organization in fetal and infant cartilage fibrils. J.Biol.Chem. 263(32): 16911-16917.

Bruckner P & van der Rest M (1994) Structure and function of cartilage collagens. Microsc.Res.Tech. 28(5): 378-384.

Bruckner P, Vaughan L & Winterhalter KH (1985) Type IX collagen from sternal cartilage of chicken embryo contains covalently bound glycosaminoglycans. Proc.Natl.Acad.Sci.U.S.A. 82(9): 2608-2612.

Buckwalter JA (1995) Aging and degeneration of the human intervertebral disc. Spine 20(11): 1307-1314.

Buckwalter JA & Mankin HJ (1998) Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr.Course Lect. 47: 477-486.

Budde B, Blumbach K, Ylostalo J, Zaucke F, Ehlen HW, Wagener R, Ala-Kokko L, Paulsson M, Bruckner P & Grassel S (2005) Altered integration of matrilin-3 into cartilage extracellular matrix in the absence of collagen IX. Mol.Cell.Biol. 25(23): 10465-10478.

Bulleid NJ, Dalley JA & Lees JF (1997) The C-propeptide domain of procollagen can be replaced with a transmembrane domain without affecting trimer formation or collagen triple helix folding during biosynthesis. EMBO J. 16(22): 6694-6701.

Bulleid NJ, Wilson R & Lees JF (1996) Type-III procollagen assembly in semi-intact cells: chain association, nucleation and triple-helix folding do not require formation of inter-chain disulphide bonds but triple-helix nucleation does require hydroxylation. Biochem.J. 317 (Pt 1)(Pt 1): 195-202.

Burgeson RE, Hebda PA, Morris NP & Hollister DW (1982) Human cartilage collagens. Comparison of cartilage collagens with human type V collagen. J.Biol.Chem. 257(13): 7852-7856.

Burkhardt H, Koller T, Engstrom A, Nandakumar KS, Turnay J, Kraetsch HG, Kalden JR & Holmdahl R (2002) Epitope-specific recognition of type II collagen by rheumatoid arthritis antibodies is shared with recognition by antibodies that are arthritogenic in collagen-induced arthritis in the mouse. Arthritis Rheum. 46(9): 2339-2348.

Camper L, Holmvall K, Wangnerud C, Aszodi A & Lundgren-Akerlund E (2001) Distribution of the collagen-binding integrin alpha10beta1 during mouse development. Cell Tissue Res. 306(1): 107-116.

Canty EG & Kadler KE (2005) Procollagen trafficking, processing and fibrillogenesis. J.Cell.Sci. 118(Pt 7): 1341-1353.

Canty EG, Lu Y, Meadows RS, Shaw MK, Holmes DF & Kadler KE (2004) Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. J.Cell Biol. 165(4): 553-563.

Carlsen S, Nandakumar KS & Holmdahl R (2006) Type IX collagen deficiency enhances the binding of cartilage-specific antibodies and arthritis severity. Arthritis Res.Ther. 8(4): R102.

Castagnola P, Tavella S, Gerecke DR, Dublet B, Gordon MK, Seyer J, Cancedda R, van der Rest M & Olsen BR (1992) Tissue-specific expression of type XIV collagen--a member of the FACIT class of collagens. Eur.J.Cell Biol. 59(2): 340-347.

Chapman JA (1989) The regulation of size and form in the assembly of collagen fibrils in vivo. Biopolymers 28(8): 1367-1382.

Chapman KL, Briggs MD & Mortier GR (2003) Review: clinical variability and genetic heterogeneity in multiple epiphyseal dysplasia. Pediatr.Pathol.Mol.Med. 22(1): 53-75.

Chapman KL, Mortier GR, Chapman K, Loughlin J, Grant ME & Briggs MD (2001) Mutations in the region encoding the von Willebrand factor A domain of matrilin-3 are associated with multiple epiphyseal dysplasia. Nat.Genet. 28(4): 393-396.

Charni N, Juillet F & Garnero P (2005) Urinary type II collagen helical peptide (HELIX-II) as a new biochemical marker of cartilage degradation in patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum. 52(4): 1081-1090.

Charriere G, Hartmann DJ, Vignon E, Ronziere MC, Herbage D & Ville G (1988) Antibodies to types I, II, IX, and XI collagen in the serum of patients with rheumatic diseases. Arthritis Rheum. 31(3): 325-332.

Chernousov MA, Rothblum K, Tyler WA, Stahl RC & Carey DJ (2000) Schwann cells synthesize type V collagen that contains a novel alpha 4 chain. Molecular cloning, biochemical characterization, and high affinity heparin binding of alpha 4(V) collagen. J.Biol.Chem. 275(36): 28208-28215.

Chou MY & Li HC (2002) Genomic organization and characterization of the human type XXI collagen (COL21A1) gene. Genomics 79(3): 395-401.

Clague RB & Moore LJ (1984) IgG and IgM antibody to native type II collagen in rheumatoid arthritis serum and synovial fluid. Evidence for the presence of collagen-anticollagen immune complexes in synovial fluid. Arthritis Rheum. 27(12): 1370-1377.

Collins I, Morgan K, Clague RB, Brenchley PE & Holt PJ (1988) IgG subclass distribution of antinative type II collagen and antidenatured type II collagen antibodies in patients with rheumatoid arthritis. J.Rheumatol. 15(5): 770-774.

Colorado PC, Torre A, Kamphaus G, Maeshima Y, Hopfer H, Takahashi K, Volk R, Zamborsky ED, Herman S, Sarkar PK, Ericksen MB, Dhanabal M, Simons M, Post M, Kufe DW, Weichselbaum RR, Sukhatme VP & Kalluri R (2000) Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res. 60(9): 2520-2526.

Connors WL, Jokinen J, White DJ, Puranen JS, Kankaanpaa P, Upla P, Tulla M, Johnson MS & Heino J (2007) Two synergistic activation mechanisms of alpha2beta1 integrin-mediated collagen binding. J.Biol.Chem. 282(19): 14675-14683.

Cook AD, Gray R, Ramshaw J, Mackay IR & Rowley MJ (2004) Antibodies against the CB10 fragment of type II collagen in rheumatoid arthritis. Arthritis Res.Ther. 6(5): R477-83.

Cook AD, Rowley MJ, Mackay IR, Gough A & Emery P (1996) Antibodies to type II collagen in early rheumatoid arthritis. Correlation with disease progression. Arthritis Rheum. 39(10): 1720-1727.

Cook AD, Rowley MJ, Stockman A, Muirden KD & Mackay IR (1994) Specificity of antibodies to type II collagen in early rheumatoid arthritis. J.Rheumatol. 21(7): 1186-1191.

Cremer MA, Griffiths MM, Terato K & Kang AH (1995) Type XI and II collagen-induced arthritis in rats: characterization of inbred strains of rats for arthritis-susceptibility and immune-responsiveness to type XI and II collagen. Autoimmunity 20(3): 153-161.

Cremer MA, Terato K, Watson WC, Griffiths MM, Townes AS & Kang AH (1992) Collagen-induced arthritis in rats. Examination of the epitope specificities of circulating and cartilage-bound antibodies produced by outbred and inbred rats using cyanogen bromide-derived peptides purified from heterologous and homologous type II collagens. J.Immunol. 149(3): 1045-1053.

Cremer MA, Ye XJ, Terato K, Griffiths MM, Watson WC & Kang AH (1998) Immunity to type IX collagen in rodents: a study of type IX collagen for autoimmune and arthritogenic activities. Clin.Exp.Immunol. 112(3): 375-382.

Cremer MA, Ye XJ, Terato K, Owens SW, Seyer JM & Kang AH (1994) Type XI collagen-induced arthritis in the Lewis rat. Characterization of cellular and humoral immune responses to native types XI, V, and II collagen and constituent alpha-chains. J.Immunol. 153(2): 824-832.

Crombie DE, Turer M, Zuasti BB, Wood B, McNaughton D, Nandakumar KS, Holmdahl R, Van Damme MP & Rowley MJ (2005) Destructive effects of murine arthritogenic antibodies to type II collagen on cartilage explants in vitro. Arthritis Res.Ther. 7(5): R927-37.

Czarny-Ratajczak M, Lohiniva J, Rogala P, Kozlowski K, Perala M, Carter L, Spector TD, Kolodziej L, Seppanen U, Glazar R, Krolewski J, Latos-Bielenska A & Ala-Kokko L (2001) A mutation in COL9A1 causes multiple epiphyseal dysplasia: further evidence for locus heterogeneity. Am.J.Hum.Genet. 69(5): 969-980.

Deak F, Wagener R, Kiss I & Paulsson M (1999) The matrilins: a novel family of oligomeric extracellular matrix proteins. Matrix Biol. 18(1): 55-64.

Delot E, Brodie SG, King LM, Wilcox WR & Cohn DH (1998) Physiological and pathological secretion of cartilage oligomeric matrix protein by cells in culture. J.Biol.Chem. 273(41): 26692-26697.

Diab M (1993) The role of type IX collagen in osteoarthritis and rheumatoid arthritis. Orthop.Rev. 22(2): 165-170.

Diab M, Wu JJ & Eyre DR (1996) Collagen type IX from human cartilage: a structural profile of intermolecular cross-linking sites. Biochem.J. 314 (Pt 1)(Pt 1): 327-332.

Dion AS & Myers JC (1987) COOH-terminal propeptides of the major human procollagens. Structural, functional and genetic comparisons. J.Mol.Biol. 193(1): 127-143.

Douglas SP, Jenkins JM & Kadler KE (1998) Collagen IX: evidence for a structural association between NC4 domains in cartilage and a novel cleavage site in the alpha 1(IX) chain. Matrix Biol. 16(8): 497-505.

Duance VC, Wotton SF, Voyle CA & Bailey AJ (1984) Isolation and characterization of the precursor of type M collagen. Biochem.J. 221(3): 885-889.

Eble JA, Golbik R, Mann K & Kuhn K (1993) The alpha 1 beta 1 integrin recognition site of the basement membrane collagen molecule [alpha 1(IV)]2 alpha 2(IV). EMBO J. 12(12): 4795-4802.

Edwards CJ (2005) Immunological therapies for rheumatoid arthritis. Br.Med.Bull. 73-74: 71-82.

Eikenberry EF, Mendler M, Bürgin R, Winterhalter K & Bruckner P (1992) Fibrillar organization in cartilage. In: Kuettner K, Schleyerbach R, Peyron JG & Hascall VC (eds) Articular Cartilage and Osteoarthritis. Raven Press, New York, 133-148.

Emsley J, King SL, Bergelson JM & Liddington RC (1997) Crystal structure of the I domain from integrin alpha2beta1. J.Biol.Chem. 272(45): 28512-28517.

Emsley J, Knight CG, Farndale RW & Barnes MJ (2004) Structure of the integrin alpha2beta1-binding collagen peptide. J.Mol.Biol. 335(4): 1019-1028.

Emsley J, Knight CG, Farndale RW, Barnes MJ & Liddington RC (2000) Structural basis of collagen recognition by integrin alpha2beta1. Cell 101(1): 47-56.

Engel J & Prockop DJ (1991) The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. Annu.Rev.Biophys.Biophys.Chem. 20: 137-152.

Eyre D (2002) Collagen of articular cartilage. Arthritis Res. 4(1): 30-35.

Eyre DR (1991) The collagens of articular cartilage. Semin.Arthritis Rheum. 21(3 Suppl 2): 2-11.

Eyre DR (1979) Biochemistry of the intervertebral disc. Int.Rev.Connect.Tissue Res. 8: 227-291.

Eyre DR, Apon S, Wu JJ, Ericsson LH & Walsh KA (1987) Collagen type IX: evidence for covalent linkages to type II collagen in cartilage. FEBS Lett. 220(2): 337-341.

Eyre DR, Paz MA & Gallop PM (1984) Cross-linking in collagen and elastin. Annu.Rev.Biochem. 53: 717-748.

Eyre DR, Pietka T, Weis MA & Wu JJ (2004) Covalent cross-linking of the NC1 domain of collagen type IX to collagen type II in cartilage. J.Biol.Chem. 279(4): 2568-2574.

Eyre DR, Wu JJ, Fernandes RJ, Pietka TA & Weis MA (2002) Recent developments in cartilage research: matrix biology of the collagen II/IX/XI heterofibril network. Biochem.Soc.Trans. 30(Pt 6): 893-899.

Eyre DR, Wu JJ & Woods P (1992) Cartilage specific collagens. In: Kuettner K, Schleyerbach R, Peyron JG & Hascall VC (eds) Articular Cartilage and Osteoarthritis. Raven Press, New York, 119-131.

Fassler R, Schnegelsberg PN, Dausman J, Shinya T, Muragaki Y, McCarthy MT, Olsen BR & Jaenisch R (1994) Mice lacking alpha 1 (IX) collagen develop noninflammatory degenerative joint disease. Proc.Natl.Acad.Sci.U.S.A. 91(11): 5070-5074.

Feldmann M (1996a) The cytokine network in rheumatoid arthritis: definition of TNF alpha as a therapeutic target. J.R.Coll.Physicians Lond. 30(6): 560-570.

Feldmann M, Brennan FM, Foxwell BM, Taylor PC, Williams RO & Maini RN (2005) Anti-TNF therapy: where have we got to in 2005? J.Autoimmun. 25 Suppl: 26-28.

Feldmann M, Brennan FM & Maini RN (1996b) Rheumatoid arthritis. Cell 85(3): 307-310.

Fertala A, Sieron AL, Adachi E & Jimenez SA (2001) Collagen II containing a Cys substitution for Arg-alpha1-519: abnormal interactions of the mutated molecules with collagen IX. Biochemistry 40(48): 14422-14428.

Fessler LI, Chapin S, Brosh S & Fessler JH (1986) Intracellular transport and tyrosine sulfation of procollagens V. Eur.J.Biochem. 158(3): 511-518.

Fichard A, Kleman JP & Ruggiero F (1995) Another look at collagen V and XI molecules. Matrix Biol. 14(7): 515-531.

Fiedler J, Stove J, Heber F & Brenner RE (2002) Clinical phenotype and molecular diagnosis of multiple epiphyseal dysplasia with relative hip sparing during childhood (EDM2). Am.J.Med.Genet. 112(2): 144-153.

Fitzgerald J & Bateman JF (2001) A new FACIT of the collagen family: COL21A1. FEBS Lett. 505(2): 275-280.

Frank S, Boudko S, Mizuno K, Schulthess T, Engel J & Bachinger HP (2003) Collagen triple helix formation can be nucleated at either end. J.Biol.Chem. 278(10): 7747-7750.

Fugger L, Rothbard JB & Sonderstrup-McDevitt G (1996) Specificity of an HLA-DRB1*0401-restricted T cell response to type II collagen. Eur.J.Immunol. 26(4): 928-933.

Fujii K, Tsuji M, Kitamura A & Murota K (1992) The diagnostic significance of anti-type II collagen antibody assay in rheumatoid arthritis. Int.Orthop. 16(3): 272-276.

Gadher SJ, Eyre DR, Duance VC, Wotton SF, Heck LW, Schmid TM & Woolley DE (1988) Susceptibility of cartilage collagens type II, IX, X, and XI to human synovial collagenase and neutrophil elastase. Eur.J.Biochem. 175(1): 1-7.

Garnero P, Gineyts E, Christgau S, Finck B & Delmas PD (2002) Association of baseline levels of urinary glucosyl-galactosyl-pyridinoline and type II collagen C-telopeptide with progression of joint destruction in patients with early rheumatoid arthritis. Arthritis Rheum. 46(1): 21-30.

Gelse K, Poschl E & Aigner T (2003) Collagens--structure, function, and biosynthesis. Adv.Drug Deliv.Rev. 55(12): 1531-1546.

Golbik R, Eble JA, Ries A & Kuhn K (2000) The spatial orientation of the essential amino acid residues arginine and aspartate within the alpha1beta1 integrin recognition site of collagen IV has been resolved using fluorescence resonance energy transfer. J.Mol.Biol. 297(2): 501-509.

Graham HK, Holmes DF, Watson RB & Kadler KE (2000) Identification of collagen fibril fusion during vertebrate tendon morphogenesis. The process relies on unipolar fibrils and is regulated by collagen-proteoglycan interaction. J.Mol.Biol. 295(4): 891-902.

Gran JT (1987) The epidemiology of rheumatoid arthritis. Monogr.Allergy 21: 162-196.

Gray RE, Seng N, Mackay IR & Rowley MJ (2004) Measurement of antibodies to collagen II by inhibition of collagen fibril formation in vitro. J.Immunol.Methods 285(1): 55-61.

Gregersen PK, Silver J & Winchester RJ (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum. 30(11): 1205-1213.

Gullberg DE (2003) I domains in integrins. Landes Bioscience, Georgetown, Texas, USA.

Gullberg DE & Lundgren-Akerlund E (2002) Collagen-binding I domain integrins--what do they do? Prog.Histochem.Cytochem. 37(1): 3-54.

Hagg R, Bruckner P & Hedbom E (1998) Cartilage fibrils of mammals are biochemically heterogeneous: differential distribution of decorin and collagen IX. J.Cell Biol. 142(1): 285-294.

Hagg R, Hedbom E, Mollers U, Aszodi A, Fassler R & Bruckner P (1997) Absence of the alpha1(IX) chain leads to a functional knock-out of the entire collagen IX protein in mice. J.Biol.Chem. 272(33): 20650-20654.

Haimes HB, Jimenez PA, Li Y, Shinya T & Olsen BR (1995) Overexpression of the NC4 domain of type IX collagen induces osteoarthritis in mice. Inflamm.Res. 44 Suppl 2: S127-8.

Hakala M, Aman S, Luukkainen R, Risteli L, Kauppi M, Nieminen P & Risteli J (1995) Application of markers of collagen metabolism in serum and synovial fluid for assessment of disease process in patients with rheumatoid arthritis. Ann.Rheum.Dis. 54(11): 886-890.

Hansen U & Bruckner P (2003) Macromolecular specificity of collagen fibrillogenesis: fibrils of collagens I and XI contain a heterotypic alloyed core and a collagen I sheath. J.Biol.Chem. 278(39): 37352-37359.

Har-el R, Sharma YD, Aguilera A, Ueyama N, Wu JJ, Eyre DR, Juricic L, Chandrasekaran S, Li M & Nah HD (1992) Cloning and developmental expression of the alpha 3 chain of chicken type IX collagen. J.Biol.Chem. 267(14): 10070-10076.

Hayashi M, Hayashi K, Iyama K, Trelstad RL, Linsenmayer TF & Mayne R (1992) Notochord of chick embryos secretes short-form type IX collagen prior to the onset of vertebral chondrogenesis. Dev.Dyn. 194(3): 169-176.

He X, Kang AH & Stuart JM (2001) Anti-Human type II collagen CD19+ B cells are present in patients with rheumatoid arthritis and healthy individuals. J.Rheumatol. 28(10): 2168-2175.

Heino J (2007) The collagen family members as cell adhesion proteins. Bioessays 29(10): 1001-1010.

Heino J (2000) The collagen receptor integrins have distinct ligand recognition and signaling functions. Matrix Biol. 19(4): 319-323.

Heliovaara M (1989) Risk factors for low back pain and sciatica. Ann.Med. 21(4): 257-264.

Heliovaara M, Impivaara O, Sievers K, Melkas T, Knekt P, Korpi J & Aromaa A (1987) Lumbar disc syndrome in Finland. J.Epidemiol.Community Health 41(3): 251-258.

Hietala MA, Nandakumar KS, Persson L, Fahlen S, Holmdahl R & Pekna M (2004) Complement activation by both classical and alternative pathways is critical for the effector phase of arthritis. Eur.J.Immunol. 34(4): 1208-1216.

Hogarth PM (2002) Fc receptors are major mediators of antibody based inflammation in autoimmunity. Curr.Opin.Immunol. 14(6): 798-802.

Holden P, Canty EG, Mortier GR, Zabel B, Spranger J, Carr A, Grant ME, Loughlin JA & Briggs MD (1999) Identification of novel pro-alpha2(IX) collagen gene mutations in two families with distinctive oligo-epiphyseal forms of multiple epiphyseal dysplasia. Am.J.Hum.Genet. 65(1): 31-38.

Holden P, Meadows RS, Chapman KL, Grant ME, Kadler KE & Briggs MD (2001) Cartilage oligomeric matrix protein interacts with type IX collagen, and disruptions to these interactions identify a pathogenetic mechanism in a bone dysplasia family. J.Biol.Chem. 276(8): 6046-6055.

Holmdahl R, Andersson M, Goldschmidt TJ, Gustafsson K, Jansson L & Mo JA (1990a) Type II collagen autoimmunity in animals and provocations leading to arthritis. Immunol.Rev. 118: 193-232.

Holmdahl R, Bockermann R, Backlund J & Yamada H (2002) The molecular pathogenesis of collagen-induced arthritis in mice--a model for rheumatoid arthritis. Ageing Res.Rev. 1(1): 135-147.

Holmdahl R, Jansson L, Larsson A & Jonsson R (1990b) Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies. Scand.J.Immunol. 31(2): 147-157.

Hoppe HJ, Barlow PN & Reid KB (1994) A parallel three stranded alpha-helical bundle at the nucleation site of collagen triple-helix formation. FEBS Lett. 344(2-3): 191-195.

Horton WA (1993) Morphology of connective tissue cartilage. In: Royce PM & Steinmann B (eds) Connective tissue and its heritable disorders: molecular genetics and medical aspects. Wiley-Liss, New York, 641-675.

Hu K, Xu L, Cao L, Flahiff CM, Brussiau J, Ho K, Setton LA, Youn I, Guilak F, Olsen BR & Li Y (2006) Pathogenesis of osteoarthritis-like changes in the joints of mice deficient in type IX collagen. Arthritis Rheum. 54(9): 2891-2900.

Huber S, van der Rest M, Bruckner P, Rodriguez E, Winterhalter KH & Vaughan L (1986) Identification of the type IX collagen polypeptide chains. The alpha 2(IX) polypeptide carries the chondroitin sulfate chain(s). J.Biol.Chem. 261(13): 5965-5968.

Huber S, Winterhalter KH & Vaughan L (1988) Isolation and sequence analysis of the glycosaminoglycan attachment site of type IX collagen. J.Biol.Chem. 263(2): 752-756.

Hughes AL (2001) Evolution of the integrin alpha and beta protein families. J.Mol.Evol. 52(1): 63-72.

Hulmes DJ (2002) Building collagen molecules, fibrils, and suprafibrillar structures. J.Struct.Biol. 137(1-2): 2-10.

Hulmes DJ, Kadler KE, Mould AP, Hojima Y, Holmes DF, Cummings C, Chapman JA & Prockop DJ (1989) Pleomorphism in type I collagen fibrils produced by persistence of the procollagen N-propeptide. J.Mol.Biol. 210(2): 337-345.

Humphries MJ (2000) Integrin structure. Biochem.Soc.Trans. 28(4): 311-339.

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110(6): 673-687.

Iozzo RV (1997) The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. Crit.Rev.Biochem.Mol.Biol. 32(2): 141-174.

Irwin MH, Silvers SH & Mayne R (1985) Monoclonal antibody against chicken type IX collagen: preparation, characterization, and recognition of the intact form of type IX collagen secreted by chondrocytes. J.Cell Biol. 101(3): 814-823.

Ivaska J, Kapyla J, Pentikainen O, Hoffren AM, Hermonen J, Huttunen P, Johnson MS & Heino J (1999) A peptide inhibiting the collagen binding function of integrin alpha2I domain. J.Biol.Chem. 274(6): 3513-3521.

Jackson GC, Barker FS, Jakkula E, Czarny-Ratajczak M, Makitie O, Cole WG, Wright MJ, Smithson SF, Suri M, Rogala P, Mortier GR, Baldock C, Wallace A, Elles R, Ala-Kokko L & Briggs MD (2004) Missense mutations in the beta strands of the single A-domain of matrilin-3 result in multiple epiphyseal dysplasia. J.Med.Genet. 41(1): 52-59.

Jakkula E (2005a) Defects in the genes coding for cartilage extracellular matrix proteins as a cause of osteoarthritis and multiple epiphyseal dysoplasia. Acta Univ Oul D 832.

Jakkula E, Makitie O, Czarny-Ratajczak M, Jackson GC, Damignani R, Susic M, Briggs MD, Cole WG & Ala-Kokko L (2005b) Mutations in the known genes are not the major cause of MED; distinctive phenotypic entities among patients with no identified mutations. Eur.J.Hum.Genet. 13(3): 292-301.

Jakkula E, Melkoniemi M, Kiviranta I, Lohiniva J, Raina SS, Perala M, Warman ML, Ahonen K, Kroger H, Goring HH & Ala-Kokko L (2005c) The role of sequence variations within the genes encoding collagen II, IX and XI in non-syndromic, early-onset osteoarthritis. Osteoarthritis Cartilage 13(6): 497-507.

Jasin HE (1985) Autoantibody specificities of immune complexes sequestered in articular cartilage of patients with rheumatoid arthritis and osteoarthritis. Arthritis Rheum. 28(3): 241-248.

Jim JJ, Noponen-Hietala N, Cheung KM, Ott J, Karppinen J, Sahraravand A, Luk KD, Yip SP, Sham PC, Song YQ, Leong JC, Cheah KS, Ala-Kokko L & Chan D (2005) The TRP2 allele of COL9A2 is an age-dependent risk factor for the development and severity of intervertebral disc degeneration. Spine 30(24): 2735-2742.

Jokinen J, Dadu E, Nykvist P, Kapyla J, White DJ, Ivaska J, Vehvilainen P, Reunanen H, Larjava H, Hakkinen L & Heino J (2004) Integrin-mediated cell adhesion to type I collagen fibrils. J.Biol.Chem. 279(30): 31956-31963.

Juvonen M, Sandberg M & Pihlajaniemi T (1992) Patterns of expression of the six alternatively spliced exons affecting the structures of the COL1 and NC2 domains of the alpha 1(XIII) collagen chain in human tissues and cell lines. J.Biol.Chem. 267(34): 24700-24707.

Kadler K (1995) Extracellular matrix 1: Fibril-forming collagens. Protein Profile 2(5): 491-619.

Kadler KE, Hojima Y & Prockop DJ (1987) Assembly of collagen fibrils de novo by cleavage of the type I pC-collagen with procollagen C-proteinase. Assay of critical concentration demonstrates that collagen self-assembly is a classical example of an entropy-driven process. J.Biol.Chem. 262(32): 15696-15701.

Kadler KE, Holmes DF, Trotter JA & Chapman JA (1996) Collagen fibril formation. Biochem.J. 316 (Pt 1)(Pt 1): 1-11.

Kammerer RA (1997) Alpha-helical coiled-coil oligomerization domains in extracellular proteins. Matrix Biol. 15(8-9): 555-65; discussion 567-8.

Kamphaus GD, Colorado PC, Panka DJ, Hopfer H, Ramchandran R, Torre A, Maeshima Y, Mier JW, Sukhatme VP & Kalluri R (2000) Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J.Biol.Chem. 275(2): 1209-1215.

Kapyla J, Ivaska J, Riikonen R, Nykvist P, Pentikainen O, Johnson M & Heino J (2000) Integrin alpha(2)I domain recognizes type I and type IV collagens by different mechanisms. J.Biol.Chem. 275(5): 3348-3354.

Kary S, Fritz J, Scherer HU & Burmester GR (2004) Do we still miss the chance of effectively treating early rheumatoid arthritis? New answers from a new study. Rheumatology (Oxford) 43(7): 819-820.

Kassner A, Hansen U, Miosge N, Reinhardt DP, Aigner T, Bruckner-Tuderman L, Bruckner P & Grassel S (2003) Discrete integration of collagen XVI into tissue-specific collagen fibrils or beaded microfibrils. Matrix Biol. 22(2): 131-143.

Keene DR, Oxford JT & Morris NP (1995) Ultrastructural localization of collagen types II, IX, and XI in the growth plate of human rib and fetal bovine epiphyseal cartilage: type XI collagen is restricted to thin fibrils. J.Histochem.Cytochem. 43(10): 967-979.

Kern A, Eble J, Golbik R & Kuhn K (1993) Interaction of type IV collagen with the isolated integrins alpha 1 beta 1 and alpha 2 beta 1. Eur.J.Biochem. 215(1): 151-159.

Kerwar SS & Oronsky AL (1988) Passive collagen arthritis induced by anticollagen IgG. Int.Rev.Immunol. 4(1): 17-23.

Kidd IM & Emery VC (1993) The use of baculoviruses as expression vectors. Appl.Biochem.Biotechnol. 42(2-3): 137-159.

Kielty CM & Grant ME (2002) The collagen family: structure, assembly, and organization in the extracellular matrix. In: Royce PM & Steinmann B (eds) Connective Tissue and Its Heritable Disorders. Wiley-Liss, New York, 159-221.

Kim WU, Cho ML, Jung YO, Min SY, Park SW, Min DJ, Yoon JH & Kim HY (2004) Type II collagen autoimmunity in rheumatoid arthritis. Am.J.Med.Sci. 327(4): 202-211.

Kim WU, Yoo WH, Park W, Kang YM, Kim SI, Park JH, Lee SS, Joo YS, Min JK, Hong YS, Lee SH, Park SH, Cho CS & Kim HY (2000) IgG antibodies to type II collagen reflect inflammatory activity in patients with rheumatoid arthritis. J.Rheumatol. 27(3): 575-581.

Kimura T, Mattei MG, Stevens JW, Goldring MB, Ninomiya Y & Olsen BR (1989) Molecular cloning of rat and human type IX collagen cDNA and localization of the alpha 1(IX) gene on the human chromosome 6. Eur.J.Biochem. 179(1): 71-78.

Kimura T, Nakata K, Tsumaki N, Miyamoto S, Matsui Y, Ebara S & Ochi T (1996) Progressive degeneration of articular cartilage and intervertebral discs. An experimental study in transgenic mice bearing a type IX collagen mutation. Int.Orthop. 20(3): 177-181.

Kivirikko KI & Pihlajaniemi T (1998) Collagen hydroxylases and the protein disulfide isomerase subunit of prolyl 4-hydroxylases. Adv.Enzymol.Relat.Areas Mol.Biol. 72: 325-398.

Kleinau S, Martinsson P & Heyman B (2000) Induction and suppression of collagen-induced arthritis is dependent on distinct fcgamma receptors. J.Exp.Med. 191(9): 1611-1616.

Knight CG, Morton LF, Onley DJ, Peachey AR, Messent AJ, Smethurst PA, Tuckwell DS, Farndale RW & Barnes MJ (1998) Identification in collagen type I of an integrin alpha2 beta1-binding site containing an essential GER sequence. J.Biol.Chem. 273(50): 33287-33294.

Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW & Barnes MJ (2000) The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. J.Biol.Chem. 275(1): 35-40.

Koch M, Foley JE, Hahn R, Zhou P, Burgeson RE, Gerecke DR & Gordon MK (2001) alpha 1(Xx) collagen, a new member of the collagen subfamily, fibril-associated collagens with interrupted triple helices. J.Biol.Chem. 276(25): 23120-23126.

Koch M, Laub F, Zhou P, Hahn RA, Tanaka S, Burgeson RE, Gerecke DR, Ramirez F & Gordon MK (2003) Collagen XXIV, a vertebrate fibrillar collagen with structural features of invertebrate collagens: selective expression in developing cornea and bone. J.Biol.Chem. 278(44): 43236-43244.

Koch M, Schulze J, Hansen U, Ashwodt T, Keene DR, Brunken WJ, Burgeson RE, Bruckner P & Bruckner-Tuderman L (2004) A novel marker of tissue junctions, collagen XXII. J.Biol.Chem. 279(21): 22514-22521.

Koivula MK, Aman S, Karjalainen A, Hakala M & Risteli J (2005) Are there autoantibodies reacting against citrullinated peptides derived from type I and type II collagens in patients with rheumatoid arthritis? Ann.Rheum.Dis. 64(10): 1443-1450.

Koivula MK, Heliovaara M, Ramberg J, Knekt P, Rissanen H, Palosuo T & Risteli J (2007) Autoantibodies binding to citrullinated telopeptide of type II collagen and to cyclic citrullinated peptides predict synergistically the development of seropositive rheumatoid arthritis. Ann.Rheum.Dis.

Kojima T, Mwale F, Yasuda T, Girard C, Poole AR & Laverty S (2001) Early degradation of type IX and type II collagen with the onset of experimental inflammatory arthritis. Arthritis Rheum. 44(1): 120-127.

Konomi H, Seyer JM, Ninomiya Y & Olsen BR (1986) Peptide-specific antibodies identify the alpha 2 chain as the proteoglycan subunit of type IX collagen. J.Biol.Chem. 261(15): 6742-6746.

Konttinen YT, Li TF, Lassus J, Waris V, Santavirta S & Virtanen I (2001) Removal of hyaline articular cartilage reduces lymphocyte infiltration and activation in rheumatoid synovial membrane. J.Rheumatol. 28(10): 2184-2189.

Korpela M, Laasonen L, Hannonen P, Kautiainen H, Leirisalo-Repo M, Hakala M, Paimela L, Blafield H, Puolakka K, Mottonen T & FIN-RACo Trial Group (2004) Retardation of joint damage in patients with early rheumatoid arthritis by initial aggressive treatment with disease-modifying antirheumatic drugs: five-year experience from the FIN-RACo study. Arthritis Rheum. 50(7): 2072-2081.

Kroot EJ, de Jong BA, van Leeuwen MA, Swinkels H, van den Hoogen FH, van't Hof M, van de Putte LB, van Rijswijk MH, van Venrooij WJ & van Riel PL (2000) The prognostic value of anti-cyclic citrullinated peptide antibody in patients with recent-onset rheumatoid arthritis. Arthritis Rheum. 43(8): 1831-1835.

Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH & Holers VM (2006) Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. J.Clin.Invest. 116(4): 961-973.

Labourdette L & van der Rest M (1993) Analysis of the role of the COL1 domain and its adjacent cysteine-containing sequence in the chain assembly of type IX collagen. FEBS Lett. 320(3): 211-214.

Lamberg A, Helaakoski T, Myllyharju J, Peltonen S, Notbohm H, Pihlajaniemi T & Kivirikko KI (1996) Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. J.Biol.Chem. 271(20): 11988-11995.

Landewe RB, Geusens P, van der Heijde DM, Boers M, van der Linden SJ & Garnero P (2006) Arthritis instantaneously causes collagen type I and type II degradation in patients with early rheumatoid arthritis: a longitudinal analysis. Ann.Rheum.Dis. 65(1): 40-44.

Latvanlehto A, Snellman A, Tu H & Pihlajaniemi T (2003) Type XIII collagen and some other transmembrane collagens contain two separate coiled-coil motifs, which may function as independent oligomerization domains. J.Biol.Chem. 278(39): 37590-37599.

Lees JF, Tasab M & Bulleid NJ (1997) Identification of the molecular recognition sequence which determines the type-specific assembly of procollagen. EMBO J. 16(5): 908-916.

Leitinger B & Hohenester E (2007) Mammalian collagen receptors. Matrix Biol. 26(3): 146-155.

Lim AL, Doyle SA, Balian G & Smith BD (1998) Role of the pro-alpha2(I) COOH-terminal region in assembly of type I collagen: truncation of the last 10 amino acid residues of pro-alpha2(I) chain prevents assembly of type I collagen heterotrimer. J.Cell.Biochem. 71(2): 216-232.

Linsenmayer TF, Gibney E, Igoe F, Gordon MK, Fitch JM, Fessler LI & Birk DE (1993) Type V collagen: molecular structure and fibrillar organization of the chicken alpha 1(V) NH2-terminal domain, a putative regulator of corneal fibrillogenesis. J.Cell Biol. 121(5): 1181-1189.

Liu CY, Olsen BR & Kao WW (1993) Developmental patterns of two alpha 1(IX) collagen mRNA isoforms in mouse. Dev.Dyn. 198(2): 150-157.

Lohiniva J, Paassilta P, Seppanen U, Vierimaa O, Kivirikko S & Ala-Kokko L (2000) Splicing mutations in the COL3 domain of collagen IX cause multiple epiphyseal dysplasia. Am.J.Med.Genet. 90(3): 216-222.

Loutis N, Bruckner P & Pataki A (1988) Induction of erosive arthritis in mice after passive transfer of anti-type II collagen antibodies. Agents Actions 25(3-4): 352-359.

Lu S, Carlsen S, Hansson AS & Holmdahl R (2002) Immunization of rats with homologous type XI collagen leads to chronic and relapsing arthritis with different genetics and joint pathology than arthritis induced with homologous type II collagen. J.Autoimmun. 18(3): 199-211.

Lu S & Holmdahl R (1999) Different therapeutic and bystander effects by intranasal administration of homologous type II and type IX collagens on the collagen-induced arthritis and pristane-induced arthritis in rats. Clin.Immunol. 90(1): 119-127.

Mabuchi A, Haga N, Maeda K, Nakashima E, Manabe N, Hiraoka H, Kitoh H, Kosaki R, Nishimura G, Ohashi H & Ikegawa S (2004) Novel and recurrent mutations clustered in the von Willebrand factor A domain of MATN3 in multiple epiphyseal dysplasia. Hum.Mutat. 24(5): 439-440.

Maciewicz RA & Wotton SF (1991) Degradation of cartilage matrix components by the cysteine proteinases, cathepsins B and L. Biomed.Biochim.Acta 50(4-6): 561-564.

Maddox BK, Keene DR, Sakai LY, Charbonneau NL, Morris NP, Ridgway CC, Boswell BA, Sussman MD, Horton WA, Bachinger HP & Hecht JT (1997) The fate of cartilage oligomeric matrix protein is determined by the cell type in the case of a novel mutation in pseudoachondroplasia. J.Biol.Chem. 272(49): 30993-30997.

Maeshima Y, Colorado PC, Torre A, Holthaus KA, Grunkemeyer JA, Ericksen MB, Hopfer H, Xiao Y, Stillman IE & Kalluri R (2000) Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J.Biol.Chem. 275(28): 21340-21348.

Magnusson SE, Engstrom M, Jacob U, Ulfgren AK & Kleinau S (2007) High synovial expression of the inhibitory FcgammaRIIb in rheumatoid arthritis. Arthritis Res.Ther. 9(3): R51.

Mallein-Gerin F, Ruggiero F, Quinn TM, Bard F, Grodzinsky AJ, Olsen BR & van der Rest M (1995) Analysis of collagen synthesis and assembly in culture by immortalized mouse chondrocytes in the presence or absence of alpha 1(IX) collagen chains. Exp.Cell Res. 219(1): 257-265.

Mann K (1992) Isolation of the alpha 3-chain of human type V collagen and characterization by partial sequencing. Biol.Chem.Hoppe Seyler 373(2): 69-75.

Mathsson L, Lampa J, Mullazehi M & Ronnelid J (2006) Immune complexes from rheumatoid arthritis synovial fluid induce FcgammaRIIa dependent and rheumatoid factor correlated production of tumour necrosis factor-alpha by peripheral blood mononuclear cells. Arthritis Res.Ther. 8(3): R64.

Matrisian LM (1992) The matrix-degrading metalloproteinases. Bioessays 14(7): 455-463.

Matsui H, Kanamori M, Ishihara H, Yudoh K, Naruse Y & Tsuji H (1998) Familial predisposition for lumbar degenerative disc disease. A case-control study. Spine 23(9): 1029-1034.

Matsui Y, Wu JJ, Weis MA, Pietka T & Eyre DR (2003) Matrix deposition of tryptophan-containing allelic variants of type IX collagen in developing human cartilage. Matrix Biol. 22(2): 123-129.

Mayne R & Brewton RG (1993) New members of the collagen superfamily. Curr.Opin.Cell Biol. 5(5): 883-890.

Mayne R, van der Rest M, Ninomiya Y & Olsen BR (1985) The structure of type IX collagen. Ann.N.Y.Acad.Sci. 460: 38-46.

Mazzorana M, Cogne S, Goldschmidt D & Aubert-Foucher E (2001) Collagenous sequence governs the trimeric assembly of collagen XII. J.Biol.Chem. 276(30): 27989-27998.

Mazzorana M, Giry-Lozinguez C & van der Rest M (1995) Trimeric assembly of collagen XII: effect of deletion of the C-terminal part of the molecule. Matrix Biol. 14(7): 583-588.

Mazzorana M, Gruffat H, Sergeant A & van der Rest M (1993) Mechanisms of collagen trimer formation. Construction and expression of a recombinant minigene in HeLa cells reveals a direct effect of prolyl hydroxylation on chain assembly of type XII collagen. J.Biol.Chem. 268(5): 3029-3032.

Mazzorana M, Snellman A, Kivirikko KI, van der Rest M & Pihlajaniemi T (1996) Involvement of prolyl 4-hydroxylase in the assembly of trimeric minicollagen XII. Study in a baculovirus expression system. J.Biol.Chem. 271(46): 29003-29008.

McAlinden A, Crouch EC, Bann JG, Zhang P & Sandell LJ (2002) Trimerization of the amino propeptide of type IIA procollagen using a 14-amino acid sequence derived from the coiled-coil neck domain of surfactant protein D. J.Biol.Chem. 277(43): 41274-41281.

McAlinden A, Smith TA, Sandell LJ, Ficheux D, Parry DA & Hulmes DJ (2003) Alpha-helical coiled-coil oligomerization domains are almost ubiquitous in the collagen superfamily. J.Biol.Chem. 278(43): 42200-42207.

McCarty DJ (1989) Arthritis and Allied Conditions. Lea & Febiger, Philadelphia.

McCawley LJ & Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! Curr.Opin.Cell Biol. 13(5): 534-540.

McCormick D, van der Rest M, Goodship J, Lozano G, Ninomiya Y & Olsen BR (1987) Structure of the glycosaminoglycan domain in the type IX collagen-proteoglycan. Proc.Natl.Acad.Sci.U.S.A. 84(12): 4044-4048.

McLaughlin SH & Bulleid NJ (1998) Molecular recognition in procollagen chain assembly. Matrix Biol. 16(7): 369-377.

Mechling DE, Gambee JE, Morris NP, Sakai LY, Keene DR, Mayne R & Bachinger HP (1996) Type IX collagen NC1 domain peptides can trimerize in vitro without forming a triple helix. J.Biol.Chem. 271(23): 13781-13785.

Mendler M, Eich-Bender SG, Vaughan L, Winterhalter KH & Bruckner P (1989) Cartilage contains mixed fibrils of collagen types II, IX, and XI. J.Cell Biol. 108(1): 191-197.

Michishita M, Videm V & Arnaout MA (1993) A novel divalent cation-binding site in the A domain of the beta 2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell 72(6): 857-867.

Miles CA, Knott L, Sumner IG & Bailey AJ (1998) Differences between the thermal stabilities of the three triple-helical domains of type IX collagen. J.Mol.Biol. 277(1): 135-144.

Miller EJ, Epstein EH,Jr & Piez KA (1971) Identification of three genetically distinct collagens by cyanogen bromide cleavage of insoluble human skin and cartilage collagen. Biochem.Biophys.Res.Commun. 42(6): 1024-1029.

Miller EJ & Gay S (1987) The collagens: an overview and update. Methods Enzymol. 144: 3-41.

Miller EJ & Matukas VJ (1969) Chick cartilage collagen: a new type of alpha 1 chain not present in bone or skin of the species. Proc.Natl.Acad.Sci.U.S.A. 64(4): 1264-1268.

Miller LK (1993) Baculoviruses: high-level expression in insect cells. Curr.Opin.Genet.Dev. 3(1): 97-101.

Morgan K, Buckee C, Collins I, Ayad S, Clague RB & Holt PJ (1988) Antibodies to type II and XI collagens: evidence for the formation of antigen specific as well as cross reacting antibodies in patients with rheumatoid arthritis. Ann.Rheum.Dis. 47(12): 1008-1013.

Morgan K, Clague RB, Collins I, Ayad S, Phinn SD & Holt PJ (1989) A longitudinal study of anticollagen antibodies in patients with rheumatoid arthritis. Arthritis Rheum. 32(2): 139-145.

Morgan K, Clague RB, Collins I, Ayad S, Phinn SD & Holt PJ (1987) Incidence of antibodies to native and denatured cartilage collagens (types II, IX, and XI) and to type I collagen in rheumatoid arthritis. Ann.Rheum.Dis. 46(12): 902-907.

Morgan K, Evans HB, Firth SA, Smith MN, Ayad S, Weiss JB & Lennox Holt PJ (1983) 1 Alpha 2 alpha 3 alpha collagen is arthritogenic. Ann.Rheum.Dis. 42(6): 680-683.

Mostert AK, Dijkstra PF, Jansen BR, van Horn JR, de Graaf B, Heutink P & Lindhout D (2003) Familial multiple epiphyseal dysplasia due to a matrilin-3 mutation: further delineation of the phenotype including 40 years follow-up. Am.J.Med.Genet.A. 120(4): 490-497.

Mottonen T, Hannonen P, Korpela M, Nissila M, Kautiainen H, Ilonen J, Laasonen L, Kaipiainen-Seppanen O, Franzen P, Helve T, Koski J, Gripenberg-Gahmberg M, Myllykangas-Luosujarvi R, Leirisalo-Repo M & FIN-RACo Trial Group. FINnish Rheumatoid Arthritis Combination therapy (2002) Delay to institution of therapy and induction of remission using single-drug or combination-disease-modifying antirheumatic drug therapy in early rheumatoid arthritis. Arthritis Rheum. 46(4): 894-898.

Mottonen T, Hannonen P, Leirisalo-Repo M, Nissila M, Kautiainen H, Korpela M, Laasonen L, Julkunen H, Luukkainen R, Vuori K, Paimela L, Blafield H, Hakala M, Ilva K, Yli-Kerttula U, Puolakka K, Jarvinen P, Hakola M, Piirainen H, Ahonen J, Palvimaki I, Forsberg S, Koota K & Friman C (1999) Comparison of combination therapy with single-drug therapy in early rheumatoid arthritis: a randomised trial. FIN-RACo trial group. Lancet 353(9164): 1568-1573.

Mullazehi M, Mathsson L, Lampa J & Ronnelid J (2007) High anti-collagen type-II antibody levels and induction of proinflammatory cytokines by anti-collagen antibody-containing immune complexes in vitro characterise a distinct rheumatoid arthritis phenotype associated with acute inflammation at the time of disease onset. Ann.Rheum.Dis. 66(4): 537-541.

Mullazehi M, Mathsson L, Lampa J & Ronnelid J (2006) Surface-bound anti-type II collagen-containing immune complexes induce production of tumor necrosis factor alpha, interleukin-1beta, and interleukin-8 from peripheral blood monocytes via Fc gamma receptor IIA: a potential pathophysiologic mechanism for humoral anti-type II collagen immunity in arthritis. Arthritis Rheum. 54(6): 1759-1771.

Muller A, Jakob K & Hein GE (2003) Evaluation of free and peptide bound collagen crosslink excretion in different skeletal diseases. Ann.Rheum.Dis. 62(1): 65-67.

Muller-Glauser W, Humbel B, Glatt M, Strauli P, Winterhalter KH & Bruckner P (1986) On the role of type IX collagen in the extracellular matrix of cartilage: type IX collagen is localized to intersections of collagen fibrils. J.Cell Biol. 102(5): 1931-1939.

Munakata H, Takagaki K, Majima M & Endo M (1999) Interaction between collagens and glycosaminoglycans investigated using a surface plasmon resonance biosensor. Glycobiology 9(10): 1023-1027.

Muragaki Y, Kimura T, Ninomiya Y & Olsen BR (1990a) The complete primary structure of two distinct forms of human alpha 1 (IX) collagen chains. Eur.J.Biochem. 192(3): 703-708.

Muragaki Y, Mariman EC, van Beersum SE, Perala M, van Mourik JB, Warman ML, Olsen BR & Hamel BC (1996) A mutation in the gene encoding the alpha 2 chain of the fibril-associated collagen IX, COL9A2, causes multiple epiphyseal dysplasia (EDM2). Nat.Genet. 12(1): 103-105.

Muragaki Y, Nishimura I, Henney A, Ninomiya Y & Olsen BR (1990b) The alpha 1 (IX) collagen gene gives rise to two different transcripts in both mouse embryonic and human fetal RNA. Proc.Natl.Acad.Sci.U.S.A. 87(7): 2400-2404.

Myers JC, Li D, Amenta PS, Clark CC, Nagaswami C & Weisel JW (2003) Type XIX collagen purified from human umbilical cord is characterized by multiple sharp kinks delineating collagenous subdomains and by intermolecular aggregates via globular, disulfide-linked, and heparin-binding amino termini. J.Biol.Chem. 278(34): 32047-32057.

Myers JC, Li D, Bageris A, Abraham V, Dion AS & Amenta PS (1997) Biochemical and immunohistochemical characterization of human type XIX defines a novel class of basement membrane zone collagens. Am.J.Pathol. 151(6): 1729-1740.

Myers LK, Brand DD, Ye XJ, Cremer MA, Rosloniec EF, Bodo M, Myllyharju J, Helaakoski T, Nokelainen M, Pihlajaniemi T, Kivirikko K, Yang CL, Ala-Kokko L, Prockop DJ, Notbohm H, Fietzek P, Stuart JM & Kang AH (1998) Characterization of recombinant type II collagen: arthritogenicity and tolerogenicity in DBA/1 mice. Immunology 95(4): 631-639.

Myers LK, Myllyharju J, Nokelainen M, Brand DD, Cremer MA, Stuart JM, Bodo M, Kivirikko KI & Kang AH (2004) Relevance of posttranslational modifications for the arthritogenicity of type II collagen. J.Immunol. 172(5): 2970-2975.

Myers LK, Pihlajamaa T, Brand DD, Cremer MA, Bodo M, Ala-Kokko L & Kang AH (2002) Immunogenicity of recombinant type IX collagen in murine collagen-induced arthritis. Arthritis Rheum. 46(4): 1086-1093.

Myers LK, Rosloniec EF, Cremer MA & Kang AH (1997) Collagen-induced arthritis, an animal model of autoimmunity. Life Sci. 61(19): 1861-1878.

Myllyharju J & Kivirikko KI (2004) Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet. 20(1): 33-43.

Myllyharju J & Kivirikko KI (2001) Collagens and collagen-related diseases. Ann.Med. 33(1): 7-21.

Myllyharju J, Lamberg A, Notbohm H, Fietzek PP, Pihlajaniemi T & Kivirikko KI (1997) Expression of wild-type and modified proalpha chains of human type I procollagen in insect cells leads to the formation of stable [alpha1(I)]2alpha2(I) collagen heterotrimers and [alpha1(I)]3 homotrimers but not [alpha2(I)]3 homotrimers. J.Biol.Chem. 272(35): 21824-21830.

Nakashima E, Kitoh H, Maeda K, Haga N, Kosaki R, Mabuchi A, Nishimura G, Ohashi H & Ikegawa S (2005) Novel COL9A3 mutation in a family with multiple epiphyseal dysplasia. Am.J.Med.Genet.A. 132(2): 181-184.

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 alpha 1(IX) collagen chains with a central deletion. Proc.Natl.Acad.Sci.U.S.A. 90(7): 2870-2874.

Nandakumar KS, Andren M, Martinsson P, Bajtner E, Hellstrom S, Holmdahl R & Kleinau S (2003) Induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and enhancement of arthritis in mice lacking inhibitory FcgammaRIIB. Eur.J.Immunol. 33(8): 2269-2277.

Nandakumar KS, Backlund J, Vestberg M & Holmdahl R (2004) Collagen type II (CII)-specific antibodies induce arthritis in the absence of T or B cells but the arthritis progression is enhanced by CII-reactive T cells. Arthritis Res.Ther. 6(6): R544-50.

Nandakumar KS & Holmdahl R (2005) Efficient promotion of collagen antibody induced arthritis (CAIA) using four monoclonal antibodies specific for the major epitopes recognized in both collagen induced arthritis and rheumatoid arthritis. J.Immunol.Methods 304(1-2): 126-136.

Navarro-Sarabia F, Ariza-Ariza R, Hernandez-Cruz B & Villanueva I (2005) Adalimumab for treating rheumatoid arthritis. Cochrane Database Syst.Rev. (3)(3): CD005113.

Nell VP, Machold KP, Eberl G, Stamm TA, Uffmann M & Smolen JS (2004) Benefit of very early referral and very early therapy with disease-modifying anti-rheumatic drugs in patients with early rheumatoid arthritis. Rheumatology (Oxford) 43(7): 906-914.

Nerlich AG, Boos N, Wiest I & Aebi M (1998) Immunolocalization of major interstitial collagen types in human lumbar intervertebral discs of various ages. Virchows Arch. 432(1): 67-76.

Newall JF & Ayad S (1995) Collagen IX isoforms in the intervertebral disc. Biochem.Soc.Trans. 23(4): 517S.

Ninomiya Y & Olsen BR (1984) Synthesis and characterization of cDNA encoding a cartilage-specific short collagen. Proc.Natl.Acad.Sci.U.S.A. 81(10): 3014-3018.

Nishida N, Sumikawa H, Sakakura M, Shimba N, Takahashi H, Terasawa H, Suzuki EI & Shimada I (2003) Collagen-binding mode of vWF-A3 domain determined by a transferred cross-saturation experiment. Nat.Struct.Biol. 10(1): 53-58.

Nishimura I, Muragaki Y, Hayashi M, Ninomiya Y & Olsen BR (1990) Tissue-specific expression of type IX collagen. Ann.N.Y.Acad.Sci. 580: 112-119.

Nishimura I, Muragaki Y & Olsen BR (1989) Tissue-specific forms of type IX collagen-proteoglycan arise from the use of two widely separated promoters. J.Biol.Chem. 264(33): 20033-20041.

Nishiyama T, McDonough AM, Bruns RR & Burgeson RE (1994) Type XII and XIV collagens mediate interactions between banded collagen fibers in vitro and may modulate extracellular matrix deformability. J.Biol.Chem. 269(45): 28193-28199.

Nokelainen M, Helaakoski T, Myllyharju J, Notbohm H, Pihlajaniemi T, Fietzek PP & Kivirikko KI (1998) Expression and characterization of recombinant human type II collagens with low and high contents of hydroxylysine and its glycosylated forms. Matrix Biol. 16(6): 329-338.

Noponen-Hietala N, Kyllonen E, Mannikko M, Ilkko E, Karppinen J, Ott J & Ala-Kokko L (2003) Sequence variations in the collagen IX and XI genes are associated with degenerative lumbar spinal stenosis. Ann.Rheum.Dis. 62(12): 1208-1214.

Noponen-Hietala N, Virtanen I, Karttunen R, Schwenke S, Jakkula E, Li H, Merikivi R, Barral S, Ott J, Karppinen J & Ala-Kokko L (2005) Genetic variations in IL6 associate with intervertebral disc disease characterized by sciatica. Pain 114(1-2): 186-194.

Noro A, Kimata K, Oike Y, Shinomura T, Maeda N, Yano S, Takahashi N & Suzuki S (1983) Isolation and characterization of a third proteoglycan (PG-Lt) from chick embryo cartilage which contains disulfide-bonded collagenous polypeptide. J.Biol.Chem. 258(15): 9323-9331.

Nykvist P, Tu H, Ivaska J, Kapyla J, Pihlajaniemi T & Heino J (2000) Distinct recognition of collagen subtypes by alpha(1)beta(1) and alpha(2)beta(1) integrins. Alpha(1)beta(1) mediates cell adhesion to type XIII collagen. J.Biol.Chem. 275(11): 8255-8261.

O'Dell JR (2002) Treating rheumatoid arthritis early: a window of opportunity? Arthritis Rheum. 46(2): 283-285.

Okada Y, Shinmei M, Tanaka O, Naka K, Kimura A, Nakanishi I, Bayliss MT, Iwata K & Nagase H (1992) Localization of matrix metalloproteinase 3 (stromelysin) in osteoarthritic cartilage and synovium. Lab.Invest. 66(6): 680-690.

Olsen BR (1997) Collagen IX. Int.J.Biochem.Cell Biol. 29(4): 555-558.

O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR & Folkman J (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88(2): 277-285.

Ortega N & Werb Z (2002) New functional roles for non-collagenous domains of basement membrane collagens. J.Cell.Sci. 115(Pt 22): 4201-4214.

Paassilta P, Lohiniva J, Annunen S, Bonaventure J, Le Merrer M, Pai L & Ala-Kokko L (1999a) COL9A3: A third locus for multiple epiphyseal dysplasia. Am.J.Hum.Genet. 64(4): 1036-1044.

Paassilta P, Lohiniva J, Goring HH, Perala M, Raina SS, Karppinen J, Hakala M, Palm T, Kroger H, Kaitila I, Vanharanta H, Ott J & Ala-Kokko L (2001) Identification of a novel common genetic risk factor for lumbar disk disease. JAMA 285(14): 1843-1849.

Paassilta P, Pihlajamaa T, Annunen S, Brewton RG, Wood BM, Johnson CC, Liu J, Gong Y, Warman ML, Prockop DJ, Mayne R & Ala-Kokko L (1999b) Complete sequence of the 23-kilobase human COL9A3 gene. Detection of Gly-X-Y triplet deletions that represent neutral variants. J.Biol.Chem. 274(32): 22469-22475.

Pace JM, Atkinson M, Willing MC, Wallis G & Byers PH (2001) Deletions and duplications of Gly-Xaa-Yaa triplet repeats in the triple helical domains of type I collagen chains disrupt helix formation and result in several types of osteogenesis imperfecta. Hum.Mutat. 18(4): 319-326.

Pace JM, Corrado M, Missero C & Byers PH (2003) Identification, characterization and expression analysis of a new fibrillar collagen gene, COL27A1. Matrix Biol. 22(1): 3-14.

Pai S, Pai L & Birkenfeldt R (1998) Correlation of serum IgA rheumatoid factor levels with disease severity in rheumatoid arthritis. Scand.J.Rheumatol. 27(4): 252-256.

Patterson RM, Selkirk JK & Merrick BA (1995) Baculovirus and insect cell gene expression: review of baculovirus biotechnology. Environ.Health Perspect. 103(7-8): 756-759.

Perala M, Hanninen M, Hastbacka J, Elima K & Vuorio E (1993) Molecular cloning of the human alpha 2(IX) collagen cDNA and assignment of the human COL9A2 gene to chromosome 1. FEBS Lett. 319(1-2): 177-180.

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

Pereira RS, Black CM, Duance VC, Jones VE, Jacoby RK & Welsh KI (1985) Disappearing collagen antibodies in rheumatoid arthritis. Lancet 2(8453): 501-502.

Piez KA & Sherman MR (1970) Characterization of the product formed by renaturation of alpha 1-CB2, a small peptide from collagen. Biochemistry 9(21): 4129-4133.

Pihlajamaa T, Lankinen H, Ylostalo J, Valmu L, Jaalinoja J, Zaucke F, Spitznagel L, Gosling S, Puustinen A, Morgelin M, Peranen J, Maurer P, Ala-Kokko L & Kilpelainen I (2004) Characterization of recombinant amino-terminal NC4 domain of human collagen IX: interaction with glycosaminoglycans and cartilage oligomeric matrix protein. J.Biol.Chem. 279(23): 24265-24273.

Pihlajamaa T, Perala M, Vuoristo MM, Nokelainen M, Bodo M, Schulthess T, Vuorio E, Timpl R, Engel J & Ala-Kokko L (1999) Characterization of recombinant human type IX collagen. Association of alpha chains into homotrimeric and heterotrimeric molecules. J.Biol.Chem. 274(32): 22464-22468.

Pihlajamaa T, Vuoristo MM, Annunen S, Perala M, Prockop DJ & Ala-Kokko L (1998) Human COL9A1 and COL9A2 genes. Two genes of 90 and 15 kb code for similar polypeptides of the same collagen molecule. Matrix Biol. 17(3): 237-241.

Popova SN, Barczyk M, Tiger CF, Beertsen W, Zigrino P, Aszodi A, Miosge N, Forsberg E & Gullberg D (2007) Alpha11 beta1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor. Mol.Cell.Biol. 27(12): 4306-4316.

Postacchini F, Lami R & Pugliese O (1988) Familial predisposition to discogenic low-back pain. An epidemiologic and immunogenetic study. Spine 13(12): 1403-1406.

Prockop DJ & Hulmes DJS (1994) Assembly of collagen fibrils *de novo* from soluble precursors. In: Yurchenko PD, Birk DE, and Mecham RP (eds) Extracellular Matrix Assembly and Structure. Academic Press, San Diego, 47–90.

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

Puolakka K, Kautiainen H, Mottonen T, Hannonen P, Korpela M, Julkunen H, Luukkainen R, Vuori K, Paimela L, Blafield H, Hakala M & Leirisalo-Repo M (2004) Impact of initial aggressive drug treatment with a combination of disease-modifying antirheumatic drugs on the development of work disability in early rheumatoid arthritis: a five-year randomized followup trial. Arthritis Rheum. 50(1): 55-62.

Quinn MA, Conaghan PG & Emery P (2001) The therapeutic approach of early intervention for rheumatoid arthritis: what is the evidence? Rheumatology (Oxford) 40(11): 1211-1220.

Ramchandran R, Dhanabal M, Volk R, Waterman MJ, Segal M, Lu H, Knebelmann B & Sukhatme VP (1999) Antiangiogenic activity of restin, NC10 domain of human collagen XV: comparison to endostatin. Biochem.Biophys.Res.Commun. 255(3): 735-739.

Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U & van Venrooij WJ (2003) Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 48(10): 2741-2749.

Reese CA & Mayne R (1981) Minor collagens of chicken hyaline cartilage. Biochemistry 20(19): 5443-5448.

Reese CA, Wiedemann H, Kuhn K & Mayne R (1982) Characterization of a highly soluble collagenous molecule isolated from chicken hyaline cartilage. Biochemistry 21(5): 826-830.

Ricard-Blum S, Hartmann DJ, Herbage D, Payen-Meyran C & Ville G (1982) Biochemical properties and immunolocalization of minor collagens in foetal calf cartilage. FEBS Lett. 146(2): 343-347.

Ricard-Blum S & Ruggiero F (2005) The collagen superfamily: from the extracellular matrix to the cell membrane. Pathol.Biol.(Paris) 53(7): 430-442.

Richardson C & Emery P (1996) Laboratory markers of disease activity. J.Rheumatol.Suppl. 44: 23-30.

Rokos I, Muragaki Y, Warman M & Olsen BR (1994) Assembly and sequencing of a cDNA covering the entire mouse alpha 1(IX) collagen chain. Matrix Biol. 14(1): 1-8.

Ronnelid J, Lysholm J, Engstrom-Laurent A, Klareskog L & Heyman B (1994) Local anti-type II collagen antibody production in rheumatoid arthritis synovial fluid. Evidence for an HLA-DR4-restricted IgG response. Arthritis Rheum. 37(7): 1023-1029.

Ronnelid J, Wick MC, Lampa J, Lindblad S, Nordmark B, Klareskog L & van Vollenhoven RF (2005) Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression. Ann.Rheum.Dis. 64(12): 1744-1749.

Rosenberg K, Olsson H, Morgelin M & Heinegard D (1998) Cartilage oligomeric matrix protein shows high affinity zinc-dependent interaction with triple helical collagen. J.Biol.Chem. 273(32): 20397-20403.

Rosloniec EF, Brand DD, Myers LK, Esaki Y, Whittington KB, Zaller DM, Woods A, Stuart JM & Kang AH (1998) Induction of autoimmune arthritis in HLA-DR4 (DRB1*0401) transgenic mice by immunization with human and bovine type II collagen. J.Immunol. 160(6): 2573-2578.

Rosloniec EF, Brand DD, Myers LK, Whittington KB, Gumanovskaya M, Zaller DM, Woods A, Altmann DM, Stuart JM & Kang AH (1997) An HLA-DR1 transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen. J.Exp.Med. 185(6): 1113-1122.

Roughley PJ & Lee ER (1994) Cartilage proteoglycans: structure and potential functions. Microsc.Res.Tech. 28(5): 385-397.

Rowley MJ, Gershwin ME & Mackay IR (1988) Collagen antibodies in juvenile arthritis and adult rheumatoid arthritis: differences in levels and type-specificity. J.Rheumatol. 15(2): 289-294.

Rudolphi U, Rzepka R, Batsford S, Kaufmann SH, von der Mark K, Peter HH & Melchers I (1997) The B cell repertoire of patients with rheumatoid arthritis. II. Increased frequencies of IgG+ and IgA+ B cells specific for mycobacterial heat-shock protein 60 or human type II collagen in synovial fluid and tissue. Arthritis Rheum. 40(8): 1409-1419.

Sacca B, Fiori S & Moroder L (2003) Studies of the local conformational properties of the cell-adhesion domain of collagen type IV in synthetic heterotrimeric peptides. Biochemistry 42(12): 3429-3436.

Sato K, Yomogida K, Wada T, Yorihuzi T, Nishimune Y, Hosokawa N & Nagata K (2002) Type XXVI collagen, a new member of the collagen family, is specifically expressed in the testis and ovary. J.Biol.Chem. 277(40): 37678-37684.

Scapinelli R (1993) Lumbar disc herniation in eight siblings with a positive family history for disc disease. Acta Orthop.Belg. 59(4): 371-376.

Shimokomaki M, Duance VC & Bailey AJ (1981) Identification of two further collagenous fractions from articular cartilage. Biosci.Rep. 1(7): 561-570.

Shimokomaki M, Duance VC & Bailey AJ (1980) Identification of a new disulphide bonded collagen from cartilage. FEBS Lett. 121(1): 51-54.

Sieper J, Kary S, Sorensen H, Alten R, Eggens U, Huge W, Hiepe F, Kuhne A, Listing J, Ulbrich N, Braun J, Zink A & Mitchison NA (1996) Oral type II collagen treatment in early rheumatoid arthritis. A double-blind, placebo-controlled, randomized trial. Arthritis Rheum. 39(1): 41-51.

Siljander PR, Hamaia S, Peachey AR, Slatter DA, Smethurst PA, Ouwehand WH, Knight CG & Farndale RW (2004) Integrin activation state determines selectivity for novel recognition sites in fibrillar collagens. J.Biol.Chem. 279(46): 47763-47772.

Smith C, Estavillo D, Emsley J, Bankston LA, Liddington RC & Cruz MA (2000) Mapping the collagen-binding site in the I domain of the glycoprotein Ia/IIa (integrin alpha(2)beta(1)). J.Biol.Chem. 275(6): 4205-4209.

Snellman A, Tu H, Vaisanen T, Kvist AP, Huhtala P & Pihlajaniemi T (2000) 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(19): 5051-5059.

Spayde EC, Joshi AP, Wilcox WR, Briggs M, Cohn DH & Olsen BR (2000) Exon skipping mutation in the COL9A2 gene in a family with multiple epiphyseal dysplasia. Matrix Biol. 19(2): 121-128.

Steplewski A, Brittingham R, Jimenez SA & Fertala A (2005) Single amino acid substitutions in the C-terminus of collagen II alter its affinity for collagen IX. Biochem.Biophys.Res.Commun. 335(3): 749-755.

Steplewski A, Hintze V & Fertala A (2007) Molecular basis of organization of collagen fibrils. J.Struct.Biol. 157(2): 297-307.

Stuart JM, Cremer MA, Townes AS & Kang AH (1982) Type II collagen-induced arthritis in rats. Passive transfer with serum and evidence that IgG anticollagen antibodies can cause arthritis. J.Exp.Med. 155(1): 1-16.

Stuart JM & Dixon FJ (1983) Serum transfer of collagen-induced arthritis in mice. J.Exp.Med. 158(2): 378-392.

Suzuki A, Yamada R, Ohtake-Yamanaka M, Okazaki Y, Sawada T & Yamamoto K (2005) Anti-citrullinated collagen type I antibody is a target of autoimmunity in rheumatoid arthritis. Biochem.Biophys.Res.Commun. 333(2): 418-426.

Svoboda KK, Nishimura I, Sugrue SP, Ninomiya Y & Olsen BR (1988) Embryonic chicken cornea and cartilage synthesize type IX collagen molecules with different amino-terminal domains. Proc.Natl.Acad.Sci.U.S.A. 85(20): 7496-7500.

Swedler W, Wallman J, Froelich CJ & Teodorescu M (1997) Routine measurement of IgM, IgG, and IgA rheumatoid factors: high sensitivity, specificity, and predictive value for rheumatoid arthritis. J.Rheumatol. 24(6): 1037-1044.

Swiderski RE & Solursh M (1992) Differential co-expression of long and short form type IX collagen transcripts during avian limb chondrogenesis in ovo. Development 115(1): 169-179.

Tarkowski A, Klareskog L, Carlsten H, Herberts P & Koopman WJ (1989) Secretion of antibodies to types I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. Arthritis Rheum. 32(9): 1087-1092.

Terato K, DeArmey DA, Ye XJ, Griffiths MM & Cremer MA (1996) The mechanism of autoantibody formation to cartilage in rheumatoid arthritis: possible cross-reaction of antibodies to dietary collagens with autologous type II collagen. Clin.Immunol.Immunopathol. 79(2): 142-154.

Terato K, Hasty KA, Reife RA, Cremer MA, Kang AH & Stuart JM (1992) Induction of arthritis with monoclonal antibodies to collagen. J.Immunol. 148(7): 2103-2108.

Terato K, Shimozuru Y, Katayama K, Takemitsu Y, Yamashita I, Miyatsu M, Fujii K, Sagara M, Kobayashi S & Goto M (1990) Specificity of antibodies to type II collagen in rheumatoid arthritis. Arthritis Rheum. 33(10): 1493-1500.

Thur J, Rosenberg K, Nitsche DP, Pihlajamaa T, Ala-Kokko L, Heinegard D, Paulsson M & Maurer P (2001) Mutations in cartilage oligomeric matrix protein causing pseudoachondroplasia and multiple epiphyseal dysplasia affect binding of calcium and collagen I, II, and IX. J.Biol.Chem. 276(9): 6083-6092.

Tiger CF, Fougerousse F, Grundstrom G, Velling T & Gullberg D (2001) Alpha11beta1 Integrin is a Receptor for Interstitial Collagens Involved in Cell Migration and Collagen Reorganization on Mesenchymal Nonmuscle Cells. Dev.Biol. 237(1): 116-129.

Tiller GE, Warman ML, Gong Y, Knoll JH, Mayne R & Brewton RG (1998) Physical and linkage mapping of the gene for the alpha3 chain of type IX collagen, COL9A3, to human chromosome 20q13.3. Cytogenet.Cell Genet. 81(3-4): 205-207.

Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, Hafler DA & Weiner HL (1993) Effects of oral administration of type II collagen on rheumatoid arthritis. Science 261(5129): 1727-1730.

Tuckwell D (2002) Identification and analysis of collagen alpha 1(XXI), a novel member of the FACIT collagen family. Matrix Biol. 21(1): 63-66.

Tulla M, Huhtala M, Jaalinoja J, Kapyla J, Farndale RW, Ala-Kokko L, Johnson MS & Heino J (2007) Analysis of an ascidian integrin provides new insight into early evolution of collagen recognition. FEBS Lett. 581(13): 2434-2440.

Tulla M, Pentikainen OT, Viitasalo T, Kapyla J, Impola U, Nykvist P, Nissinen L, Johnson MS & Heino J (2001) Selective binding of collagen subtypes by integrin alpha 1I, alpha 2I, and alpha 10I domains. J.Biol.Chem. 276(51): 48206-48212.

Vallbracht I & Helmke K (2005) Additional diagnostic and clinical value of anti-cyclic citrullinated peptide antibodies compared with rheumatoid factor isotypes in rheumatoid arthritis. Autoimmun.Rev. 4(6): 389-394.

Van Camp G, Snoeckx RL, Hilgert N, van den Ende J, Fukuoka H, Wagatsuma M, Suzuki H, Smets RM, Vanhoenacker F, Declau F, Van de Heyning P & Usami S (2006) A new autosomal recessive form of Stickler syndrome is caused by a mutation in the COL9A1 gene. Am.J.Hum.Genet. 79(3): 449-457.

van der Rest M & Mayne R (1988) Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. J.Biol.Chem. 263(4): 1615-1618.

van der Rest M & Mayne R (1987) Type IX collagen. In: Mayne R & Burgeson RE (eds) Structure and Function of Collagen Types. Academic Press, Orlando, 195-221.

van Gaalen FA, Linn-Rasker SP, van Venrooij WJ, de Jong BA, Breedveld FC, Verweij CL, Toes RE & Huizinga TW (2004) Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. Arthritis Rheum. 50(3): 709-715.

van Mourik JB, Buma P & Wilcox WR (1998) Electron microscopical study in multiple epiphyseal dysplasia type II. Ultrastruct.Pathol. 22(3): 249-251.

Varlotta GP, Brown MD, Kelsey JL & Golden AL (1991) Familial predisposition for herniation of a lumbar disc in patients who are less than twenty-one years old. J.Bone Joint Surg.Am. 73(1): 124-128.

Vasios G, Nishimura I, Konomi H, van der Rest M, Ninomiya Y & Olsen BR (1988) Cartilage type IX collagen-proteoglycan contains a large amino-terminal globular domain encoded by multiple exons. J.Biol.Chem. 263(5): 2324-2329.

Vaughan L, Mendler M, Huber S, Bruckner P, Winterhalter KH, Irwin MI & Mayne R (1988) D-periodic distribution of collagen type IX along cartilage fibrils. J.Cell Biol. 106(3): 991-997.

Vaughan L, Winterhalter KH & Bruckner P (1985) Proteoglycan Lt from chicken embryo sternum identified as type IX collagen. J.Biol.Chem. 260(8): 4758-4763.

Visser H, le Cessie S, Vos K, Breedveld FC & Hazes JM (2002) How to diagnose rheumatoid arthritis early: a prediction model for persistent (erosive) arthritis. Arthritis Rheum. 46(2): 357-365.

von Delwig A, Altmann DM, Isaacs JD, Harding CV, Holmdahl R, McKie N & Robinson JH (2006) The impact of glycosylation on HLA-DR1-restricted T cell recognition of type II collagen in a mouse model. Arthritis Rheum. 54(2): 482-491.

von der Mark K, van Menxel M & Wiedemann H (1984) Isolation and characterization of a precursor form of M collagen from embryonic chicken cartilage. Eur.J.Biochem. 138(3): 629-633.

von der Mark K, van Menxel M & Wiedemann H (1982) Isolation and characterization of new collagens from chick cartilage. Eur.J.Biochem. 124(1): 57-62.

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

Wang Y, Rollins SA, Madri JA & Matis LA (1995) Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. Proc.Natl.Acad.Sci.U.S.A. 92(19): 8955-8959.

Warman M, Kimura T, Muragaki Y, Castagnola P, Tamei H, Iwata K & Olsen BR (1993a) Monoclonal antibodies against two epitopes in the human alpha 1 (IX) collagen chain. Matrix 13(2): 149-156.

Warman ML, McCarthy MT, Perala M, Vuorio E, Knoll JH, McDaniels CN, Mayne R, Beier DR & Olsen BR (1994) The genes encoding alpha 2(IX) collagen (COL9A2) map to human chromosome 1p32.3-p33 and mouse chromosome 4. Genomics 23(1): 158-162.

Warman ML, Tiller GE, Polumbo PA, Seldin MF, Rochelle JM, Knoll JH, Cheng SD & Olsen BR (1993b) Physical and linkage mapping of the human and murine genes for the alpha 1 chain of type IX collagen (COL9A1). Genomics 17(3): 694-698.

Watson WC, Brown PS, Pitcock JA & Townes AS (1987) Passive transfer studies with type II collagen antibody in B10.D2/old and new line and C57Bl/6 normal and beige (Chediak-Higashi) strains: evidence of important roles for C5 and multiple inflammatory cell types in the development of erosive arthritis. Arthritis Rheum. 30(4): 460-465.

Watt SL, Lunstrum GP, McDonough AM, Keene DR, Burgeson RE & Morris NP (1992) Characterization of collagen types XII and XIV from fetal bovine cartilage. J.Biol.Chem. 267(28): 20093-20099.

White DJ, Puranen S, Johnson MS & Heino J (2004) The collagen receptor subfamily of the integrins. Int.J.Biochem.Cell Biol. 36(8): 1405-1410.

Williams RO (2004) Collagen-induced arthritis as a model for rheumatoid arthritis. Methods Mol.Med. 98: 207-216.

Williams RO, Paleolog E & Feldmann M (2007) Cytokine inhibitors in rheumatoid arthritis and other autoimmune diseases. Curr.Opin.Pharmacol.

Winterbottom N, Tondravi MM, Harrington TL, Klier FG, Vertel BM & Goetinck PF (1992) Cartilage matrix protein is a component of the collagen fibril of cartilage. Dev.Dyn. 193(3): 266-276.

Wooley PH, Luthra HS, Singh SK, Huse AR, Stuart JM & David CS (1984) Passive transfer of arthritis to mice by injection of human anti-type II collagen antibody. Mayo Clin.Proc. 59(11): 737-743.

Wotton SF, Dieppe PA & Duance VC (1999) Type IX collagen immunoreactive peptides in synovial fluids from arthritis patients. Rheumatology (Oxford) 38(4): 338-345.

Wotton SF, Duance VC & Fryer PR (1988) Type IX collagen: a possible function in articular cartilage. FEBS Lett. 234(1): 79-82.

Wu JJ & Eyre DR (1989) Covalent interactions of type IX collagen in cartilage. Connect.Tissue Res. 20(1-4): 241-246.

Wu JJ, Lark MW, Chun LE & Eyre DR (1991) Sites of stromelysin cleavage in collagen types II, IX, X, and XI of cartilage. J.Biol.Chem. 266(9): 5625-5628.

Wu JJ, Woods PE & Eyre DR (1992) Identification of cross-linking sites in bovine cartilage type IX collagen reveals an antiparallel type II-type IX molecular relationship and type IX to type IX bonding. J.Biol.Chem. 267(32): 23007-23014.

Xu Y, Gurusiddappa S, Rich RL, Owens RT, Keene DR, Mayne R, Hook A & Hook M (2000) Multiple binding sites in collagen type I for the integrins alpha1beta1 and alpha2beta1. J.Biol.Chem. 275(50): 38981-38989.

Yamada Y, Avvedimento VE, Mudryj M, Ohkubo H, Vogeli G, Irani M, Pastan I & de Crombrugghe B (1980) The collagen gene: evidence for its evolutinary assembly by amplification of a DNA segment containing an exon of 54 bp. Cell 22(3): 887-892.

Yoshida M, Tsuji M, Kurosaka D, Kurosaka D, Yasuda J, Ito Y, Nishizawa T & Yamada A (2006) Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. Mod.Rheumatol. 16(5): 276-281.

Yoshino S, Quattrocchi E & Weiner HL (1995) Suppression of antigen-induced arthritis in Lewis rats by oral administration of type II collagen. Arthritis Rheum. 38(8): 1092-1096.

Young BB, Gordon MK & Birk DE (2000) Expression of type XIV collagen in developing chicken tendons: association with assembly and growth of collagen fibrils. Dev.Dyn. 217(4): 430-439.

Zhang HG, Yang P, Xie J, Liu Z, Liu D, Xiu L, Zhou T, Wang Y, Hsu HC & Mountz JD (2002) Depletion of collagen II-reactive T cells and blocking of B cell activation prevents collagen II-induced arthritis in DBA/1j mice. J.Immunol. 168(8): 4164-4172.

Zhang P, McAlinden A, Li S, Schumacher T, Wang H, Hu S, Sandell L & Crouch E (2001) The amino-terminal heptad repeats of the coiled-coil neck domain of pulmonary surfactant protein d are necessary for the assembly of trimeric subunits and dodecamers. J.Biol.Chem. 276(23): 19862-19870.

Zhang WM, Kapyla J, Puranen JS, Knight CG, Tiger CF, Pentikainen OT, Johnson MS, Farndale RW, Heino J & Gullberg D (2003) alpha 11beta 1 integrin recognizes the GFOGER sequence in interstitial collagens. J.Biol.Chem. 278(9): 7270-7277.

Original articles

I Jäälinoja J, Ylöstalo J, Beckett W, Hulmes DJS & Ala-Kokko L (2007) Trimerization of collagen IX α chains does not require the presence of the COL1 and NC1 domains. Biochem J. In press.

II Käpylä J, Jäälinoja J, Tulla M, Ylöstalo J, Nissinen L, Viitasalo T, Vehviläinen P, Marjomäki V, Nykvist P, Säämänen AM, Farndale RW, Birk DE, Ala-Kokko L & Heino J (2004) The fibril-associated collagen IX provides a novel mechanism for cell adhesion to cartilaginous matrix. J Biol Chem 279: 51677-87.

III Jäälinoja J, Nissilä M, Kauppi M, Hakala M, Laiho, K, Karttunen R & Ala-Kokko L (2007) Serum antibodies against intact human collagen IX are elevated at the onset of RA but are not related to the development of erosions. Manuscript.

940. Tuohimäki, Carita (2007) The use of coercion in the Finnish civil psychiatric inpatients. A part of the Nordic project Paternalism and Autonomy

941. Karvonen, Juha T. (2007) Somatization in young adults. The Northern Finland 1966 Birth Cohort Study

942. Poutanen, Raija (2007) Boys and girls as health-promoting actors—determinants of oral health-related lifestyle among 11- to 12-year-old schoolchildren

943. Arpiainen, Satu (2007) Transcriptional regulation of the hepatic cytochrome *P450 2a5* gene

944. Päiväläinen-Jalonen, Satu (2007) Expression and stability of myelin-associated elements

945. Tiainen, Johanna (2007) Bioresorbable plain and ciprofloxacin-releasing self-reinforced PLGA 80/20 implants' suitability for craniofacial surgery. Histological and mechanical assessment

946. Aaltonen, Vesa (2007) PKC and neurofibromin in the molecular pathology of urinary bladder carcinoma. The effect of PKC inhibitors on carcinoma cell junctions, movement and death

947. Kuvaja, Paula (2007) The prognostic role of matrix metalloproteinases MMP-2 and -9 and their tissue inhibitors TIMP-1 and -2 in primary breast carcinoma

948. Siira, Virva (2007) Vulnerability signs of mental disorders in adoptees with genetic liability to schizophrenia and their controls measured with Minnesota Multiphasic Personality Inventory

949. Komulainen, Silja (2007) Effect of antihypertensive drugs on blood pressure during exposure to cold. Experimental study in normotensive and hypertensive subjects

950. Anttonen, Vuokko (2007) Laser fluorescence in detecting and monitoring the progression of occlusal dental caries lesions and for screening persons with unfavourable dietary habits

951. Torppa, Kaarina (2007) Managerialismi suomalaisen julkisen erikoissairaanhoidon johtamisessa. Tutkimus yksityissektorin johtamisoppien soveltamisesta neljässä yliopistollisessa sairaanhoitopiirissä ja arvio managerialismin soveltuvuudesta julkisen erikoissairaanhoidon uudistamiseen

952. Hokkanen, Eero (2007) Neurologian kehitysvaiheita Oulussa ja Pohjois-Suomessa

953. Lahtinen, Jarmo (2007) Predictors of immediate outcome after coronary artery bypass surgery

Book orders:
OULU UNIVERSITY PRESS
P.O. Box 8200, FI-90014
University of Oulu, Finland

Distributed by
OULU UNIVERSITY LIBRARY
P.O. Box 7500, FI-90014
University of Oulu, Finland

UNIVERSITY OF OULU P.O. Box 7500 FI-90014 UNIVERSITY OF OULU FINLAND

ACTA UNIVERSITATIS OULUENSIS

ISBN 978-951-42-8653-7 (Paperback)
ISBN 978-951-42-8654-4 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)