Susanne Grässel · Attila Aszódi *Editors*

Cartilage

Volume 1: Physiology and Development

Springer

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Preface

Volume one of a book series comprised of three volumes is dedicated to provide an overview about physiology and biology of permanent cartilage tissue and its role as a template in development and skeletal growth.

The text is designed to be of use to multiple medical and basic science disciplines as orthopedics, rheumatology, and trauma surgery and all basic investigators working in the field of cartilage and joint physiology and development.

Three types of cartilage (hyaline, elastic, and fibrous) have been characterized on the basis of histological criteria and mechanical properties. The most prevalent type is hyaline cartilage which is a visually uniform, translucent tissue found in the skeleton of all vertebrates. Articular cartilage, the most familiar hyaline permanent cartilage, forms the smooth gliding surface of joints, such as the knee and hip that permits locomotion in humans and animals. Injuries to this tissue and degradative diseases as osteoarthritis impair joint mobility and are a great challenge of modern regenerative medicine. Hyaline cartilage also comprises the growth plate, the transient and temporary template required for endochondral bone formation in fetal development, skeletal growth, and repair processes, i.e., after fracture. In addition, hyaline cartilage occurs as a permanent structural tissue in costal cartilage and tracheal reinforcing rings.

Cartilage is a matrix-dominated tissue, and with regard to its abundance, the matrix is mainly composed of collagens and proteoglycans. These two main components form suprastructures interconnected by plenty of proteins that way forming a kind of alloy. Cartilage fibrils vary in their molecular organization, their width, and their orientation in the tissue in order to resist forces generated by external load. Proteoglycans, especially the lectican family, provide the required tissue elasticity and resilience by dissipating load. The interconnecting molecules, sometimes also referred to as adaptor proteins, are from a biochemical point of view mainly non-collagenous glycoproteins and small leucine-rich repeat proteoglycans which closely regulate the assembly and connection of the fibrillar and extrafibrillar matrices. Chapters 1, 2, and 3 of this volume summarize information about the impact of proteoglycans, forming the extrafibrillar matrix, on cartilage physiology and integrity; the role of the different collagens in cartilage matrix homeostasis and formation of fibrillar suprastructures; and the role of non-collagenous matrix adaptor proteins in growth factor binding, mediation of inflammatory and immune responses, and their use as biomarkers in cartilage-associated diseases.

In long bones, a specialized structure called the growth plate is responsible for the linear growth and forms just below the epiphysis at both ends of the cartilaginous mold. The growth plate is organized into zones which reflect the sequential differentiation stages of chondrocyte proliferation, maturation, and hypertrophy. The differentiation process is accompanied by the establishment of cellular anisotropy and planar polarity that generates the unique spatial structure of the tissue. Chondrocyte differentiation and polarity are essential and mutually interacting foundations of the normal growth plate function, and their disturbance results in chondrodysplasias with impaired longitudinal growth. Chapter 4 will focus on the mechanisms responsible for the establishment and maintenance of the structural polarity of the cartilaginous growth plate.

The cell fate of hypertrophic growth plate chondrocytes at the chondro-osseous junction has been a subject of discussion for several decades: on the one hand, there is ample evidence for programmed cell death by apoptosis or other mechanisms in the lower hypertrophic zone; on the other hand, several studies have indicated that some hypertrophic chondrocytes may not be "terminally differentiated" but are able to further differentiate into osteoblasts. Comprehensive insight into this novel concept of the fate of hypertrophic chondrocytes is provided by Chap. 5. Hypoxia-driven pathways, governed by the hypoxia-inducible factors (HIFs), are absolutely essential for the survival and functioning of chondrocytes in these challenging conditions. HIF-mediated signaling has also been implicated in joint formation and the integrity of the adult articular cartilage. Thus, the oxygen-regulated genetic program mediated by HIFs is key to the controlled development, growth, health, and disease of endochondral bone summarized in Chap. 6.

Chapter 7 focuses on our current understanding at the cellular and molecular levels, from creation to maturation of a synovial joint. Morphologically, we know there is the formation of interzone regions at the presumptive sites of the future joint. Molecularly, we have some insights into signals that direct the initiation and progression of interzone regions toward a joint. And through innovative technologies in mouse genetics and genomics, we are beginning to understand the developmental processes, with the identification of progenitor cell pools, and to trace origin of cells and track the fate of descendent cells from initiation to formation of the complete joint.

Chapter 8 provides an overview about signaling factors which control cartilage formation, development, and the differentiation and maturation of chondrocytes during embryonic skeletal development. The orchestrated formation, differentiation, and degradation of cartilage and bone are regulated by a multitude of signaling systems and transcription factors. The identified signaling molecules include Ihh, PTHrP, FGF, BMP, Wnt, IGF, CNP, and CCN proteins. One essential group of regulators of chondrogenesis comprises members of the Hedgehog (Hh) morphogen family. Hedgehogs act as long-range morphogens during chondrocyte development and endochondral ossification. Mutations in Hh effectors, receptors, and co-receptors, as well as in ciliary proteins that act as modulators of Hh reception, result in skeletal and craniofacial deformities. Chapter 9 summarizes the current understanding of Hh production and signaling in chondrocytes in development and disease. Wnt signals play important regulatory roles in those processes. In the vertebrate genome,

a total of 19 different Wnt ligands are encoded which can utilize diverse signaling pathways acting either positively or negatively on chondrogenesis and during cartilage development, forming a highly interactive system addressed by Chap. 10.

Chondrogenesis, e.g., the formation of cartilage from precursor cells, is characterized by drastic changes in cell shape and size. This involves major reorganization of the cytoskeleton, in particular, the actin network. Recent years have provided new insights into both the regulation of actin organization during chondrogenesis and into the downstream mechanisms connecting actin dynamics to chondrocyte gene expression which is addressed by Chap. 11.

Bringing together international experts from diverse fields of musculoskeletal research was a demanding task requiring patience and persistence. For that we are very grateful to our authors of this volume who managed to complete their chapters on time and who dedicated their spare free time to writing their reviews.

Regensburg, Germany — Susanne Grässel
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Cartilage Proteoglycans

1

Anders Aspberg

Abstract

Proteoglycans are key components of the cartilage extracellular matrix and essential for normal tissue function. The core protein and the glycosaminoglycan chains both contribute to function and provide different properties of the individual proteoglycans. This review is focused on the two main families of cartilage proteoglycans.

The first of these is the lectican family, including aggrecan, versican, and the cartilage link protein. The aggregating proteoglycan network formed by aggrecan, link protein, and hyaluronan provides biomechanical properties that give the tissue its ability to withstand and distribute load.

The second group discussed is the small leucine-rich repeat proteoglycan family, which includes decorin, biglycan, asporin, fibromodulin, lumican, keratocan, osteoadherin, proline-/arginine-rich end leucine-rich repeat protein, epiphycan, mimecan, opticin, chondroadherin, and chondroadherin-like. These proteoglycans bind collagens and are important regulators of cartilage extracellular matrix assembly. In addition, some of these proteoglycans bind and regulate growth factors and their receptors and regulate innate immunity through interactions with Toll-like receptors or the complement system.

This review will give an overview of the structure and function of the different aggregating proteoglycans and small leucine-rich repeat proteoglycans in normal cartilage extracellular matrix.

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1.1 Introduction

Articular cartilage function depends on the molecular composition and organization of its extracellular matrix (ECM). This complex protein network fills the space between the cells and provides a structural scaffold, giving the tissue its unique biomechanical properties.

Disturbed cartilage ECM composition or organization, either by failure to produce and assemble the ECM building blocks or dysregulated ECM degradation, is a key factor in the development of joint disease such as osteoarthritis.

The components of cartilage ECM are usually grouped into proteoglycans, collagens, and non-collagenous proteins, each providing specific functionalities to the composite ECM material. This chapter will give an overview of cartilage proteoglycans, while the latter molecular classes will be discussed in Chaps. 2 and 3, respectively.

1.2 Proteoglycans

A proteoglycan is a protein posttranslationally modified with one or several glycosaminoglycan (GAG) chains, a type of linear carbohydrate polymers. The GAG chains are composed of repeating disaccharide units, with different specific disaccharides used in the different types of GAGs: hyaluronan, chondroitin/dermatan sulfate (CS/DS), heparan sulfate (HS)/heparin, and keratan sulfate (KS). The proteoglycan-forming GAGs CS/DS and HS are attached to the core protein by linkage to serine residues in Ser-Gly sequence motifs through a specific tetrasaccharide linker. Keratan sulfate is either O-linked (KS type II) to serine or threonine residues or N-linked (KS type I). Unlike other GAGs, hyaluronan is not attached to a protein core but is extruded into the extracellular environment by transmembrane hyaluronan synthases. Further variation and specificity in GAG structure are achieved through sulfation at different positions of the individual disaccharide units and through epimerization of uronic acid residues in DS and HS. The cellular synthesis of GAGs is complex and not yet entirely understood, with a large number of different enzymes involved in producing and modifying the GAG chains. The details of structural variation and synthesis of GAGs are beyond the scope of this chapter and have been the subject of many excellent recent reviews; see, for example, (Mikami and Kitagawa 2013).

In cartilage, a key function of proteoglycan is to provide swelling pressure, allowing the tissue to take up and distribute mechanical load. This is achieved by the aggrecan-hyaluronan matrix (see below). Other cartilage proteoglycans play vital roles in guiding the ECM assembly, functioning as tissue reservoirs for soluble factors or as cell surface receptors. Additional functions of proteoglycans include regulating the innate immune system through interaction with complement components and Toll-like receptors (TLRs), which may lead to an inflammatory response and contribute to osteoarthritis pathogenesis (Orlowsky and Kraus 2015).

All proteoglycans found in cartilage are present in other tissues as well. Even aggrecan, the quintessential cartilage proteoglycan is prominent in the central nervous system ECM. This chapter will give an overview of the structure and function of the main proteoglycans of cartilage, including some functional information gained from studies in other tissues. Other proteoglycans with important functions are present in cartilage, including the basement membrane proteoglycan perlecan and cell surface proteoglycans such as syndecans. In addition, several part-time proteoglycans such as lubricin (proteoglycan 4) are important for cartilage and joint function. For space reasons, these are not discussed, and this chapter is focused on the two main extracellular matrix proteoglycan groups – the aggregating proteoglycan network and small leucine-rich repeat proteoglycans (SLRPs).

1.3 The Aggregating Proteoglycan Network

The large aggregating cartilage proteoglycan aggrecan is perhaps the most studied of all proteoglycans. Aggrecan, together with versican, neurocan, and brevican, forms the lectican family (Ruoslahti 1996), also known as hyalectins. These proteoglycans have an elongated core protein carrying CS and KS chains, with globular interaction domains at the N- and C-termini (Fig. 1.1). The N-terminal G1 domain interacts with hyaluronan and the hyaluronan-proteoglycan link proteins. The C-terminal G3 domain binds multimeric ECM molecules such as tenascins and fibulins. The result of these interactions is that the PG is organized into an enormous molecular network, by G1 hyaluronan anchorage and G3 cross-linking.

Aggrecan and cartilage link protein are fundamental for cartilage function. Versican is also found in cartilage and may play a role during development, whereas neurocan and brevican are only found in the nervous system.

1.3.1 Aggrecan

The aggrecan domain structure was first revealed by molecular electron microscopy studies, showing an N-terminal G1 domain separated from a second globular domain (G2) by a short interglobular domain, an elongated domain carrying KS and CS chains, and a C-terminal globular G3 domain (Fig. 1.1). Subsequent sequencing of the aggrecan cDNA identified the structural protein repeats forming the globular domains and the homologies between the G1 and G2 domains with proteoglycan hyaluronan link protein (LP) and between the G3 domain and selectins; see (Aspberg 2012) for references.

The 250 kDa human aggrecan core protein carries approximately 100 CS chains and multiple additional KS chains, resulting in a molecular weight of roughly 2.5 MDa for the proteoglycan. The CS chains are attached to serine residues in Ser-Gly motif containing repeats in the CS-attachment domain. Keratan sulfate chains are O-linked to serine residues in the region between the G2 domain and the CS-attachment region. These KS chains have been shown to interact with fibrillar

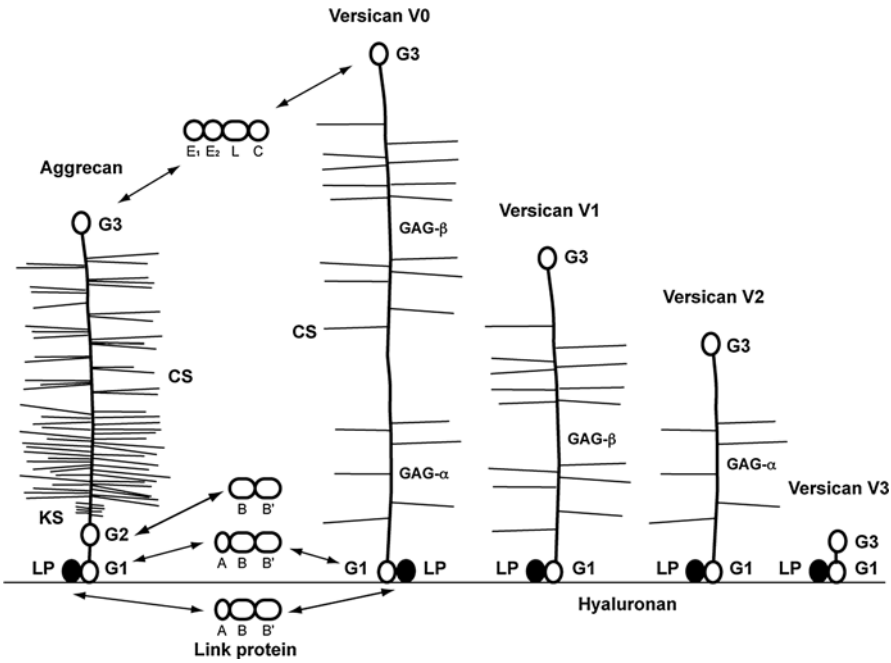

Fig. 1.1 The aggregating proteoglycans of cartilage. The figure shows aggrecan and the four splice forms of versican (*V0 to V3*) bound to hyaluronan in ternary complexes with cartilage link protein (*LP*). The subunit structure of the core protein globular domains is indicated. The G1 domain and link protein contain Ig-like repeat (*A*) and proteoglycan tandem repeats (*B, B′*); the latter also found in the G2 domain. The G3 domain consists of two EGF-like repeats (*E1, E2*), a C-type lectin repeat (*C*), and a complement regulatory protein-like repeat (*C*). Chondroitin sulfate (*CS*) and keratan sulfate (*KS*) glycosaminoglycans are indicated, and the alternatively spliced CS substituted domains of versican are labeled GAG-α and GAG-β

collagen (Hedlund et al. 1999). Interestingly, this region is polymorphic in human, with varying number of KS attachment repeats. Other O- and N-linked KS glycans are present in the interglobular domain (Barry et al. 1995). Taken together, each aggrecan molecule carries about 10,000 fixed negative charges in the form of carboxyl and sulfate groups of the CS chains.

The name aggrecan is derived from this proteoglycan's ability to form large aggregates with hyaluronan in the extracellular matrix. Seminal work by Sajdera, Hascall, Heinegård, and Hardingham clarified the mechanism of aggregation and formation of the supramolecular complexes with hyaluronan and link protein, as reviewed in (Heinegård 2009). In addition, interactions of the G3 domain with multimeric ECM proteins such as tenascins and fibulins allow complex formation through the C-terminal end of the proteoglycan (Aspberg 2012).

The formation of the aggrecan-hyaluronan network results in the immobilization of a vast number of negative charges in the tissue. By attracting counterions, this results in the formation of a Donnan equilibrium; thus, by osmotic processes, the PG draws water into cartilage tissue. This provides the biomechanical properties

critical for articular cartilage function, namely, the ability to absorb and distribute mechanical load over the joint surface. Recent technical advances in atomic force microscopy have allowed detailed analysis of the nanomechanical properties of cartilage microdomains. This has emphasized corresponding changes in aggrecan GAG chain; for review, see (Han et al. 2011)

The vital importance of aggrecan for cartilage development and function is emphasized by the identification of a variety of mutations in the aggrecan gene or affecting aggrecan posttranslational modification; see (Aspberg 2012) for review. Aggrecan null or functional null mutations have been described in a number of species, including humans. These dominant mutations result in skeletal dysplasia in heterozygous individuals and animals, whereas homozygosity for null mutation results in perinatal lethality in mice, cattle, and chicken. Missense mutations in the aggrecan G3 domain impair interactions with other ECM proteins, with clinical phenotypes ranging from short stature and osteochondritis dissecans with early-onset osteoarthritis to severe chondrodysplasia (Nilsson et al. 2014; Stattin et al. 2010; Tompson et al. 2009). A third group of mutations affect glycosyltransferases and transporter enzymes involved in GAG synthesis and sulfation. When homozygous, these mutations result in a spectrum of phenotypes ranging from short stature to lethal chondrodysplasia.

Aggrecan turnover in the tissue is relatively fast, with a reported half-life of 3.4 years for the full-length proteoglycan (Maroudas et al. 1998). Aggrecan degradation is achieved through matrix metalloproteinases (MMPs), aggrecanases, and other proteases (Troeberg and Nagase 2012). In mature cartilage, a mixture of truncated aggrecan fragments is found, reflecting cleavage at progressively more N-terminal sites and gradual loss of the more C-terminal fragments of the proteoglycan. Thus, G1 domain concentration is higher than G3 concentration, and G1 half-life in the tissue is around 24 years (Maroudas et al. 1998; Verzijl et al. 2001).

A key step in osteoarthritis disease progression is the loss of aggrecan by metalloproteinase and aggrecanase degradation. Aggrecan degradation occurs relatively early in the disease progression, and proteoglycan loss exposes the collagen fibrils to degradation. It is now becoming clear that the aggrecan fragments released by proteolysis have biological activities in regulating innate immune responses. Thus, the aggrecan G3 domain binds complement factors C1q and C3 and activates the classical and alternative complement pathways (Melin Fürst et al. 2013). Furthermore, the 32mer aggrecan fragment released by MMP and aggrecanase cleavage between the G1 and G2 domain activates TLR2 (Lees et al. 2015). Such fragments may thus maintain inflammatory processes and lead to further cartilage degradation.

1.3.2 Link Protein

The cartilage link protein is not a proteoglycan in itself but critical for cartilage proteoglycan function. It forms a ternary complex with hyaluronan and aggrecan G1 domain, stabilizing the proteoglycan aggregate (Heinegård and Hascall 1974;

Hardingham 1979). Interestingly, the link protein and the G1 domain are homologous, sharing a domain organization with an immunoglobulin-like domain followed by two proteoglycan tandem repeats (Deak et al. 1986; Doege et al. 1986, 1987; Neame et al. 1986). Four different members of the link protein family have been identified, but of these, only the classical cartilage link protein appears to be present in cartilage (Spicer et al. 2003). Mice lacking link protein showed perinatal lethality due to respiratory problems, disorganized growth plates, and shortened limbs, a phenotype similar to but milder than the aggrecan-deficient cmd mice (Watanabe and Yamada 1999). Interestingly, an N-terminal 16 amino acid long peptide, released from cartilage link protein by proteolysis, binds bone morphogenetic protein (BMP)-receptor type II and stimulates aggrecan and collagen production in chondrocytes (Wang et al. 2013; Liu et al. 2000; McKenna et al. 1998).

1.3.3 Versican

Versican is a widely expressed lectican. Although lacking the G2 domain, the overall molecular organization of versican is similar to that of aggrecan (Fig. 1.1), with an amino-terminal G1 domain that binds hyaluronan and link proteins, a central region carrying up to 23 CS chains, and a C-terminal G3 domain that binds multimeric ECM proteins like tenascins, fibulins, and fibrillin (Aspberg 2012; Zimmermann and Ruoslahti 1989; LeBaron et al. 1992). The CS region is encoded on two large exons, and alternative splicing gives rise to four different splice variants with both (V0), either (V1, V2), or none (V3) of these exons (Dours-Zimmermann and Zimmermann 1994).

Versican is important in the condensing mesenchyme preceding the formation of cartilage bone anlagen during skeletal development (Williams et al. 2005). In mice, versican was found present in the interterritorial matrix of the growth plate, at the sites of joint formation and superficially in articular cartilage (Choocheep et al. 2010; Matsumoto et al. 2006). Conditional versican gene targeting interfered with joint formation in a transforming growth factor (TGF)-β dependent mechanism (Choocheep et al. 2010). In adult human tissues, versican was found to be present in elastic and fibrous cartilage, but was not detectable in hyaline cartilage (Bode-Lesniewska et al. 1996). More recent quantitative proteomic studies show versican presence in a number of different human hyaline cartilages, with a preferentially superficial location in articular cartilage (Müller et al. 2014; Önnerfjord et al. 2012). In osteoarthritic cartilage, versican was upregulated and showed pericellular localization in chondrocyte clusters (Nishida et al. 1994; Cs-Szabo et al. 1997).

Versican activates myeloid cells in a TLR2 dependent manner, although the details of the interaction remain unclear (Kim et al. 2009). Furthermore, proteolytic cleavage of versican has been shown to produce a bioactive G1-containing fragment regulating interdigital web apoptosis (McCulloch et al. 2009; Nandadasa et al. 2014).

1.4 Small Leucine-Rich Repeat Proteoglycans (SLRPs)

The SLRPs are small ECM proteoglycans or glycoproteins of the leucine-rich repeat (LRR) protein superfamily (Fig. 1.2). They are characterized by a central region of LRR repeats, which is stabilized by N-terminal and C-terminal cysteine bridges. All SLRPs contain an N-terminal LRRNT cysteine knot motif with two disulfide bonds. The canonical SLRPs (see below) have a characteristic LRRCE motif, whereas noncanonical SLRPs have the LRRCT motif shared by many other types of LRR proteins (Park et al. 2008). Additional N-terminal or C-terminal protein extensions provide unique properties to the different SLRPs. Based on their primary structure, the SLRPs can be divided into five classes. Classes I to III constitute the canonical SLRPs and class IV and V the noncanonical SLRPs (Park et al. 2008; Iozzo and Schaefer 2015). Class V SLRPs have not been detected in cartilage and will not be discussed further in this chapter.

Many, if not all, SLRPs bind collagen, and some, like decorin, remain bound to the mature collagen fibrils. The SLRPs are in fact important regulators of collagen fibril assembly. Different SLRPs bind at different positions along the forming fibril and regulate the rate of polymerization as well as the diameter of the final fibril (Kalamajski and Oldberg 2010). Positioned at the fibril surfaces, the SLRPs can interact with other proteins present in the ECM. This allows them to function as linkers between different fibrils, and other supramolecular assemblies, as exemplified by biglycan bridging between collagen type II and type VI fibrils. Furthermore, SLRPs bound to collagen fibrils can protect the fibril against collagenase degradation (Geng et al. 2006). The functions of SLRPs are not limited to providing and regulating ECM structure. Thus, many SLRPs bind and regulate the activity of growth factors, or their receptors. Furthermore, some SLRPs, or SLRP fragments, regulate innate immunity through interaction with TLRs or the complement system.

1.4.1 Class I SLRPs

Class I SLRPs include decorin, biglycan, and asporin. Apart from asporin, these have an N-terminal extension carrying DS chain(s). They are synthesized as preproteins, with a propeptide of unknown function, and have the LRRNT cysteine knot motif, 12 LRR repeats, and the LRRCE-terminating cysteine bridge motif. Interestingly, the propeptides of biglycan and decorin are cleaved by the same bone morphogenetic protein-1/mTolloid proteases that remove fibrillar collagen propeptides (Scott et al. 2000; von Marschall and Fisher 2010).

Decorin is widely expressed and is present in cartilage, with one DS chain on its N-terminal extension (Krusius and Ruoslahti 1986; Rosenberg et al. 1985). It was named in reference to its appearance in electron micrographs, where it appears to decorate the surface of collagen fibrils (Ruoslahti 1988). Decorin also modulates collagen fibril assembly in vitro, resulting in slower fibrillar assembly (Vogel et al. 1984). Gene targeting of decorin led to disorganized collagen fibrils with irregular fibrillar diameters. This resulted in mechanically weaker skin of the mutant mice

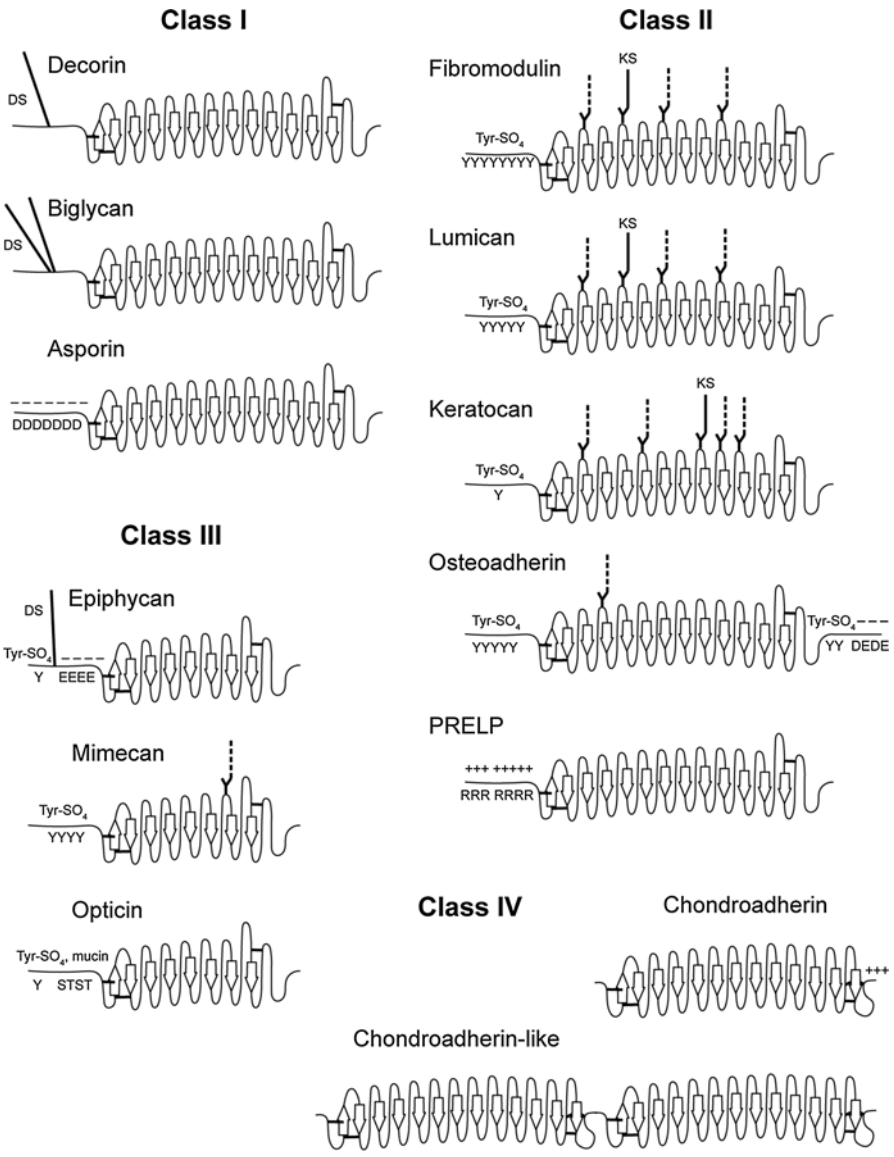

Fig. 1.2 Cartilage small leucine-rich repeat proteoglycans (SLRPs). The figure shows a generalized overview of the cartilage SLRPs grouped according to class (*I to IV*). Important structural properties are illustrated on a schematic core protein structure. Acidic and basic amino acid residue clusters providing negative or positive charges as well as dermatan sulfate (*DS*), keratan sulfate (*KS*), mucin-type O-glycosylation, and tyrosine O-sulfation (Tyr-SO4) posttranslational modifications are indicated. For details, see the main text

(Danielson et al. 1997). Decorin acts as a growth suppressor by interacting with TGF-β (Yamaguchi et al. 1990) and a number of different growth factor receptors; see (Iozzo and Schaefer 2015) for a recent review.

The structure of decorin was the first SLRP structure to be determined by X-ray crystal diffraction, which showed a curved solenoid structure with its concave face formed by a beta sheet and its convex face formed by loops and helices (Scott et al. 2004). This study also showed that decorin can form homodimers, which had been suggested from biophysical studies on decorin in solution (Scott et al. 2003) and was confirmed in later studies (Scott et al. 2006). The dimers formed by antiparallel binding of the concave surfaces of the monomers, which was difficult to reconcile with collagen binding through these surfaces. Although sites on the decorin monomer mediating interaction with collagen (Kalamajski et al. 2007; Svensson et al. 1995), as well as binding sites for decorin along the collagen molecule and fibril (Scott and Orford 1981), have been identified, the details of decorin binding to collagen remain unclear. Further mutational and biophysical studies have shown that decorin in solution exists in a monomer/dimer equilibrium and suggest that decorin binds collagen as a monomer through its concave face (Islam et al. 2013). Indeed, molecular modeling studies support decorin monomer binding across some four collagen triple helices on the fibril surface (Orgel et al. 2009).

Biglycan, as the name implies, carries two DS chains (Fisher et al. 1989). Biglycan is prominent in cartilage (Rosenberg et al. 1985) but is also found in other connective tissues, notably tendon and bone, where it was first identified (Fisher et al. 1983). Biglycan can form dimers in solution, and its crystal structure showed strong similarities with decorin, although in biglycan the monomers were somewhat more curved than decorin (Scott et al. 2006).

Like decorin, biglycan binds fibrillar collagens. Apart from fibrillar collagens, biglycan also binds collagen type VI, organizes this into a hexagonal network, and links collagen type VI molecules with type II fibrils (Wiberg et al. 2001, 2002, 2003).

Mice lacking biglycan show a bone phenotype similar to osteoporosis (Xu et al. 1998), with altered collagen fibril formation (Corsi et al. 2002), affecting bone fracture healing (Berendsen et al. 2014). Compound knockouts with other SLRP genes show additional and more pronounced phenotypes, such as increased skin fragility in decorin-biglycan double-knockout mice (Corsi et al. 2002) and increased osteoarthritis in fibromodulin-biglycan double-knockout mice (Ameye et al. 2002).

Biglycan interacts with TGF-β, BMP-4, and vascular endothelial growth factor (VEGF) and modulates Wnt signaling, osteoblast differentiation, and angiogenesis (Nastase et al. 2012; Berendsen et al. 2014). Biglycan has been shown to activate TLR2 and TLR4 signaling and thus regulate innate immunity (Schaefer et al. 2005). This suggests a function as a tissue-derived damage-associated molecular pattern, perhaps involved in driving inflammation in the joint. On the other hand, biglycan and decorin both bind C1q and inhibit complement activation (Sjöberg et al. 2009; Groeneveld et al. 2005; Krumdieck et al. 1992).

Asporin, the third member of the class I SLRPs, is actually not a proteoglycan. Instead, the N-terminal extension, where GAG chains are attached on decorin and

biglycan, shows a sequence of consecutive aspartate residues, providing a cluster of negative charges (Henry et al. 2001; Lorenzo et al. 2001). Although first identified in cartilage, asporin is in fact widely expressed. Asporin binds collagen through its LRR domain, competing for the same binding sites as decorin, and inhibits collagen fibril formation in vitro (Kalamajski et al. 2009). The asporin N-terminal polyaspartate sequence binds calcium, and asporin is a potent inducer of collagen mineralization (Kalamajski et al. 2009).

Asporin binds TGF-β (Kizawa et al. 2005; Nakajima et al. 2007; Kou et al. 2010) and BMP-2 and inhibits their actions (Tomoeda et al. 2008) while binding fibroblast growth factor (FGF)-2 and positively regulating its activity (Awata et al. 2015). In addition, recent studies in *Xenopus* show that asporin interacts with the insulin-like growth factor (IGF) receptor and activates IGF signaling while perturbing BMP, Wnt, and activin signaling (Luehders et al. 2015). Asporin also appears to be able to regulate innate immunity by binding TLR2 and TLR4 and decreasing cytokine release in response to lipopolysaccharide ligands (Yamaba et al. 2015).

No asporin knockout mouse has yet been published. Interestingly, the human asporin gene is polymorphic, with variable lengths of the polyaspartate stretch (Lorenzo et al. 2001). Genetic studies have reported an association of one of these alleles (D14) with risk for knee osteoarthritis and lumbar disk degeneration (Kizawa et al. 2005; Nakamura et al. 2007; Song et al. 2008), although this appears to be limited to specific Asian populations (Xing et al. 2013; Song et al. 2014).

1.4.2 Class II SLRPs

The class II SLRPs include fibromodulin, lumican, keratocan, osteoadherin, and proline/arginine-rich end LRR protein (PRELP). These SLRPs contain a twelve LRR core flanked by LRRNT and LRRCE motifs, carry KS chains on their LRR domain, and are often tyrosine O-sulfated.

Fibromodulin was first purified from cartilage (Heinegård et al. 1986) but is expressed in many connective tissues, including the tendon and skin (Oldberg et al. 1989). Cartilage fibromodulin carries up to four N-linked KS chains on its LRR domain (Plaas et al. 1990; Oldberg et al. 1989), and its N-terminal extension contains a number of tyrosine residues that can be modified by O-sulfation (Antonsson et al. 1991). Fibromodulin binds collagen fibrils at the gap region (Hedlund et al. 1994) and inhibits collagen fibril formation in vitro (Hedbom and Heinegård 1989). The fibromodulin knockout mouse showed irregular collagen fibrils and lower mechanical strength of tendons (Svensson et al. 1999). Interestingly, osteoarthritis incidence was increased in the fibromodulin knockout mouse, but whether this depends directly on changes in cartilage or on altered joint loading pattern due to changes in ligaments remains unclear (Gill et al. 2002). Both in tendons and cartilage of the fibromodulin knockout mouse, levels of lumican protein, another class II SLRP, showed compensatory upregulation. Lumican and fibromodulin were found to bind the same site on collagen type I, which is different from the decorin binding site (Svensson et al. 2000). Indeed, the collagen binding sites on fibromodulin and

lumican show homologous sequence (Kalamajski and Oldberg 2009). Recently, collagen type I cross-linking was found to be dysregulated in fibromodulin knockout animals (Kalamajski et al. 2014).

Fibromodulin binds complement C1q and is an activator of the classical complement pathway (Sjöberg et al. 2005) while also binding the complement inhibitors factor H and C4b-binding protein (Happonen et al. 2009; Sjöberg et al. 2007).

The N-terminal extension of fibromodulin is posttranslationally modified with up to nine O-sulfated tyrosine residues (Önnerfjord et al. 2004). This part of the protein mimics heparin and binds several growth factors and cytokines (Tillgren et al. 2009). The tyrosine O-sulfated domain can be released from the protein through MMP-13 proteolysis (Heathfield et al. 2004).

The expression of osteoadherin (Sommarin et al. 1998; Wendel et al. 1998), sometimes called osteomodulin, was initially thought to be restricted to mineralized tissues. In developing bone, osteoadherin is found in the primary spongiosa, enriched at the border between bone and hypertrophic cartilage (Sugars et al. 2013; Wendel et al. 1998). Quantitative proteomic studies have now clearly demonstrated osteoadherin presence also in articular cartilages (Önnerfjord et al. 2012; Wilson et al. 2012). Osteoadherin is a KS proteoglycan in the bone (Wendel et al. 1998), but whether this is the case in cartilage remains unclear. Like other SLRPs, osteoadherin binds collagen and affects collagen fibril formation (Tashima et al. 2015). Osteoblasts adhere to osteoadherin through integrin αvβ3 receptors (Wendel et al. 1998). Like fibromodulin, osteoadherin contains O-sulfated tyrosine residues in its N-terminal extension (Önnerfjord et al. 2004), which mediates interactions with heparin-binding motifs in other ECM components as well as with bioactive factors such as oncostatin M (Tillgren et al. 2009). Uniquely, osteoadherin also contains a C-terminal extension rich in aspartate and glutamate residues, which likely mediates osteoadherin interaction with hydroxyapatite (Sommarin et al. 1998).

In cartilage, the corneal KS proteoglycans lumican and keratocan exist as glycoproteins, carrying short, non-sulfated poly-(N-acetyllactosamine) chains (Corpuz et al. 1996; Rees et al. 2009). Likewise, although bovine cartilage fibromodulin is a KS proteoglycan (Oldberg et al. EMBO 1989), lumican and fibromodulin from human juvenile articular cartilage carry KS chains but appear to gradually shift to glycoprotein forms with age (Grover et al. 1995; Roughley et al. 1996). Like fibromodulin and osteoadherin, lumican and probably keratocan contain sulfated tyrosine residues in their N-terminal extensions (Corpuz et al. 1996; Önnerfjord et al. 2004). The functions of lumican and keratocan in cartilage have not been studied in detail, but likely overlap with those of other SLRPs. As mentioned above, lumican and fibromodulin have homologous collagen binding sequences in their LRR domains and bind the same site on collagen, and lumican is upregulated in fibromodulin knockout cartilage. Mice lacking lumican or keratocan also show abnormal corneal collagen fibril assembly and organization (Chakravarti et al. 1998; Liu et al. 2003). Lumican interacts with CD14, promoting TLR4-mediated innate immunity (Wu et al. 2007).

The SLRP PRELP, or prolargin, was first identified in cartilage (Heinegård et al. 1986) but is present in many connective tissues. PRELP is a glycoprotein rather than a proteoglycan, although short polylactosamine chains that may be sulfated to some

degree are present on cartilage PRELP (Bengtsson et al. 1995). Unlike other SLRPs, the atypical N-terminal extension of PRELP contains a number of arginine and proline residues, providing a cluster of positive charges (Bengtsson et al. 1995; Grover and Roughley 1998, 2001). This domain binds heparin and heparan sulfate proteoglycans in vitro (Bengtsson et al. 2000), whereas the PRELP LRR domain binds collagen (Bengtsson et al. 2002). In many tissues, PRELP is located adjacent to basement membranes, and as it binds the basement membrane HSPG perlecan, it may be involved in connecting the BM to the loose connective tissue (Bengtsson et al. 2002). The PRELP N-terminal peptide shows interesting bioactive properties. This peptide binds cell surface PGs on preosteoclasts, is internalized by the cells, and counteracts osteoclast differentiation (Rucci et al. 2009). In experiments in mice, the peptide showed efficacy in preventing bone mineral loss (Rucci et al. 2013). The PRELP heparin-binding peptide also shows antimicrobial activity (Malmsten et al. 2006, 2011).

1.4.3 Class III SLRPs

The class III SLRPs epiphycan, mimecan, and opticin are smaller than class I or class II SLRPs, with only seven LRRs, flanked by LRRNT and LRRCE cysteine motifs, and carry DS or KS chains.

Epiphycan (Johnson et al. 1997), also known as dermatan sulfate proteoglycan 3 (Deere et al. 1996) or Pg-Lb (Kurita et al. 1996), is expressed in the growth plate and articular cartilage of developing bones (Johnson et al. 1997). The protein levels in cartilage decrease with maturation (Wilson et al. 2012), and quantitative proteomic investigations of adult human tissues show epiphycan in rib and tracheal cartilages, but not in articular cartilages (Önnerfjord et al. 2012). This proteoglycan carries a DS chain in its N-terminal region. Mice lacking epiphycan appear normal at birth but fall behind wild-type controls in postnatal body weight and bone length growth (Nuka et al. 2010). The epiphycan-deficient mice also develop spontaneous osteoarthritis. In epiphycan/biglycan double-knockout mice, the phenotype is aggravated (Nuka et al. 2010).

Mimecan, also referred to as osteoglycin, is widely expressed in connective tissues, including cartilages. In cornea, it carries a KS chain on its N-terminal extension, whereas mimecan from other tissues appears to be glycoproteins with unsulfated oligolactosamine glycans (Funderburgh et al. 1997). Proteomic investigations have verified mimecan presence in various different cartilages (Önnerfjord et al. 2012), although its function in these tissues remains unclear. Mice lacking mimecan show no overt phenotype but have decreased skin tensile strength and increased skin collagen fibril diameter (Tasheva et al. 2002).

Opticin was identified in the eye (Friedman et al. 2000; Hobby et al. 2000; Reardon et al. 2000) and has been reported present in cartilage (Monfort et al. 2008; Tio et al. 2014). Unlike other SLRPs, opticin contains an N-terminal mucin-like domain carrying up to 16 sialylated O-linked glycans (Reardon et al. 2000). Opticin likely binds collagen as it was purified from vitreous collagen fibril preparations (Reardon et al. 2000) and also binds HS and CS chains (Hindson et al. 2005).

1.4.4 Class IV SLRPs

The class IV SLRPs chondroadherin and chondroadherin-like diverge from the canonical SLRPs in their LRR sequence and have LRRCT C-terminal cysteine motifs instead of the LRRCE of canonical SLRPs (Park et al. 2008). Class IV SLRPs do not carry GAG chains but nevertheless have important functions in the cartilage ECM.

Chondroadherin was identified as a cartilage ECM protein mediating cell adhesion (Sommarin et al. 1989). Chondroadherin has no N-terminal extension but contains a unique C-terminal end with an additional disulfide bond, a heparin-binding sequence, and a C-terminal loop structure (Neame et al. 1994). Chondroadherin binds fibrillar collagens (Månsson et al. 2001). In addition, chondroadherin is an integrin ligand (Camper et al. 1997), binding integrin α2β1 through the amino acid sequence WLEAK in its C-terminal loop structure (Haglund et al. 2011). Interestingly, the chondroadherin heparin-binding sequence is a syndecan ligand, and chondroadherin interaction with syndecan and integrin in concert is required for firm attachment, cell spreading, and formation of focal adhesion structures (Haglund et al. 2013). The heparin-binding sequence can be released from chondroadherin by proteolysis (Neame et al. 1994). Synthetic cyclic peptides corresponding to the chondroadherin integrin binding site have been shown to inhibit osteoclastogenesis and bone resorption (Capulli et al. 2014) and to inhibit bone metastasis of breast cancer cells (Rucci et al. 2015).

Mice lacking chondroadherin were viable and fertile without macroscopic phenotype, but the chondroadherin knockout mice showed thinner cortical bone, changed trabecular bone architecture, and decreased bone mechanical strength, and proteomic analysis revealed disturbed cartilage homeostasis (Hessle et al. 2013). Further nanomechanical analysis showed significant changes in the superficial zone cartilage of chondroadherin deficient mice (Batista et al. 2014).

Chondroadherin-like, a novel SLRP expressed in cartilage, was recently characterized (Tillgren et al. 2015). This atypical SLRP has a tandem arrangement of two LRRNT and LRRCT flanked LRR domains with homology to chondroadherin, linked by an arginine and proline-rich linker. Chondroadherin-like binds collagen and inhibits collagen fibril formation. In contrast to chondroadherin, chondrocytes do not adhere to chondroadherin-like. Chondroadherin-like appears to be involved in the regulation of chondrogenic differentiation. It is expressed in the cartilage anlagen and growth plate, and chondroadherin-like knockdown accelerated ATDC5 chondroprogenitor cell differentiation.

1.5 Conclusions and Perspectives

Detailed molecular and biomechanical studies combined with gene-targeted animal models have steadily improved our understanding of proteoglycan functions as structural components of the ECM, in regulation of ECM assembly, and as regulators of different growth factor systems. A growing number of studies now show that

proteolytic fragments of proteoglycans have bioactive properties. Proteoglycans present in cartilage also act as regulators of innate immunity through TLR and complement interactions and thus can regulate inflammatory responses in the joint and osteoarthritis progression.

Technological advances in mass spectrometry and proteomics are rapidly improving our ability to detect and quantify proteins including proteoglycans. This will allow quantitative study of spatiotemporal distributions and possibly interactions of proteoglycans in different tissue compartments, during development and in disease progression. The molecular architecture of cartilage is surprisingly complex, with different cellularity and collagen fibril dimensions and orientation at different depths from the joint surface and with the ECM organized into pericellular, territorial, and interterritorial zones around each chondrocyte (Heinegård and Saxne 2011). The SLRPs are key regulators of collagen matrix assembly, and determining the regional ECM composition will help to clarify the combinatorial effects of different regulators on collagen fibril formation. Proteomic approaches also allow identification of proteolytic cleavage sites and neoepitopes resulting from tissue degradation, as well as protein fragments that may show bioactivity. Indeed, novel cartilage proteoglycans or part-time proteoglycans may even be identified (Noborn et al. 2015). In parallel, genetic studies will likely reveal novel proteoglycan gene variants with linkage to hereditary skeletal disorders, which may be instructive in elucidating normal function as well as disease mechanisms in common with nonhereditary joint disorders. Taken together, the understanding of cartilage proteoglycan function in ECM molecular organization, proteoglycan turnover, and function as signaling molecules regulating, e.g., innate immunity, can be expected to increase substantially in the near future. This will most likely provide novel diagnostic tools and therapeutic targets in joint disease.

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