Murine fibromodulin: cDNA and genomic structure, and age-related expression and distribution in the knee joint

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The genomic structure of murine fibromodulin was determined, and its age-related expression and distribution were characterized in knee epiphyses, with decorin studied for reference. Fibromodulin, as well as decorin, have roles in collagen fibrillogenesis both *in vitro* and *in vivo*. The murine fibromodulin gene, *Fmod*, was similar with that in other species, with three exons and 86 % of the translated sequence in exon 2. The 2.7 kb long cDNA contains an open reading frame of 1131 nt. Fibromodulin mRNA levels were highest in tissues rich in fibrillar collagens type I or type II. During growth, the distribution of fibromodulin mRNA was similar with that of type II collagen, with the highest levels between 5 days and 1 month of age. Thereafter, the expression of type II collagen declined to a level near the detection limit, whereas the fibromodulin expression decreased less markedly to a level of approx. 35 % of maximum, and remained constant throughout the rest of the observation period. In contrast, decorin mRNA levels were the highest in old animals. Pericellular deposition of fibromodulin was strong around the late-hyper-

trophic chondrocytes of the secondary ossification centre and in the growth plate. In young epiphyses, both fibromodulin and decorin were found interterritorially, mainly in the uncalcified and deep-calcified cartilage. In the old mice, calcified cartilage became enriched with regard to fibromodulin, while, in contrast, decorin deposition diminished, particularly near the tidemark. In the subchondral bone trabeculae, decorin was found in the endosteum of growing, but not in the mature, epiphyses. Differences in the expression and distribution profiles suggest different roles for fibromodulin and decorin in the regulation of collagen fibrillogenesis, maintenance of the fibril organization and matrix mineralization. As fibromodulin is deposited closer to cells than decorin, it may have a primary role in collagen fibrillogenesis, whereas decorin might be involved in the maintenance of fibril structures in the interterritorial matrix.

Key words: bone, cartilage, collagen, decorin.

INTRODUCTION

Fibromodulin was first isolated as a 59 kDa protein from articular cartilage, and was also shown to be present in other types of cartilages and tendon [1]. The protein belongs to a family of small leucine-rich proteoglycans/glycoproteins (SLRPs) which currently comprises 11 members [2–5]. Most of the SLRPs exist as true proteoglycans, some as part-time proteoglycans (e.g. fibromodulin) and others as glycoproteins with no glycosaminoglycan substitution (e.g. chondroadherin). The highly homologous core proteins of SLRPs are characterized by tandemly organized leucine-rich repeats (LRRs), which are found in a variety of proteins performing diverse functions [6]. These LRR domains fold into particular β -sheet– α -helix structural units that are important for extracellular interactions of SLRPs with other proteins. Based on their genomic structure, organization and evolutionary conservation, SLRPs can be placed into four classes. Characteristic features of each class of SLRPs include conservation of the number of exons and LRRs and the structure of cysteine clusters next to the LRRs. Decorin and biglycan form the first class of SLRPs. The second class is rather heterogeneous

with proteins containing tyrosine sulphate as well as keratan sulphate, the latter different between tissues and species. These proteins include fibromodulin, lumican, osteoadherin and probably keratocan. Proline/arginine-rich end leucine-rich repeat protein (PRELP), also a member of this family, differs in having none of these two substituents, whereas it contains an N-terminal active heparin-binding motif. Chondroadherin forms the third class. Epiphycan (PG-Lb), mimecan (osteoglycin) and optican are the members of the fourth class [3]. Large members of SLRP family in the first three classes contain 10 or 11 LRRs, while small members in the fourth class each contain 6 or 7 LRRs, depending upon whether the LRR in the C-terminal disulphide-bonded loop is included.

Functional studies have indicated that many SLRPs have affinity to fibrillar collagens. Decorin and fibromodulin have been shown to bind to type I and II collagens, resulting in delayed fibrillogenesis and formation of thinner fibrils *in vitro* [7–9]. Binding of fibromodulin and decorin occurs via their LRR region to separate sites on the collagen fibril [10,11]. Electron microscopic and structural modelling data on decorin indicate that the LRRs form a horseshoe-shaped structure, which appears

Abbreviations used: SLRP, small leucine-rich proteoglycan/glycoprotein; LRR, leucine-rich repeat; PRELP, proline/arginine-rich end leucine-rich repeat protein; RT-PCR, reverse transcription-PCR.

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³ The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers Y11757 for pMDcn-1 (partial murine decorin cDNA) and X94998 for pMFmn-1 (complete murine fibromodulin cDNA and partial genomic structure).

essential for interactions with collagen fibrils [3,11,12]. The assembly of collagen fibrils into bundles with correct size and proper lateral organization is fundamental for the functional properties of articular and other cartilages [13–15]. This is also essential during endochondral ossification, when cartilage is replaced by bone, and the fibrils of type I collagen constitute a template for mineral deposition [16]. Therefore decorin and fibromodulin are believed to have important roles in establishing and maintaining extracellular matrix ultrastructure in connective tissues.

Indeed, gene inactivation of fibromodulin results in altered collagen fibril assembly. Surprisingly, the abundance of thin fibrils was higher. At the same time the tissue in tendon had a severalfold-higher lumican content [17]. It appears that in the normal development of collagen fibres, lumican is replaced by fibromodulin. In support, the two proteins bind to the same site with a higher affinity for fibromodulin [18].

Although the properties of decorin and fibromodulin suggest important functions in the regulation of collagen fibrillogenesis, the specific roles of individual molecules in different types of connective tissues still remain poorly understood. There is only limited information on changes in fibromodulin distribution in tissues during fetal and neonatal development [19], and no information on changes during aging. This study was initiated to provide such information in knee joint structures during early postnatal development, growth and aging in mice. We have characterized the cDNA and genomic structure of murine fibromodulin, and compared its expression and distribution profiles in knee epiphyses with decorin as a reference. Differences in the age-related expression and distribution profiles of these small proteoglycans in murine joints suggest distinct, perhaps variable, roles in collagen-rich connective tissues.

EXPERIMENTAL

Isolation of total RNA

Total RNA was extracted from 22 different tissues of 5-day-old C57bl × DBA F1 hybrid mice. Total RNA was isolated from murine knee joints at ten time points between birth and the age of 15 months. For extraction and purification of total RNA, the samples were immediately frozen and pulverized in liquid nitrogen, homogenized in guanidinium isothiocyanate, and sedimented through 5.7 M caesium chloride, as described in [20].

Construction of cDNA and genomic clones for murine fibromodulin

Total RNA from newborn mouse epiphyseal cartilage was used as a template for reverse transcription (RT)-PCR. Oligonucleotide primers (18–20-mers) were designed for areas conserved in published sequences for human and bovine fibromodulin cDNA sequences [21–23]. The PCR products were ligated into the *EcoRV* site of the Bluescript KS⁺ vector (Stratagene), cloned, and sequenced on both strands. One of the cDNA clones (pMFmn-1) was used as a probe in subsequent Northern and *in situ* hybridizations. The 3′- and 5′-ends of the mRNA were cloned using a 5′/3′ rapid amplification of cDNA ends reagent kit (Boehringer Mannheim) and primers based on the mouse fibromodulin sequence. Genomic DNA was isolated from tails of C57bl and 129/SvJ mice, and used as template for PCR to obtain overlapping genomic clones for the characterization of the exon/intron structure of the murine fibromodulin gene.

Hybridization probes

To obtain a probe specific for murine decorin mRNA (pMDcn-1), epiphyseal cartilage RNA was used as a template in RT-PCR amplification as above, using specific primers based on published nucleotide sequences [24]. The PCR product of expected size was cloned into the *EcoRV* site of the Bluescript KS⁺ vector.

Plasmids pMCol1a1-1 and pMCol2a1-1 were used as probes for mouse type I and II collagen mRNAs respectively [25], and clone 341-1 for 28 S rRNA [26]. For Northern hybridizations, the inserts were released from the plasmids by appropriate restriction enzyme digestions, and labelled with [α -³²P]dCTP using a random prime labelling kit (Boehringer Mannheim). For *in situ* hybridizations, the plasmids were linearized with appropriate restriction enzymes, and antisense and sense riboprobes were synthesized to specific activities of $\geq 2 \times 10^8$ c.p.m./ μ g with T3 or T7 polymerases using [α -³⁵S]UTP as the radiolabelled nucleotide.

Northern and Southern hybridization

Aliquots of total RNA (10 μ g) were electrophoresed on 1% agarose gels and transferred on to Pall Biodyne nylon membranes (Pall Europe, Portsmouth, U.K.) for Northern analysis, using hybridization and washing conditions recommended by the supplier. Bound radioactivity was detected and quantified on a Molecular Imager phosphorimager (Bio-Rad Laboratories) and normalized to a constant amount of 28 S rRNA.

For Southern analysis, 10 μ g aliquots of DNA isolated from 129/SvJ mice mouse tails were digested with different restriction enzymes, electrophoresed and transferred on to Hybond filters (Amersham International). The hybridizations were performed using a protocol recommended by the supplier. Bound radioactivity was detected by exposure of imaging films (X-OMAT AR; Kodak, Rochester, NY, U.S.A.).

In situ hybridization

Knee joints of newborn and 10-day-old mice were fixed for 24 h in 4% paraformaldehyde at 4 °C. Samples from 10-day-old mice were decalcified in 10% Na₂EDTA in 0.1 M sodium phosphate buffer, pH 7.0, for 2 days at 4 °C, before dehydration with ethanol and embedding in paraffin. Details of the *in situ* hybridization technique used are described in [27,28]. Sections of 5 μ m were collected on to slides coated with Vectabond (Vector Laboratories, Burlingame, CA, U.S.A.), deparaffinized, rehydrated, and treated with 0.2 M HCl for 10 min, and proteinase K (10 μ g/ml) in PBS, pH 7.4, at 37 °C for 30 min. The sections were postfixed in fresh 1% paraformaldehyde for 5 min, dehydrated, and dried at room temperature.

Hybridizations were performed at 55 °C for 40 h. Unbound probes were removed by RNase A digestion (20 μ g/ml in 0.5 M NaCl/1 mM Na₂EDTA/0.1 M Tris, pH 8.0) by washes with 2 $\times$, 1 $\times$, and 0.5 $\times$ SSC (1 $\times$ SSC is 150 mM NaCl/15 mM trisodium citrate, pH 8.0) supplemented with 1 mM Na₂EDTA at 37 °C for 5–10 min each, and by stringent washes in 0.1 $\times$ SSC/1 mM Na₂EDTA/20 mM dithiothreitol at 53–55 °C for 30 min. Sections were processed for autoradiography, exposed for 1–2 weeks, and stained with haematoxylin.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinized, rehydrated, and digested with bovine testicular hyaluronidase

(2000 units/ml in PBS, pH 5.0). Appropriate dilutions of rabbit polyclonal antibodies raised against bovine fibromodulin [1] and murine decorin (LF-113) [29] were applied in PBS, pH 7.4, containing 1% BSA over the tissue sections, and incubated overnight at 4 °C. After rinses in PBS, bound antibodies were detected with the avidin–biotin complex procedure, using horseradish peroxidase-conjugated streptavidin and diaminobenzidine substrate for colour development, following instructions of the supplier (Histostain-Plus kit; Zymed Laboratories, South San Francisco, CA, U.S.A.). Second antibody solution was supplemented with 2% mouse normal serum (Zymed Laboratories). The sections were counterstained with haematoxylin.

RESULTS

Structure of the fibromodulin cDNA

Molecular cloning of murine fibromodulin cDNA was initiated by PCR amplification of single-stranded cDNA for epiphyseal cartilage RNA using oligonucleotide primers based, first on the published human and bovine sequences [21,23], and subsequently on the murine sequence obtained. The amplification and cloning strategy is outlined in Figure 1. The primary structure of murine fibromodulin cDNA was determined by sequencing. The cDNA is 2.7 kb long and contains an open reading frame of 1131 bases, corresponding to a polypeptide of 376 amino acids, with a predicted mass of 43 kDa. The length of the untranslated 3'-sequence is 1535 bases, with the polyadenylation signal located 1513 nt downstream of the translation stop codon. Attempts to amplify and clone the 5'-untranslated sequence of the mRNA resulted in a clone with 97 untranslated nt.

Structure of fibromodulin gene

To characterize the murine fibromodulin gene, overlapping fragments of genomic DNA were amplified by PCR, cloned and identified by sequencing. As summarized in Figure 1, the murine fibromodulin gene is approx. 9 kb long and contains three exons

Figure 1 Cloning strategy and schematic structures of murine fibromodulin gene and deduced protein

Black bars indicate the cDNA clones obtained with RT-PCR. Grey areas at the end of some clones indicate the 3'/5' rapid amplification of cDNA ends primers. Clone pMFmn-1 was used as a probe for Northern and *in situ* hybridizations. Intron/exon organization of the gene shown in the middle panel was studied by sequencing and Southern hybridization of genomic RT-PCR clones retrieved from tail DNA of 129/SvJ and C57Bl mice. Bottom panel: predicted protein structure of murine fibromodulin. S indicates the 18 amino acid signal peptide, asterisks denote the positions of two tyrosine residues. Positions of four cysteines (C) at the N-terminal domain and two at the C-terminal end are indicated as predicted loop structures. Spirals indicate the five potential sites for N-glycosylation. Ten LRRs are illustrated as grey ovals.

Figure 2 Expression of fibromodulin mRNA in different tissues

Northern hybridization analysis was performed on total RNAs isolated from 22 different tissues of 5-day-old mice. The highest mRNA levels were observed in knee epiphysis, in calvarial and diaphyseal bone, in nasal and costal cartilage, in the eye and in bladder. Lower levels of mRNA were seen in tissues from skeletal muscle, stomach, lung, brain, kidney, thorax, heart, large intestine and tongue. Expression close to detection limit was observed in skin, liver, pancreas, adipose tissue and small intestine. The levels of 28 S rRNA (28S) were determined for loading control. Fmn, fibromodulin.

and two introns. The translation initiation codon is located in exon 2, which contains 86% of the translated sequence. Based on PCR and Southern hybridization studies (results not shown), the length of the first intron is approx. 2.8 kb and that of the second intron approx. 4.1 kb.

Expression of fibromodulin in different tissues

Northern analysis of total RNAs isolated from 22 different tissues of 5-day-old mice was carried out with a probe specific for murine fibromodulin (Figure 2). The size of the mRNA is 2.7 kb. The highest fibromodulin mRNA levels were observed in knee epiphysis, in calvarial and diaphyseal bone, in nasal and costal cartilage, in the eye, and in bladder.

Immunohistochemistry of mature knee joint showed the highest staining intensity was seen in the proliferating zone of growth plate (Figure 3). Fibromodulin staining was also observed in ligaments, especially at insertion sites, in the junction between meniscus and joint capsule, in the perimysium of skeletal muscle and in the periosteum.

Developmental expression of fibromodulin and decorin mRNAs in the knee joint

Expression profiles of fibromodulin and decorin mRNAs in the murine knee joint were studied by Northern-blot analysis at ten time points between birth and the age of 15 months (Figure 4). The mRNA levels of type I and II collagens were measured as a reference for production of the different extracellular matrices in the samples, where type I collagen is typical for bone, tendon and ligaments, and type II collagen for cartilage.

During development and growth, both fibromodulin and type II collagen exhibited increasing mRNA levels, reaching their maximum at the age of 1 month (Figure 4). After cessation of rapid growth at the age of 2–4 months, the expression of both fibromodulin and type II collagen mRNAs decreased. Type II collagen mRNAs declined to a level near the detection limit, whereas fibromodulin mRNAs remained at a level corresponding to approx. 35% of the maximum through the rest of the observation period. Decorin revealed a strikingly different pattern: its expression increased with age, with the highest mRNA levels detected at 15 months of age (Figure 4). Although the changes in decorin mRNA levels initially resembled those in type I collagen mRNA, the patterns diverged after 2 months of age.

Figure 3 Immunodetection of fibromodulin in different connective tissues of murine knee joints using a bovine fibromodulin antibody and the avidin–biotin complex procedure with diaminobenzidine secondary antibody detection

Tibial growth plate (A), insertion site of patellar tendon to proximal tibia (B), perimysium of the skeletal muscle (C), junction between the meniscus and the joint capsule (D), and periosteum of tibial cortical bone (E). Positive immunostaining for fibromodulin is seen as dark areas in the extracellular matrix (indicated by arrows) and as intracellular staining of chondrocytes in (D). ac, articular cartilage; sb, subchondral bone. Samples were obtained from 10-day-old (C) or 6-month-old mice (A, B, D, E). Bar = 50 μ m.

Cellular localization of mRNAs for fibromodulin and decorin in epiphyseal heads

To identify the cellular source of fibromodulin and decorin mRNAs in the epiphyses, *in situ* hybridizations were performed on tissue sections of knee joints from newborn and 10-day-old mice (Figure 5). Chondrocytes in the epiphyseal and growth cartilages of newborn mice were found to contain mRNAs for fibromodulin and decorin, but with somewhat different patterns. Fibromodulin mRNA was restricted to chondrocytes, with the highest signal level over the proliferating zone and the early articular cartilage, whereas decorin mRNA was seen both in epiphyseal chondrocytes and in metaphyseal trabecular and cortical bone. Type II collagen mRNA was present in all chondrocytes of the epiphysis and growth plate. Type I collagen mRNA was seen in fibroblasts, osteoblasts and periosteal fibroblasts, but not in epiphyseal chondrocytes (Figure 5).

During advancing endochondral ossification at the age of 10 days, the highest expression of fibromodulin mRNA was observed in hypertrophic chondrocytes of the secondary ossification centres and growth plates (Figure 5). Fibromodulin mRNA was also seen in the cells of maturing articular cartilage, especially near ligament attachment sites and in patellar tendon (results not shown). The highest signal for type II collagen mRNA was seen over the epiphyseal chondrocytes. Decorin mRNA was observed in hypertrophic chondrocytes of the secondary ossification centres and growth plates, and also osteoblasts lining the trabecular bone. In the latter location decorin mRNA was coexpressed with type I collagen mRNA.

Immunohistochemical localization of fibromodulin in developing epiphyses

Immunohistochemical localization of fibromodulin corresponded to the localization of its mRNA, indicating that the protein was deposited on site. In newborn mice, the strongest staining for fibromodulin was seen along the articular cartilage (Figure 6). Some staining was also seen in the proliferating zone of growth plate. At the age of 5 days, intensive staining for fibromodulin was not only seen in the superficial regions of early articular cartilage, but also in the hypertrophic zone around the ossification centres. At the age of 10 days, staining for fibromodulin was strong around chondrocytes in the hypertrophic zones of the secondary ossification centres and the growth plates, and in the proliferating zone of the growth plate. Fibromodulin was deposited interterritorially in the maturing articular cartilage and proliferating zone of growth plate, whereas it was pericellular in the hypertrophic zones of the growth plate and the secondary ossification centre, and in the resting zone.

Age-related distribution of fibromodulin and decorin in knee epiphyses

The staining patterns for fibromodulin varied with age in knee articular cartilage (Figure 7). In growing animals, fibromodulin appeared both pericellularly and interterritorially, with the highest intensity in the middle and deep zones of the uncalcified cartilage. Upon aging, staining for fibromodulin in the uncalcified cartilage decreased, while staining in the calcified cartilage

Figure 4 Expression of fibromodulin, decorin and type I and II collagen mRNAs in epiphyseal samples of murine knee joints during development, growth and aging

Tetragon in the schematic graph in the top panel shows the tissue structures included in the epiphyseal sample of murine knee joint. Total RNA was isolated from the samples containing joint capsule, epiphyses of proximal tibia and distal femur with epiphyseal subchondral bone (eb) and growth plate (gp), patella, ligaments, and menisci. Relative mRNA levels of fibromodulin (A), type II collagen (B), decorin (C), and type I collagen (D) at 10 different time points between birth (day 0) and 15 months of age were studied by Northern-blot analysis with specific cDNA hybridization probes. Hybridization intensities were quantified by phosphorimaging and normalized per a constant amount of 28 S rRNA determined by hybridization. Each column represents a mean of six animals. The highest and lowest value within each group are indicated by bars to show the range of data.

increased. Pericellular staining was observed around hypertrophic chondrocytes in the calcified zone at all ages studied. In subchondral bone of developing mice, fibromodulin was observed in cartilage rudiments within the ossifying bone trabeculae of developing joints, but not in the endosteum (Figures 6J, 7A and 7C). Trabecular staining diminished and finally disappeared with maturation and aging of the epiphyses.

Decorin appeared mostly in the interterritorial matrix of developing animals (Figure 7). At 10 days of age, areas of the calcified zone/subchondral bone interphase were rich in decorin. Decorin was also observed within the cartilage rudiments, but especially at the endosteum of subchondral bone trabeculae of growing animals. After maturation, decorin disappeared from endosteum. Upon aging, decorin became more uniformly distributed in the interterritorial and territorial compartments of uncalcified cartilage. With maturation, staining intensity for decorin became lower in the calcified zone than in the uncalcified zone, particularly below the tidemark and in regions where the condyles are in contact with the menisci (Figure 7J). Pericellular staining for decorin was observed at all ages around the hypertrophic chondrocytes of calcified cartilage. The number of chondrocytes displaying intracellular decorin increased with

Figure 5 Expression of fibromodulin, type II collagen, decorin and type I collagen mRNAs in murine epiphyses

Dark-field images of *in situ* hybridizations of fibromodulin (A, B), type II collagen (C, D), decorin (E, F) and type I collagen (G, H) in newborn proximal tibial heads (left panels) and in 10-day-old distal femoral heads (right panels). Light-field images of newborn (I) and 10-day-old knee joint (J). Tissues were sectioned in the anterior-posterior plane. Arrowheads in panels (B) and (F) indicate the position of the secondary ossification centre at the central epiphysis of distal femoral head. No expression of type II collagen, decorin or fibromodulin was seen in the two most superficial cell layers of articular cartilage (arrows).

aging, especially in the uncalcified cartilage and towards the periphery of the condyles.

DISCUSSION

Murine fibromodulin has a high sequence identity with the protein in other mammalian species. Identity of the murine fibromodulin cDNA sequence with rat [GenBank® accession number X82152], bovine [23] and human [21,22,30] sequences is 95%, 88% and 88% respectively. Comparative analyses of functional modules among different SLRPs and LRR glycoproteins have been extensively covered in recent reviews [2,31–33]. The primary structure of murine fibromodulin is in accordance with these comparisons. Six cysteines are found in conserved positions also in human, bovine and rat fibromodulin. The first

Figure 6 Immunodetection of fibromodulin in developing distal femoral epiphyses

Paraffin sections of knee joints of newborn (**A**), 5-day-old (**B**) and 10-day-old (**C**) mice were incubated with a rabbit polyclonal antibody raised against bovine fibromodulin [1]. Avidin–biotin complex method with horseradish peroxidase and DAP as a substrate was used for the primary antibody detection. Control staining without the secondary antibody is shown for a 5-day-old knee joint (**D**). Higher magnifications of 10-day-old mouse knee joint are presented in panels (**E**)–(**J**): articular surface (**E**), hypertrophic zone of secondary ossification centre (**F**), hypertrophic zone of the growth plate (**G**), proliferative zone of growth plate (**H**), resting zone of secondary ossification centre (**I**), and ossifying subchondral trabecule (**J**). The bar in panel **A** = 200 μm (for **A**–**D**) and 25 μm (for **E**–**J**).

Figure 7 Immunodetection of fibromodulin and decorin in articular cartilage of murine knee joints

A rabbit polyclonal antibody against bovine fibromodulin [1] or a rabbit polyclonal antibody against murine decorin [22] and the avidin–biotin complex procedure with diaminobenzidine substrate for secondary antibody detection were used for immunodetection of these proteoglycans in paraffin-embedded tissue sections. Left panels: immunodetection of fibromodulin in knee epiphyses of 10-day (A, weight-bearing cartilage), 1-month (C, weight-bearing cartilage), 6-month (E, weight-bearing cartilage), and 15-month (G and I, weight-bearing cartilage)-old mice. Right panels: are immunodetection of decorin in 10-day (B, weight-bearing cartilage), 1-month (D, weight-bearing cartilage), 6-month (F, weight-bearing cartilage), and 15-month (H, weight-bearing cartilage; J, nonweight-bearing cartilage)-old mice. Arrowheads point at endosteum in panels (C) and (D). The arrows point at tidemark in panels (E)–(J). Bar = 50 μ m.

cluster of four cysteine residues is located at the N-terminus in a consensus sequence of $Cx_3Cx_6Cx_6C$. The central part of the protein contains ten tandemly organized LRRs, each composed of 21–26 amino acids with asparagine at position 10. Five N-x-T/S-sequences in fibromodulin that are potential acceptor sites for N-glycosylation are conserved among all species studied so far (human, bovine, rat and murine) [34]. The number of N-terminal tyrosine residues that are potential sites for sulphation is less conserved between the species. In murine fibromodulin there are two N-terminal tyrosines, whereas rat, bovine and human proteins contain up to eight [21,23].

Both the murine and human fibromodulin genes are organized into three exons and two introns, although the intron sizes are different [21]. Analogous to the fibromodulin gene, the genes for lumican, keratocan and PRELP all contain three exons with the translation initiation codon and most of the translated region in exon 2 [21,35–37]. Based on their genomic organization, these four LRR glycoproteins are placed into a distinct subfamily of SLRPs [2].

In 5-day-old mice the highest mRNA levels were observed in epiphyseal, nasal and costal cartilage, in calvarial and diaphyseal bone, bladder and the eye. Fibromodulin is present in essentially all cartilages, but has also been demonstrated in other connective tissues, such as tendon, sclera and nucleus pulposus [1]. Fibromodulin has also been observed in fetal skin and in skin fibroblasts [38,39]. When compared with the mRNA levels in cartilaginous tissues, however, the fibromodulin mRNA in the skin of a 5-day-old mouse was expressed at a much lower level. The abundance of fibromodulin in different connective tissues of the knee joint exhibiting tensile strength, such as ligaments, tendons and perimysium, suggests that it predominantly functions in these tissues in the modulation of tensional properties of collagen fibrils also *in vivo*. Electron microscopic analyses have shown fibromodulin bound at the D band on type II collagen [40]. By binding to the surface of collagen fibrils, fibromodulin could inhibit the lateral growth of the collagen fibrils, but also strengthen the fibrils by providing more opportunities for interactions with other extracellular matrix components via its negatively charged glycosaminoglycan and/or tyrosine sulphate moieties. Lack of functional fibromodulin in fibromodulin-null mice has been reported to result in abnormal assembly of collagen fibrils in tendon with fewer, thinner, and less organized fibre bundles [41]. This phenotype suggests the importance of fibromodulin as a regulator of fibrillogenesis *in vivo*. The relatively mild phenotype with irregular type I collagen fibrils in tendon, suggests partial functional redundancy of the small proteoglycans. In fibromodulin-null mice lumican most likely substituted for fibromodulin, as its abundance, albeit not synthesis, was found up-regulated in tendons. It has been shown to bind to the same sites as fibromodulin on type I collagen fibrils, but with lower affinity [18]. Targetted inactivation of lumican, decorin and biglycan have also indicated that small proteoglycans share partially overlapping functions in regulation of collagen fibrillogenesis and assembly in skin, tendon, cartilage and bone [41–43].

Age-related expression of fibromodulin mRNA was studied in epiphyseal samples of knee joints at ten different ages between birth and 15 months, and compared with the expression profiles of decorin and that of type I and II collagens. The changes in the mRNA levels observed in the epiphyseal samples reflect two different processes. First, the proportions of cartilage, bone, ligaments and menisci change during development and growth, and even during aging. In newborn mice, the epiphyses are completely cartilaginous, whereas in the mature epiphyses, most of the tissue volume consists of subchondral bone, which also includes bone marrow. In these samples, cartilage is only present

in articular surfaces and menisci, and to some extent in the growth plate remnants. Secondly, the mRNA levels reflect changes in the transcriptional activity of the corresponding genes and presumably the synthesis rates of the proteins. Higher mRNA levels of fibromodulin in mature tissue suggest faster turnover rate than that of type II collagen, or functions related to other biological processes than regulation of type II collagen fibrillogenesis. The results indicate that fibromodulin is relatively more important in growing epiphyses, while decorin seems to play a proportionally increasing role in adult epiphyses. Similar age-dependent changes in decorin mRNA levels have been observed in human articular cartilage and bovine tendon [44,45].

Fibromodulin appears associated with cells delineating the forming joint cavities in developing joints of mouse and marsupial opossum embryos [19,46]. In newborn and 5-day-old murine knee joints, fibromodulin appeared in the developing articular cartilage, with highest levels in the interterritorial matrix of the superficial zone. During endochondral ossification of the epiphyseal heads, the highest levels of mRNA and protein were observed in the late hypertrophic zone of secondary ossification centre and growth plate. In the resting and hypertrophic zones, fibromodulin appeared almost entirely pericellularly, whereas in the proliferating zone, the highest levels were observed in the interterritorial matrix. The decorin distribution was similar to fibromodulin in the hypertrophic zones of secondary ossification centre and growth plate in murine epiphyseal head. Earlier studies have also demonstrated decorin at sites of appositional growth in human growth plate [47] and fibromodulin, decorin and biglycan in marsupial opossum growth plate [46]. Fibromodulin and decorin are capable of binding transforming growth factor beta [22], which should enable them to serve as a local reservoir for growth factors, and thus possibly to function as mediators of cell differentiation control during the growth of epiphyses.

One of the proposed functions for decorin is to inhibit bone mineralization. In mineralization of rat calvariae, removal of decorin was necessary before the fusion of collagen fibrils and primary calcification of bone could occur [16]. In accordance with that, decorin was seen on the endosteal surfaces of subchondral bone trabeculae in growing epiphysis, but not in mature skeleton of the mice in the present study. Similarly, staining intensity of decorin in calcified articular cartilage decreased with age, particularly from the tidemark region. Fibromodulin was retained within the mineralizing trabeculae of primary spongy bone during endochondral ossification, but contrary to decorin, it was not observed on the endosteal surfaces. Possibly, by binding to collagens, fibromodulin may regulate the assembly of type I collagen fibrils, which functions as a template for bone mineralization. Upon aging, calcified articular cartilage of the knee epiphysis became enriched by fibromodulin, suggesting functions related to mineralization of the cartilage matrix.

In conclusion, we have shown that fibromodulin mRNA levels were the highest in the growing knee epiphyses, where the major staining localized in the hypertrophic zones of growth plate and secondary ossification centre. In articular cartilage, fibromodulin was interterritorial, but was found closer to the cells than decorin. Upon aging, fibromodulin deposition became strongest in the calcified cartilage. In contrast, decorin mRNA levels were the highest in the old animals, with strongest immunostaining in the uncalcified articular cartilage. Differences in the expression and distribution profiles suggest distinct roles for fibromodulin in collagen fibrillogenesis, and for decorin in maintaining the fibril organization and inhibition of matrix mineralization.

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