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Lumican is a major small leucine-rich proteoglycan (SLRP) in Atlantic cod (*Gadus morhua* L.) skeletal muscle

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Abstract Knowledge on fish matrix biology is important to ensure optimal fish -quality, -growth and -health in aquaculture. The aquaculture industry face major challenges related to matrix biology, such as inflammations and malformations. Atlantic cod skeletal muscle was investigated for collagen I, decorin, biglycan, and lumican expression and distribution by real-time PCR, immunohistochemical staining and Western blotting. Immunohistochemical staining and Western immunoblotting were also performed using antibodies against glycosaminoglycan side chains of these proteoglycans, in addition to fibromodulin. Real-time PCR showed highest mRNA expression of lumican and collagen I. Collagen I and proteoglycan immunohistochemical staining revealed distinct thread-like structures in the myocommata, with the exception of fibromodulin, which stained in dense structures embedded in the myocommata. Chondroitinase AC-generated epitopes stained more limited than cABC-generated epitopes, indicating a stronger presence of dermatan sulfate than chondroitin sulfate in cod muscle. Lumican and keratan sulfate distribution patterns were strong and ubiquitous in endomysia and myocommata. Western blots revealed similar SLRPs sizes in cod as are known from mammals. Staining of chondroitin/dermatan sulfate epitopes in Western blots were similar in molecular size to those of decorin and biglycan, whereas staining of keratan sulfate epitopes coincided with expected molecular sizes of lumican and fibromodulin. In conclusion, lumican was a major proteoglycan in cod muscle with ubiquitous distribution overlapping with keratan sulfate. Other leucine-rich proteoglycans were also present in cod muscle, and Western blot using antibodies developed for mammalian species showed cross reactivity with fish, demonstrating similar structures and molecular weights as in mammals.

Keywords Biglycan · Decorin · Fibromodulin · Fish · Lumican · Muscle

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Introduction

The extracellular matrix (ECM) is crucial for a series of processes taking place in cells and tissues of living organisms. Only a limited number of studies on proteoglycan expression in fish have been conducted, although the interest for using fish (*e.g.* zebrafish) in model systems is increasing. Proteoglycans such as the small, leucine-rich proteoglycans (SLRPs) may be significant contributors to fish health, growth and development as shown in zebrafish (*Danio rerio*) knock-out studies [1, 2]. The SLRPs may also influence fillet texture as they are major collagen-interacting molecules influencing collagen fibrillogenesis as well as being collagen network stabilizers [3]. Aquaculture is a fast-growing industry facing many biological and technological challenges. Cultivation of marine fish such as Atlantic cod (*Gadus morhua*) and the anadromous Atlantic salmon (*Salmo salar*) has potential for even further growth. Extensive research on the biology of these species is necessary to fully understand and prevent production-related problems and disease. Atlantic cod is a relatively

new species in fish farming and a product of somewhat variable quality due to complex factors [4, 5]. Pathologies of farmed fish, caused by parasitic, bacterial or viral infections, and consciousness of consumers and producers regarding production-related deformities, are constantly present. Thorough knowledge on fish matrix biology is important to increase understanding of the mechanisms causing these problems. The ECM composition changes with age, disease and pathological conditions, nutritional status and stress. Changes in ECM composition and turnover may induce dysfunctions in cell-matrix interactions and thus lead to diseases [6].

Macromolecules of the ECMs function as a scaffold for cells and structural components responsible for matrix assembly. Numerous studies have demonstrated that matrix components such as proteoglycans are involved in cell- and tissue development, homeostasis and regeneration [7–9]. Decorin, biglycan, fibromodulin and lumican are proteoglycans belonging to the SLRP family. They are characterized by several leucine-rich loops that fold into a helical structure, where the leucine-rich loops are thought to bend outward, facilitating collagen binding to the protein core [3]. The SLRPs carry glycosaminoglycan (GAG) chains of varying numbers and types. The GAG chains are important for proteoglycan effects on cells and tissues, due to their specific interactions with other proteins. The GAG substitution of the SLRPs may vary between species and tissues. In addition, these proteoglycans regulate collagen fibrillogenesis [10, 11], and SLRP knock-out studies have demonstrated key functions for tissue elasticity and fragility [12–14]. SLRPs may also influence tissue fibrosis through interactions with TGFβ, and regulatory mechanisms for key SLRPs during muscle formation and muscular dystrophy have been extensively studied [15]. Babelova *et al.* [16] and Iozzo and Schaefer [17] reported central roles for certain SLRPs, *e.g.* biglycan, in the regulation of inflammatory responses via macrophage activation. Moreover, Sjöberg *et al.* [18, 19] reported that several members of the SLRPs family were capable of complement activation and regulation, through binding of complement factor C1q.

Caged fish are subjected to several environmental, biological and mechanical stressors, such as handling, feeding and feed composition, infections, medication and vaccination. Moreover, fish subjected to marine aquaculture are prone to suffer from wounds as a result of mechanical stress, lice- and bacterial infections. The SLRPs, playing important roles in collagenesis, tissue repair, inflammation and fibrosis may thus be significant contributors to the textural properties as well as being health indicators in fish. To the best of our knowledge, limited data on the SLRPs expression in teleost fish species exist, with the exception of work on zebrafish [1, 2, 20]. Interestingly, evidence for the expression of certain SLRPs (*e.g.* decorin) in skin and bone tissue, on the mRNA level in Atlantic salmon (*Salmo salar*), has been presented by Skugor *et al.* [21] and Ytteborg *et al.* [22]. In the present study we report novel data on the molecular properties, expression and distribution patterns of major SLRPs in Atlantic cod skeletal muscle on both mRNA and protein levels, in addition to collagen I, which have not been studied on mRNA level in cod before.

Results

Expression of collagen I, SLRPs and their respective GAGs

The relative mRNA expression of SLRPs biglycan, decorin, lumican and collagen I was monitored by real-time PCR of RNA samples isolated from Atlantic cod white skeletal muscle. As shown in Fig. 1, a higher mRNA expression of collagen I, lumican and decorin compared to biglycan was detected. Among the SLRPs investigated on mRNA level, lumican was expressed strongest, and decorin was more highly expressed than biglycan.

The SLRP protein expression was examined by Western blotting using polyclonal antibodies against collagen I and the SLRPs biglycan, decorin, lumican and fibromodulin, in addition to monoclonal antibodies against GAG epitopes chondroitin-4-sulfate (C4S)/dermatan sulfate (DS) and keratan sulfate (KS). These experiments revealed the presence of decorin, biglycan, lumican and fibromodulin as shown in Fig. 2A. The SLRPs investigated had protein cores of varying sizes, between 35 and 50 kDa, as judged by migration in 10% bis-tris gels. Biglycan staining revealed a major band just below 50 kDa and two fainter bands of lower molecular sizes. Decorin staining resulted in

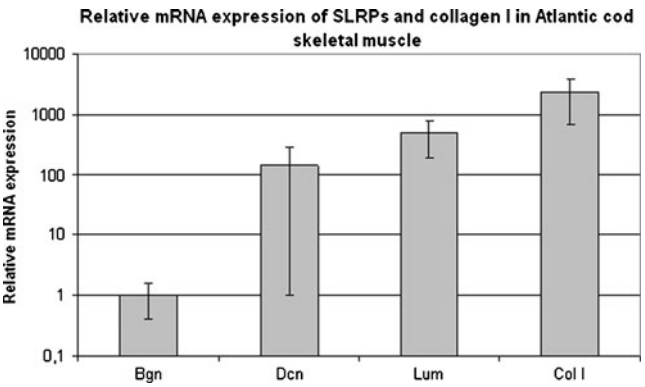

Fig. 1 Real-time PCR results showing the relative expression of three SLRPs and collagen I. Biglycan was the component with the weakest signal during the real-time PCR analysis, and delta delta CT values were for that reason related to the expression of this proteoglycan. Biglycan expression is set to 1 in this figure and the relative expression of decorin, lumican and collagen I compared to biglycan are shown on logarithmic scale. Bgn = biglycan, Dcn = decorin, Lum = lumican and Col I = collagen I. The figure is adjusted according to primer efficiency. Bars indicate STDEV

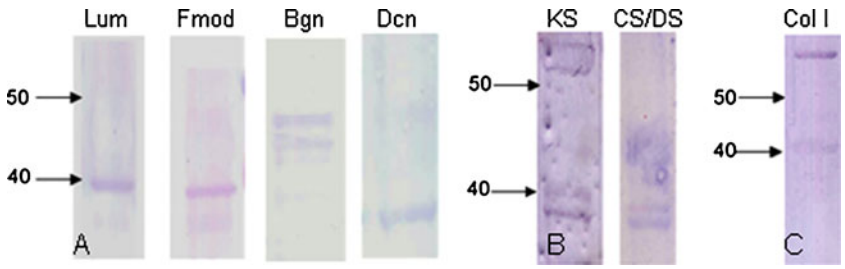

Fig. 2 Western blots of the SLRP protein cores of Dcn (decorin), Bgn (biglycan), Lum (lumican), Fmod (fibromodulin) (panel **A**), the sugar moieties KS and C4S/DS (panel **B**) and collagen I (Col I) (panel **C**). Molecular weights are indicated on the left side with arrows. Note the C4S/DS positive bands in the same areas as decorin and biglycan, as well as the positive KS bands between 40 and 50 kDa as well as just below 60 kDa, corresponding to expected molecular weights of lumican and fibromodulin

a distinct band with molecular weight between 35 and 40 kDa. Lumican staining resulted in the appearance of a single sharp band just below 40 kDa. In samples not subjected to keratanase treatment a faint band of the same size, reactive to the lumican antibody, could be seen, indicating the presence of small amounts of lumican with other glycosylations than KS substitution (results not shown). Fibromodulin staining also revealed a major band just below 40 kDa in addition to a minor band of somewhat lower size. Western immunoblotting to detect GAG epitopes, as shown in Fig. 2B, revealed that C4S/DS and KS proteoglycans of varying sizes were present between 35 and 50 kDa. Staining using the monoclonal antibody 2B6 against C4S/DS epitopes, revealed positive bands in decorin-, as well as biglycan-positive areas of the membranes. The monoclonal antibody 5D4 against highly sulfated KS resulted in bands appearing broader and with higher molecular weights, as expected, with a particularly strong band just below 60 kDa. Both C4S/DS and KS staining resulted in additional bands of higher molecular weights than what should be expected for the SLRPs (results not shown), indicating the presence of these GAG epitopes also on proteoglycans of larger size. Blotting with a polyclonal collagen I antibody, as shown in Fig. 2C, resulted in the appearance of two bands; a stronger band around 65 kDa and a weaker one around 40 kDa.

To investigate in further detail the GAG substitution of the different SLRPs, we immunoprecipitated each protein core and stained the membranes using CS/DS and KS antibodies as shown in Fig. 3. Indeed, the results showed positive staining of cABC-generated epitopes, assumed to be dermatan sulfate in both biglycan and decorin, due to no staining of cAC-generated epitopes representing CS. Moreover, both lumican and fibromodulin stained positive for KS.

Tissue expression and distribution of collagen I, SLRPs and their respective GAGs

The spatial distributions collagen I, SLRPs and GAG epitopes were investigated by using immunohistochemical (IHC) staining (Figs. 4, 5, 6, 7, 8, 9, 10 and 11). IHC staining of the corresponding proteins and GAG chains indicated that lumican was a major SLRP expressed in Atlantic cod muscle, but decorin was also strongly stained. This was in accordance with the real-time PCR results shown in Fig. 1. Biglycan antibody staining resulted in somewhat more moderate color development (Fig. 6), as did also fibromodulin (Fig. 7).

Lumican (Fig. 4) was widely distributed in the ECMs throughout the muscle sections in similar ways as collagen I (Fig. 11). Collagen I and lumican both showed ubiquitous distribution and strong staining reactions, revealing fibrillar structures in the myocommata. The staining patterns were also similar between these two ECM components. Lumican was stained strongly also in vessel walls. Decorin and biglycan (Figs. 5 and 6) were similar in their expression patterns and distributions, with distinct layers appearing as parallel oriented structures in the myocommata. Decorin was stained stronger in the endomysia and cell junctions than biglycan. Decorin also stained strongly around vessel walls. Fibromodulin (Fig. 7) showed a quite limited distribution in the cod muscle tissue, with very weak staining in endomysia. The strongest reaction for fibromodulin was seen in cell dense areas of the myocommata. cABC- and cAC digested sections were stained using the 2B6 antibody directed against C4S/DS epitopes. The staining of cABC-generated epitopes was stronger than cAC-generated epitopes as shown in Figs. 8 and 9, but the distribution of epitopes was quite similar after the two

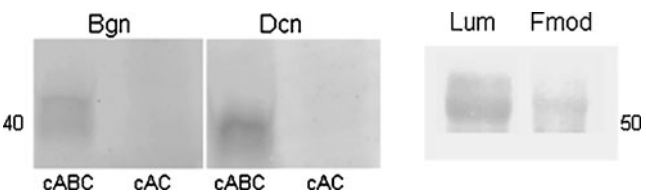

Fig. 3 Western blots of immunoprecipitated SLRP protein cores of biglycan (Bgn), decorin (Dcn), lumican (Lum) and fibromodulin (Fmod). The blots showed positive reactions for C4S only in the cABC-treated lane of biglycan and decorin. Lumican and fibromodulin both stained positive for KS

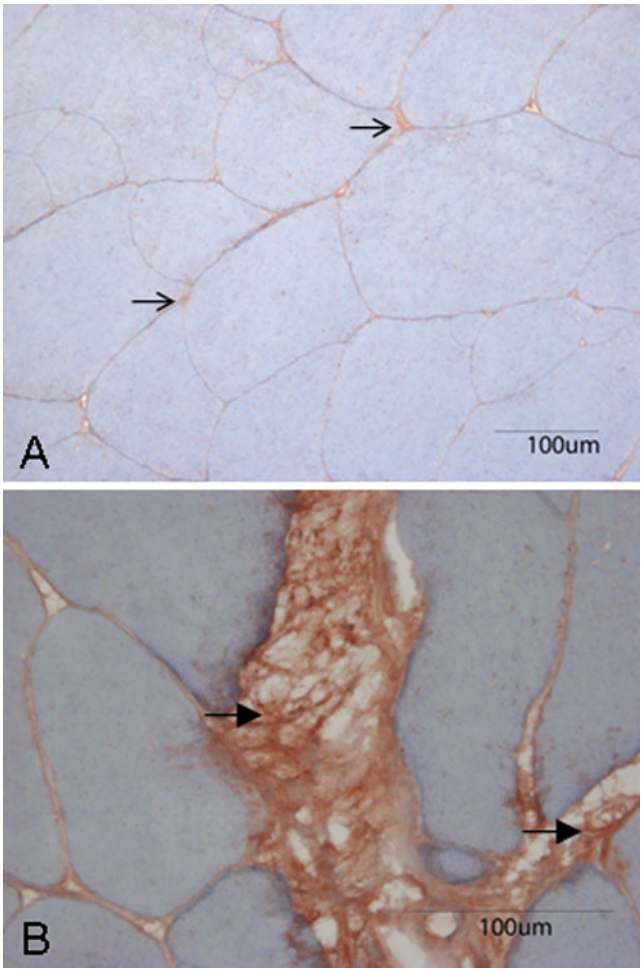

Fig. 4 Lumican staining of muscle cryo sections showed positive and strong staining ubiquitously in the extracellular environment of the tissue section. Panel **A** shows the overall muscle organization and staining of endomysia (*open arrows*). Panel **B** shows the myocommatal staining pattern, indicated by *closed arrows*

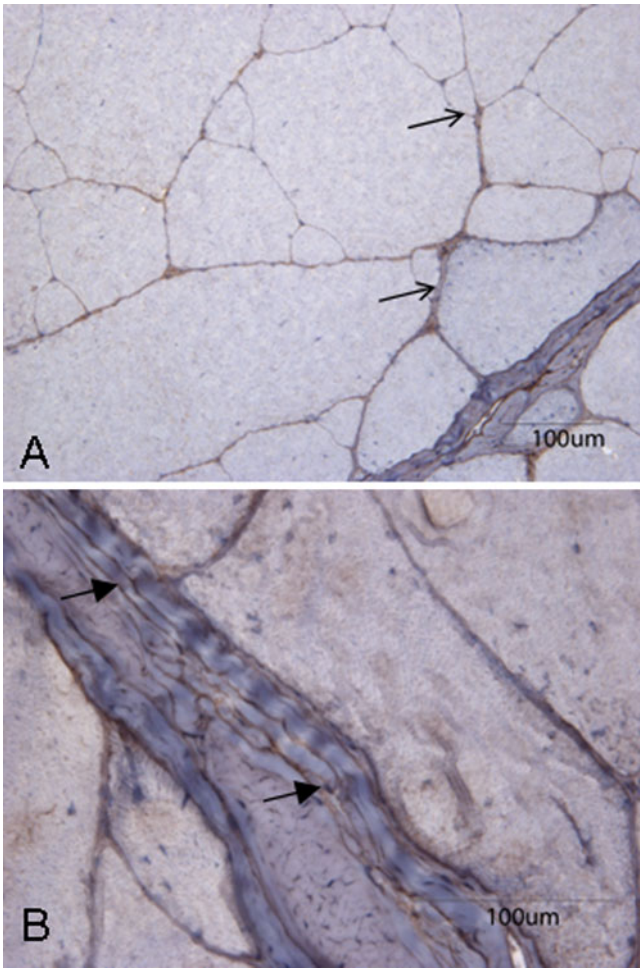

Fig. 5 Decorin staining of muscle cryo sections showed positive staining in endomysia (*open arrows*) as well as myocommata. Thread-like fibrillar structures in the myocommata were evident, indicated by *closed arrows*. Panel **A** shows the overall epitope distribution in the tissue section, whereas panel **B** shows details from the myocommata

different treatments. Staining of highly sulfated KS epitopes revealed a strong and ubiquitous presence of this GAG in cod ECM as shown in Fig. 10.

Discussion

In this study we investigated the presence, molecular properties, distribution and relative expression levels of collagen I and the SLRPs decorin, biglycan, lumican and fibromodulin on both mRNA (except fibromodulin) and protein level. Our main finding was that lumican in addition to collagen I were the most strongly expressed on the mRNA level. The tissue distributions of these two components were similar and ubiquitous in cod muscle ECM. The KS tissue distribution in the tissue sections was also ubiquitous and similar to that of lumican. Decorin and biglycan had similar staining patterns with positive staining in endomysia and cell junctions, in addition to the myocommata. Decorin was more abundant than biglycan on the mRNA level. The distribution of biglycan epitopes was somewhat different from what is reported from tetrapod skeletal muscle [9, 14], with positive staining also in the myocommata, corresponding to perimysium in mammals. Fibromodulin seemed to be most immunoreactive in segments of the myocommata, with only traces of staining in endomysia.

Staining of cABC- and cAC-generated epitopes detected by the C4S/DS antibody showed similar distribution patterns and implied that DS is a major GAG in cod muscle, as indicated by the weaker staining intensity of cAC-generated epitopes. DS attached to decorin and a large proteoglycan was previously reported to be a major GAG of striated muscle in mammals [23]. Note that the IHC-positive C4S/DS and KS staining in the present study may also originate from other, larger, proteoglycans than the SLRPs, such as aggrecan and versican.

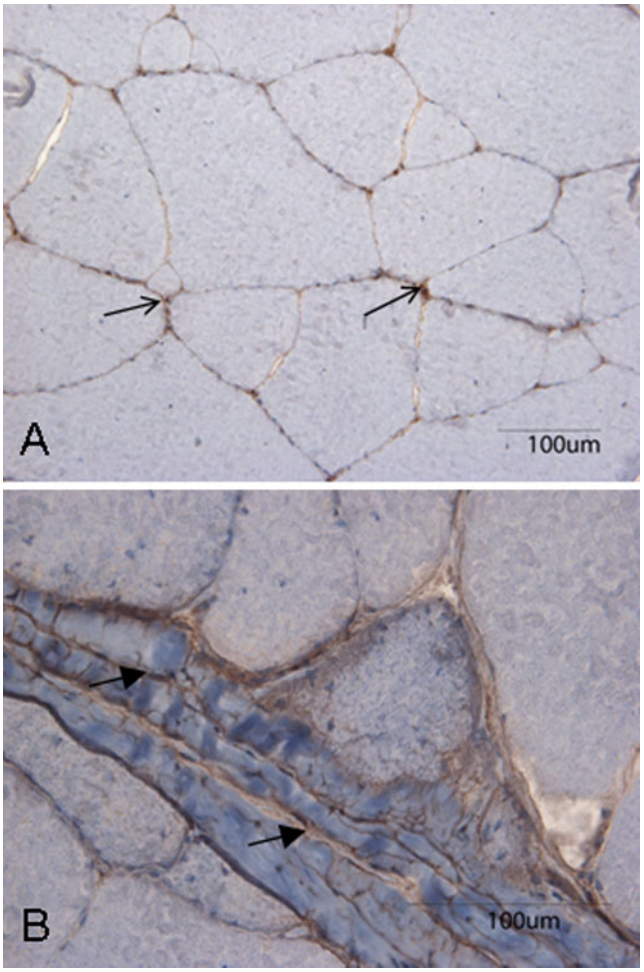

Fig. 6 Biglycan staining of muscle cryo sections showed definite regions of positive staining both in myocommata and between cells (*open arrows*). The thread-like structures of the myocommata (*closed arrows*) were evident and showed parallel oriented layers throughout the myocommata. Panel **A** shows the overall endomysial staining whereas panel **B** shows the myocommatal staining pattern

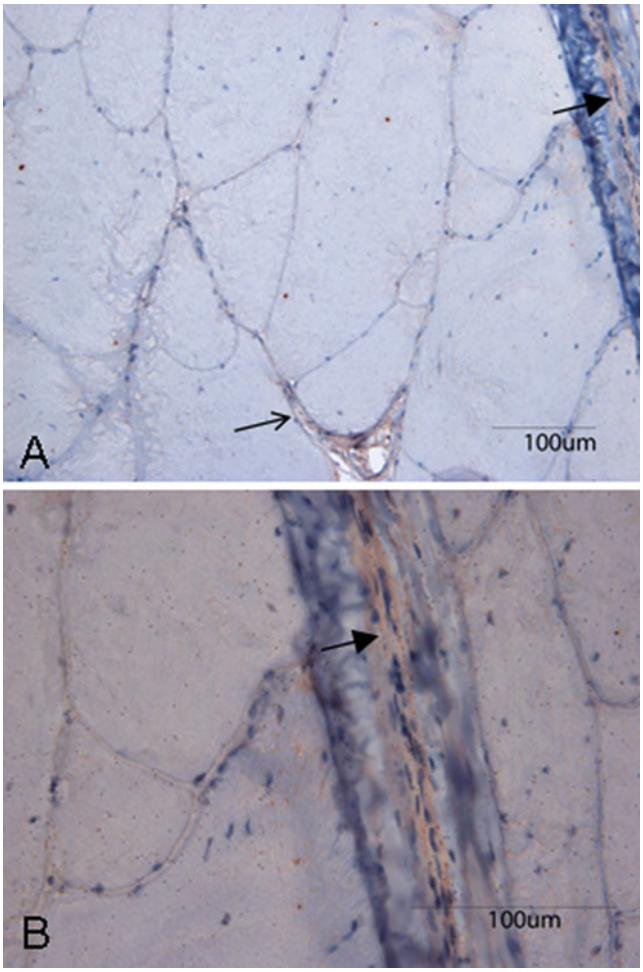

Fig. 7 Fibromodulin staining of muscle cryo sections showing thick fibromodulin-positive segment (*brown color*, *closed arrows*) in the middle of myocommata. Very weak staining could also be seen in some areas of the endomysia (*open arrow*). Panel **A** shows the overall weak endomysial staining pattern, whereas panel **B** shows the myocommatal staining pattern

Western blotting showed that the molecular weights of the SLRPs were between 35 and 50 kDa, in accordance with what has been shown in other species. Collagen I blotting resulted in two bands of approximately 45- and 60 kDa, which may reflect tissue turnover. C4S/DS staining of cABC-generated epitopes in Western blot showed positive reactions in areas corresponding to decorin and biglycan. KS staining showed positive reactions corresponding to somewhat higher molecular weights, as expected as these samples were not keratanase treated. The C4S antibody recognizes stubs remaining on the protein core after GAG removal, whereas the KS antibody recognizes the intact GAG chain attached to protein cores and thus both fibromodulin and lumican are typically found as intact proteoglycans at molecular weights of 60–90 kDa in mammals [24]. Western blotting also revealed bands of smaller sizes for biglycan and fibromodulin. This is perhaps not surprising considering the extremely high growth rate in fish. Melching *et al.* [25] reported different fragmentation of biglycan from small children than what was reported from healthy adults, suggesting that turnover in small children may resemble what is described from arthritic joints. Nevertheless, additional bands of lower molecular weights resulting from Western blotting may also be an indication of multiple isoforms of these proteoglycans in cod. Western blotting of immunoprecipitated cABC- and cAC-treated samples revealed the presence of dermatan sulfate in biglycan and decorin also in cod. Lumican and fibromodulin both carried keratan sulfate.

Collagen I was previously reported to be a major collagen of fish muscle, along with collagen V [26, 27]. In our study collagen I epitopes were expressed throughout the ECM in a similar manner as lumican. The overall distribution together with the strong expression on mRNA level, indicate that lumican is an important SLRP in fish,

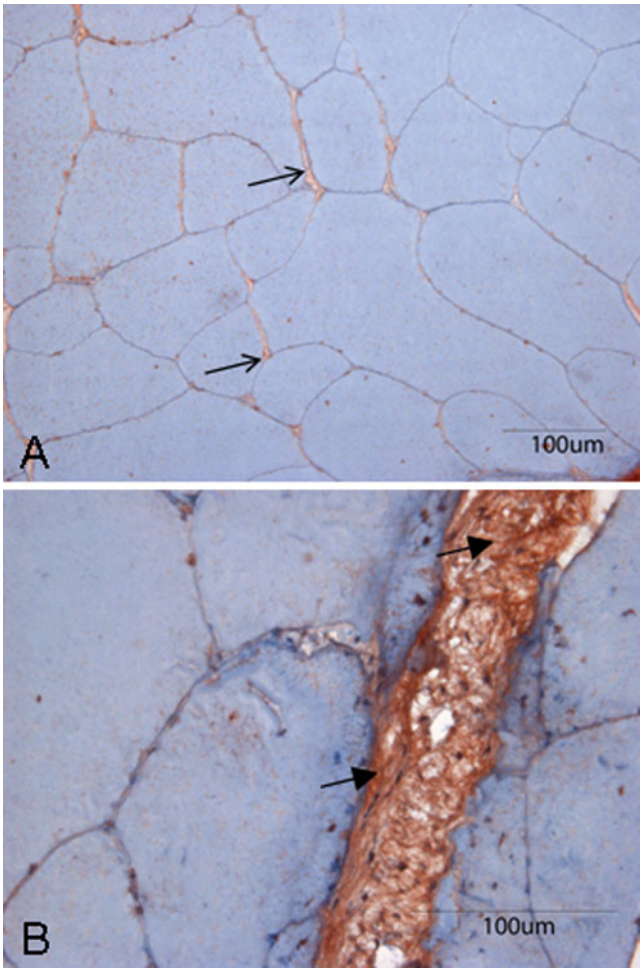

Fig. 8 C4S/DS staining of cABC-generated epitopes in the white muscle of cod. The positive staining was strong throughout the myocommata (*closed arrows*) and also in the endomysia (*open arrows*), indicating a strong presence of chondroitin-4-sulfate/dermatan-4- sulfate. Panel **A** shows the overall endomysial staining pattern, whereas panel **B** shows the myocommatal staining using GAG antibody 2B6

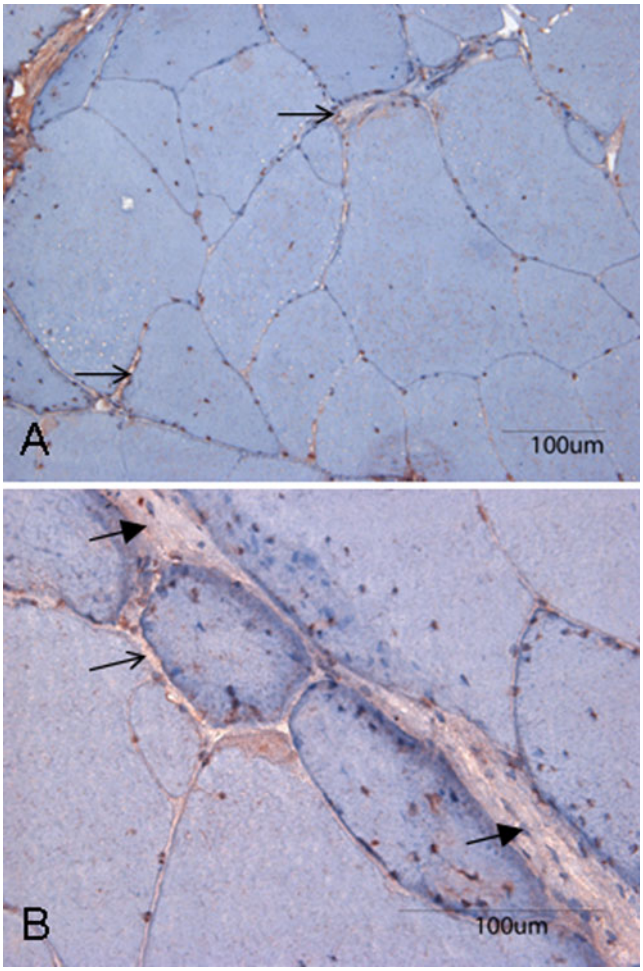

Fig. 9 CS staining of cAC-generated epitopes in cod muscle showed a weaker staining of the myocommata compared to Fig. 7. Color development could barely be seen in the endomysia (*open arrows*). A somewhat stronger color was seen in the myocommata (*closed arrows*). Panel **A** shows the overall endomysial staining pattern, whereas panel **B** shows the myocommatal staining using GAG antibody 2B6

contributing to the structure and (possible lack of) strength of the cod ECM. KS proteoglycans were suggested to contribute to looser structure of cod connective tissue also by Hannesson *et al*. [28], whereas Souza *et al*. [29] reported CS- and KS to be the major GAGs also in the adult zebrafish. We here suggest that lumican is the source of the majority of KS in fish muscle, based on strong mRNA expression and -epitope staining of both lumican core protein and KS chains in muscle cross sections. The importance of lumican in fish species was demonstrated by Yeh *et al*. [2], using knock down of the lumican gene in zebrafish. This experimental approach revealed a central function in fish development, as evidenced by severe abnormalities such as enlargement of eyes and pericardium in addition to overall deformed body shape. Interestingly, lumican has been reported to exist as a glycoprotein rather than proteoglycan in human cartilage [30]. It is also of interest to note that lumican and decorin knockdown both lead to skin fragility in mice [12, 13].

Lumican and fibromodulin belong to the same class of SLRPs and are reported to compete *in vitro* for the same attachment sites at the collagen fibrils [31]. Svensson *et al*. [31] and Jepsen [32] performed fibromodulin knock-out studies in mice resulting in abnormal collagen fibril formation and tissue organization. Kalamajski and Oldberg [3] reported that fibromodulin may be required for the formation of mechanically strong collagen cross linking in tendon. The absence of fibromodulin would allow for more lumican, resulting in mechanically weaker fibrils. Considering the rapid growth of fish in general and farmed fish in particular, the widespread distribution of lumican compared to fibromodulin on the protein level, may be a reflection of the

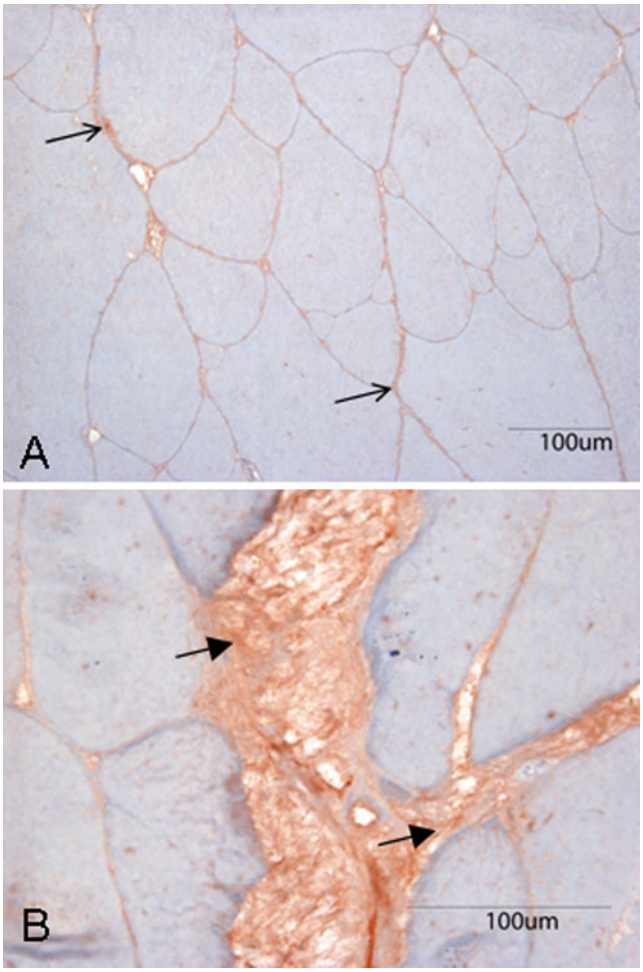

Fig. 10 KS staining of cod muscle cryo section showed a wide spread and ubiquitous distribution, as the lumican-epitopes in Fig. 3. Staining in the myocommata is indicated by *closed arrows*; endomysium staining and cell junctions by *open arrows*. Panel **A** shows the overall endomysial staining pattern, whereas panel **B** shows the myocommatal staining using monoclonal antibody 5D4

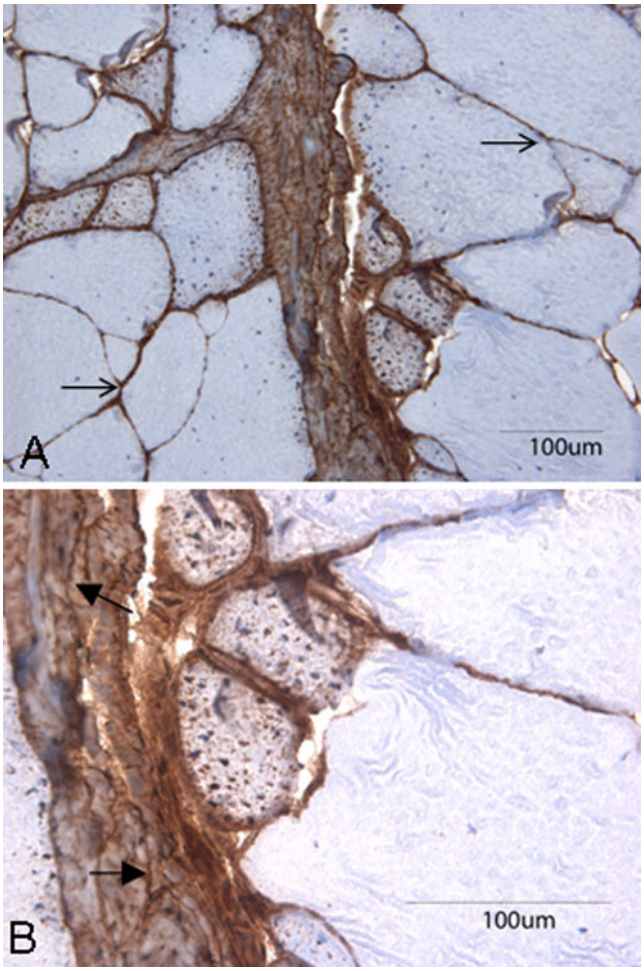

Fig. 11 Collagen I staining of cod muscle cryo sections showed a strong and ubiquitous presence of epitopes throughout the myocommatal and endomysial layers of the ECM. Fibrillar structures were evident and indicated by *closed arrows*. Strong endomysial staining is indicated by *open arrows*. Panel **A** shows the overall endomysial staining, whereas panel **B** shows details from the myocommata

formation of early and hence thinner collagen fibrils due to fast and continuous growth. The deposition of fibromodulin in the dense area of the myocommata may reflect the need for more mechanical strength in this area. Moreover, fish receive external stimuli from its surroundings due to water pressure and is probably not dependent on mechanically strong collagen fibrils to the same extent as are terrestrial animals.

Decorin and biglycan are class I SLRPs carrying CS or DS chains, and in our histology study they were similar in their distribution patterns, although decorin showed a somewhat stronger staining in the endomysia compared to biglycan, which is in accordance with reports from mammals [33]. In agreement, also the mRNA level of decorin was more abundant than that of biglycan. Decorin and biglycan are well documented to compensate for the lack of each other [3] and decorin is associated with mechanically stronger collagen fibrils than biglycan. The IHC staining results in the present study indicated that DS is potentially a major GAG in cod muscle, as the staining intensity of cAC-genereated epitopes was scarce in comparison to cABC- generated epitopes. Importantly, according to Fongmoon *et al*. [34], some oversulfated variants of CS, common in marine organisms, are resistant to cAC degradation, for instance CS-E and CS-K. Hence, the difference in staining between cABC- and cAC generated epitopes in this study may origin from the presence of either DS, CS/DS hybrids or oversulfated CS. Interestingly, Zöeller *et al*. [1] reported a decorin GAG attachment site in zebrafish, with the potential to carry heparan sulfate (HS) similar to that of perlecan, in addition to the CS/DS attachment site. We have previously described the expression and distribution of HS proteoglycans in cod and wolffish muscle [35], and showed that HS epitopes are present in cod endomysia with a somewhat limited staining

of the myocommata, thus not coincident with decorin staining in the present study.

The SLRPs were originally thought to act only as structural components. However, recent studies by Schaefer *et al.* [36], Babelova *et al.* [16], Iozzo and Schaefer [17] and Sjöberg *et al.* [18, 19], show that the functions of this group of proteoglycans are highly diverse. Schaefer *et al.* [36] reported biglycan to function as a macrophage-activating danger motif in mice, whereas Sjöberg *et al.* [18] showed that fibromodulin exhibit an active role during complement activation. Other studies have shown that lumican [37, 38] and fibromodulin [39] exhibit important functions during wound healing and tissue repair.

Cultured fish are prone to body malformations such as deformities of spine, jaws, gill lids and the heart [40]. Moreover, infectious diseases such as pancreas disease may cause chronic inflammations in *i.a.* salmon muscle [41]. The roles possible SLRP dysregulation may play in these processes are not yet clarified and should be the subjects of further studies, as well as the roles SLRPs may play during innate immunity and inflammation. The adaptive immune response of marine fish is slowly established upon infection, thus the first line defense and innate immune responses are extremely important for combating disease and preventing inflammation. Further investigations are needed to address in more detail the biological roles of SLRPs in teleosts.

Materials and methods

Sampling

Muscle tissue samples of cod (slaughter weight approximately 1 kg) were collected from the area at the base of the dorsal fin. Samples for RNA and protein analysis were immediately after slaughter transferred to and stored in RNAlater (Invitrogen life Technologies, Paisley, UK). At the same time, muscle pieces for cryo fixation were embedded in O.C.T. compound (Tissue Tek 4583, Miles Inc., Diagnostic Division, Elkhart, USA), frozen in liquid nitrogen and stored at −80°C until use.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from 18 samples using Trizol (Invitrogen Life Technologies, Paisley, UK) and Precellys® lysing tubes containing ceramic beads (Bertin technologies, Montigny-le-Bretonneux, France), according to the manufacturers' recommended procedures. RNA clean-up was thereafter performed with on-column DNase digestion using RNeasy Micro to Midi kit (Qiagen, Hilden, Germany), and RNA quality assessed by 1% agarose gel electrophoresis. 1 ug mRNA was subjected to reverse transcriptase (RT)-reaction using TaqMan Gold RT-PCR kit (Applied Biosystems, CA, USA) and then diluted 5X before application of samples (in triplicates) to real-time PCR analysis using ABI prism 7700 Sequence Detection System and TaqMan 100rx PCR core master kit (Applied Biosystems). At first Uracil-N-Glycosylase (UNG) treatment at 50°C for 2 min and UNG inactivation at 95°C for 10 min were performed, and then cDNA was amplified by 40 two-step cycles (15 s at 95°C for denaturation of DNA, 1 min at 60°C for primer annealing and extension). Cycle threshold (Ct) values were obtained graphically (Applied Biosystems, Sequence Detection System, Software version 2.2). Gene expression was presented as relative gene expression to biglycan, derived from subtraction of ΔCt values from ΔCt biglycan to give ΔΔCt, and relative gene expression calculated as $2^{-\Delta\Delta Ct}$ normalized to biglycan. The primers and TaqMan probes (Table 1) (5′ labeled-6-FAM and 3′ Quencher-TAMRA) for real-time PCR amplification of decorin, biglycan, lumican, collagen I were designed by Primer Express program (Software version 2.0, Applied Biosystems) using sequence information obtained from the cod genome database (www.codgenome.no) and beta actin (Gen Bank accession number AJ555463). Due to limited information about the Atlantic cod genome at the time of the experiments, we were not able to find sequences matching fibromodulin and thus no primer/probe pairs were available to run real-time PCR on this SLRP. Primer efficiencies were controlled and determined to be 109% for collagen I, 104% for decorin, 97% for lumican, 103% for biglycan and 104% for beta actin.

Antibodies

Antibodies used in light microscopy and Western blotting studies included the monoclonal antibodies 2B6 (against chondroitin-4-sulfate/dermatan sulfate, C4S/DS) and 5D4 (against keratan sulfate, KS) (initially kind gifts from professor Bruce Caterson, later obtained from Northstar BioProducts, MA, USA - formerly Seikagaku America) in addition to polyclonal antibodies against decorin (ab35378), lumican (ab70191), biglycan (ab58562), fibromodulin (ab67596) and collagen I (ab20033) (Abcam, Cambridge, UK). Secondary antibodies were alkaline phosphatase (AP)-conjugated monoclonal anti mouse (Promega Corp., MI, USA), polyclonal donkey anti goat (Jackoson ImmunoResearch Labs, PA, USA) and polyclonal donkey anti rabbit (Promega).

Immunohistochemistry (IHC)

Cross-sections of 8 μm were cut in a cryostat (Leitz 1720 Digital, Leica Instruments GMBH, Heidelberg, Germany), and mounted on poly-L-lysine coated glass slides and kept in −20°C until use. 0.3% H_2O_2 in methanol was used to block endogenous peroxidase activity in the tissue. Immu-

Table 1 Oligonucleotides used in this study

Gene		Sequence	Product (bp)
β-actin	F	5′-GGCTCAGAGCAAGAGAGGTATCC-3′	72
	R	5′-TCGTCCCAGTTGGTCACGAT-3′	
	P	5′-CCCTGAAGTACCCCATCGAGCATGG-3′	
Decorin	F	5′-AGATCAGCATGGTGGAGAATGG-3′	63
	R	5′-CCAGGTGCAGCTCCCTCAT-3′	
	P	5′-ACCCTGGCCAACGTCCCCCA-3′	
Biglycan	F	5′-GGGTTGTTGAACAGGCTAATGC-3′	81
	R	5′-CGTGGGAGTGGACGACTTCT-3′	
	P	5′-TGTCCTCTTCATCCCAGAGCCCCTG-3′	
Collagen I	F	5′-CACGCCGAATTCCTGATCA-3′	64
	R	5′-CCGCCTGCCAATCATTG-3′	
	P	5′-CAGCGCCAACATCCATAGGAGCAATG-3′	
Lumican	F	5′-GACCAAACTGCCACGTTGAGT-3′	73
	R	5′-AGCTTGCGGCTGTCACAGTA-3′	
	P	5′-CCATCGGCTTCCCCACAGCCAT-3′	

F = forward primer, R = reverse primer, P = probe

nostaining with mouse and rabbit antibodies was performed using DakoCytomation EnVision + System-HRP (AEC) kits for use with respectively mouse and rabbit primary antibodies (Dako Denmark AS, Glostrup, Denmark). Immunostaining with goat antibodies was performed using Vectastain Elite ABC kit (Vector Laboratories Inc., CA, USA). Peroxidase activity was revealed by use of AEC + substrate chromogen for mouse antibodies resulting in lighter brown color. Binding of goat and rabbit antibodies was visualized by use of DAB + chromogen substrate, based on the method of Graham and Karnovsky [42], resulting in darker brown color. Sections were counter-stained using Lilly's hematoxylin. A Spot RT Color Camera (Diagnostic Instruments Inc.) photographed the sections in a LEICA DMLB microscope (Leica Microsystems, Nussloch, Germany). Non-specific binding of antibodies was tested by replacing primary and secondary antibody with non-immune serum and dilution buffer, respectively.

Protein fractions

Trizol fractions remaining after RNA and DNA precipitations were washed and precipitated in 80% ethanol according to the manufacturer's recommendations. Further, samples for western blotting of proteoglycan protein cores were pooled and subjected to various digestions to remove the GAG chains (described in paragraph 4.6 GAG removal). The removal of GAG chains was necessary to achieve sufficient separation of the SLRP protein cores during SDS-PAGE, prior to Western blotting.

GAG removal

Removal of potential heparan sulfate chains from the protein cores was performed by using nitrous acid digestion at pH 1.5, according Shively and Conrad [43]. Samples were precipitated in ethanol after nitrous acid digestion to eliminate salt and were then resuspended in 0.05 M Tris–HCl pH 7.4 prior to enzymatic digestions. Chondroitinase ABC lyase (cABC) from *Proteus vulgaris* (EC 4.2.2.4, Sigma-Aldrich Chemie, Steinheim, Germany) [44] and chondroitinase AC (cAC) I from *Flavobacterium heparinum* (EC 4.2.2.5, Sigma-Aldrich) digestions were performed to create the neoepitopes reactive to the 2B6 monoclonal antibody. cABC cleaves both CS and DS, whereas cAC only cleaves the CS chains, the latter therefore creating neoepitopes only of CS origin. This method [45] produces a terminal disaccharide, consisting of an unsaturated uronic or iduronic acid residue adjacent to the *N*-acetylgalactosamine that may be unsulfated or sulfated in the C4 or C6 positions. cABC does not cleave keratan sulfate [46]; keratanase I from *Pseudomonas* sp (EC 3.2.1.103, Sigma Aldrich) and keratanase II (from *Bacillus* sp. Ks 36, EC 3.2.1.103, Seikagaku/Northstar BioProducts) were therefore used to remove keratan sulfate chains prior to Western blotting of protein cores. No reactivity to the KS antibody 5D4 was observed after keratanase treatment.

Western blotting

Protein content was measured using a BioRad kit (BioRad Laboratories, CA, USA) and 8 ug sample per lane were applied. The gels (10% uniform NuPage bis-tris gels, Invitrogen) were blotted to nitrocellulose or PVDF membranes using the iBlot dry blotting system (Invitrogen). Blocking was performed using 5% teleost gelatin (Sigma-Aldrich) in 0.1 M TBS, pH 7.4. Secondary antibodies were AP-conjugated, and the substrate used was a chromogenic ready-to-use alkaline phosphatase yellow liquid substrate system for Western Blot (Invitrogen).

Immunoprecipitation

Untreated, cAC-treated and cABC-treated samples were subjected to immunoprecipitation using Dynabeads (Invitrogen) antibody coupling kit coated with the SLRPs antibodies mentioned in Antibodies section. The immunoprecipitated proteins were then subjected to Western blot and stained for the presence of GAG epitopes using antibody 2B6 and 5D4. 2B6 recognizes either CS alone (in cAC-treated samples) or CS and DS together (in cABC-treated samples). Thus, reactivity in cABC but not cAC-treated samples would imply DS substitution, whereas reactivity in cAC (and cABC) would imply CS. Samples used for for KS staining were not enzyme treated. The binding of GAG antibodies was detected using ECL Plex goat-α-mouse IgG-Cy3 secondary antibody (GE Healthcare Life Sciences, Buckinghamshire, UK) and visualized using Ettan DIGE imager (GE Healthcare) for the 2B6 antibody. Binding of 5D4 was visualized using AP-conjugated secondary antibody as described in 4.7 Western blotting.

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