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Functions of lumican and fibromodulin: Lessons from knockout mice

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Lumican and fibromodulin are collagen-binding leucine-rich proteoglycans widely distributed in interstitial connective tissues. The phenotypes of lumican-null (*Lum*$^{-/-}$), Fibromodulin-null (*Fmod*$^{-/-}$) and compound double-null (*Lum*$^{-/-}$*Fmod*$^{-/-}$) mice identify a broad range of tissues where these two proteoglycans have overlapping and unique roles in modulating the extracellular matrix and cellular behavior. The lumican-deficient mice have reduced corneal transparency and skin fragility. The *Lum*$^{-/-}$*Fmod*$^{-/-}$ mice are smaller than their wildtype littermates, display gait abnormality, joint laxity and age-dependent osteoarthritis. Misaligned knee patella, severe knee dysmorphogenesis and extreme tendon weakness are the likely cause for joint-laxity. Fibromodulin deficiency alone leads to significant reduction in tendon stiffness in the *Lum*$^{+/+}$*Fmod*$^{-/-}$ mice, with further loss in stiffness in a lumican gene dose-dependent way. At the level of ultrastructure, the *Lum*$^{-/-}$ cornea, skin and tendon show irregular collagen fibril contours and increased fibril diameter. The *Fmod*$^{-/-}$ tendon contains irregular contoured collagen fibrils, with increased frequency of small diameter fibrils. The tendons of *Lum*$^{-/-}$*Fmod*$^{-/-}$ have an abnormally high frequency of small and large diameter fibrils indicating a deregulation of collagen fibril formation and maturation. In tissues like the tendon, where both proteoglycans are present, fibromodulin may be required early in collagen fibrillogenesis to stabilize small-diameter fibril-intermediates and lumican may be needed at a later stage, primarily to limit lateral growth of fibrils.
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Introduction

Lumican and fibromodulin are members of the structurally and functionally related family of small leucine-rich proteoglycans (SLRP) [1,2]. The eleven SLRPs, identified so far are further grouped into four classes based on amino acid sequence identity, presence of cysteine-rich clusters at the N- and C-termini, and number and spacing of the leucine-rich repeats [1–4]. Lumican and fibromodulin, class II members, are related closely by structure; the core proteins contain four N- and two C-terminal cysteines and a central domain of 11–12 leucine-rich motifs that are sites of protein-protein interactions [5,6]. Lumican and fibromodulin, each with three exons, have similar genomic organization and may have evolved by duplication of tandem-repeat supermotifs [7]. The gene for lumican (*Lum*) is localized on human chromosome 12q21.2–q22 and to a syntenic region on distal mouse chromosome 10 (www.ncbi.nlm.nih.gov/LocusLink/) [8,9]. The gene for fibromodulin (*Fmod*) is localized on human chromosome 1q32 and to a syntenic region on mouse chromosome 1 (www.ncbi.nlm.nih.gov/LocusLink/) [5,10]. Lumican is a component of the cornea, skin, sclera, tendon and cartilage [5,9,11–13]; fibromodulin is expressed at high levels in all of these tissues except the skin and the cornea [14]. *In vitro* lumican and fibromodulin bind to the same region on collagen type I and affect collagen fibrillogenesis [15–17], implying that *in vivo* they are likely to have significant functional overlap in tissues where they are co-expressed. In the current review, I will present our recent studies on the lumican-, fibromodulin- and lumican-fibromodulin-null mice, and discuss studies by others, that provide insight into regulation of gene expression and functions of these two proteoglycans *in vivo*.

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Keratan sulfate side chains

Both lumican and fibromodulin contain five potential N-glycosylation sites. Bovine fibromodulin contains four keratan sulfate (KS) side chains that are attached by N-glycosylation to asparagine residues at positions 109, 147, 182, and 272 [18].

Lumican isolated from chick cornea is estimated to be glycosylated at all five asparagine residues with only two to three of these receiving KS side chains [19–21]. KS that is N-linked to Asn residues of the core protein is termed KS-I, and is the major form of corneal KS. While fibromodulin is a non-corneal SLRP, it also contains KS-I [22]). There is some controversy about whether murine KS is different from that of the chicken and bovine KS. The oversulfated KS found in the thicker corneas of these larger animals is reportedly absent from the thinner mouse corneas. Nevertheless, anti-bovine KS antibodies recognize KS in developing mouse tissues [24]. Also, lumican undergoes gel-shift after keratanase digestion, indicating enzyme susceptibility of the KS side chains in the mouse [12]. Thus, in the mouse, lumican is substituted with KS side chains, although the number and specific asparagine residues that are KS-substituted are not known. The developmentally temporal appearance of KS in tissues and possible functions that are KS-dependent will be discussed in the following section.

Keratan sulfate in tissues

KS-I is widely present in the cornea and increases as the cornea develops and acquires transparency [13,25,26]. Loss of corneal transparency during wounding is associated with a decrease, while healing and restoration of transparency occur with concomitant KS elevation [27–29]. In the mouse cornea, KS-containing proteoglycans are detected at postnatal day 20 and later [12,24]. Insights into the pathogenesis of macular corneal dystrophy also point to KS playing a central role in corneal transparency. These dystrophies have been linked to mutations in genes for sulfotransferase enzymes. The underlying biochemical defects include poor sulfation and precipitation of the under-sulfated KS in the cornea that may be less adept in regulating the structure of collagen fibrils conducive to transparency [30–35]. The cornea of lumican-null mice is remarkably less transparent than wild type mice. Although we have not determined the effect of lumican-deficiency on corneal KS content, we estimated it to be reduced by 25% in whole eyes [40]. These observations underscore a crucial role for KS in corneal transparency. KS-I is also found in other non-corneal tissues such as eosinophil-specific granules in the mouse, implying a broad scope of its biological functions [36].

Lumican is primarily a glycoprotein with non-sulfated polylactosamine side chains in the 9 day-old developing chick embryo, but sulfated in the adult cornea [37]. Lumican from other tissues, such as arterial lumican lacks the highly sulfated chains and is often dubbed a "part-time" proteoglycan [38]. This is also likely to be the case for fibromodulin. Thus, the impact of their deficiencies in different tissues of the null mutants is the outcome of the lack of the core proteins only in certain tissues and combined lack of the core proteins and KS in others. However, to date, the lumican- and fibromodulin-null mice have been studied primarily in the context of core protein deficiencies, their impact on the KS pool of different tissues and functional consequences await further studies.

Expression of lumican and fibromodulin in tissues

We elucidated the presence of lumican transcript in the developing mouse embryo by *in situ* hybridization. Lumican is expressed in the head and lateral mesenchyme as early as E9.5 [12]. By E.13.5, it is expressed in mesenchymal tissues, particularly in areas adjacent to the epithelium, and is detectable in the developing cornea, peri-ocular mesenchyme, in the eyelid interstitium and developing dermis (Figure 1). In sagittal sections of E15.5 embryos, the expression of lumican is strong in the pulmonary and aortic valves of the heart, diaphragm, developing intestine, mesentery, urinary bladder, rib primordium and in the developing limb digits (Figure 2). At these stages, the lumican message is very specifically expressed in the mesenchyme in close proximity to the epithelial cells of the vibrissae follicles

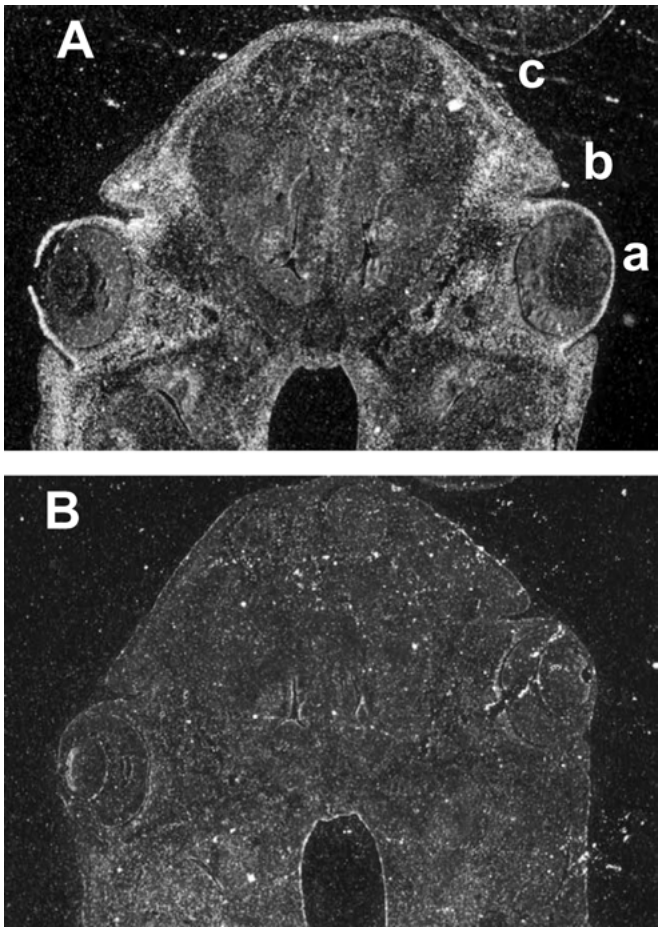

Figure 1. A dark field view of *in situ* hybridization of antisense lumican RNA (A) and sense RNA control (B). Hybridization with antisense RNA (A) shows expression of lumican in a transverse section of a wild type E13.5 embryonic head. Experimental procedures are as described earlier [12]. Lumican expression is strong in the developing cornea (a), eye-lid and sub-epidermal layer of the skin (c).

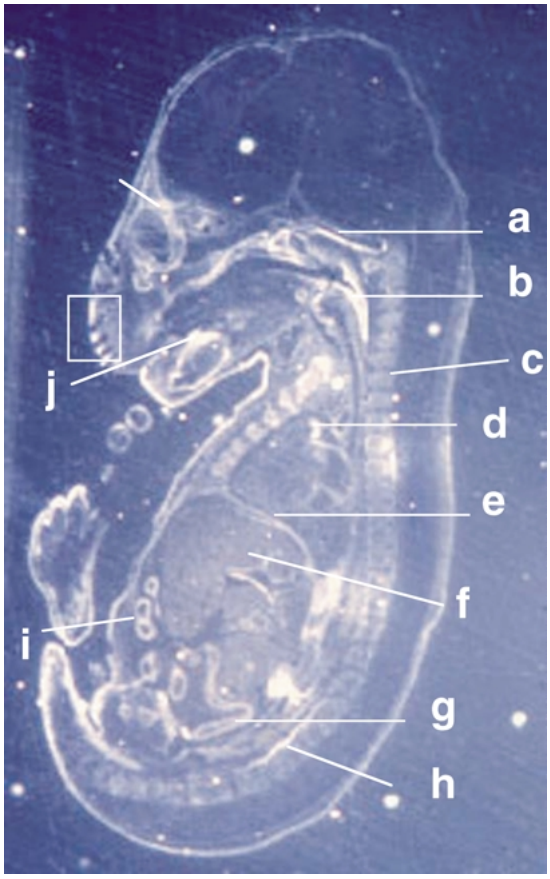

Figure 2. Parasagittal section of E15.5 embryo showing a dark field view of an *in situ* hybridization of anisense lumican. Lumican expression in the cartilage primordium of the basisphenoid bone (a), cartilage lining of the entrance to the esophagus (b), rib primordium (c), aortic valve (d), diaphragm (e), liver (minimal staining (f), lumen of the urogenital sinus (g), floor plate of the spinal cord (h), intestine (i) and developing lower incisor (j). Note that the strong staining in the boxed area is in mesenchymal tissue between the follicles of vibrissae. A higher magnification of this area is shown in Figure 3.

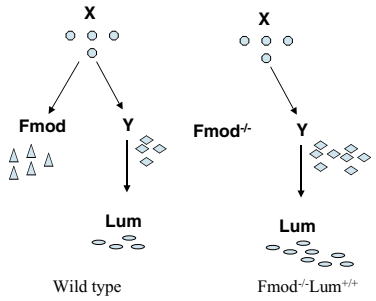

Figure 5. Hypothetical regulation of lumican and fibromodulin levels in tissues. A regulatory product X regulates expression of *Fmod* and another unknown gene Y. The Y gene product regulates expression of lumican. It is proposed that in the Fmod-null mice higher levels of X elevates lumican levels. The following symbols are defined as: unknown regulatory gene products of X and Y are ▫ and ◇ respectively: *Fmod* ⁾; lumican ▭.

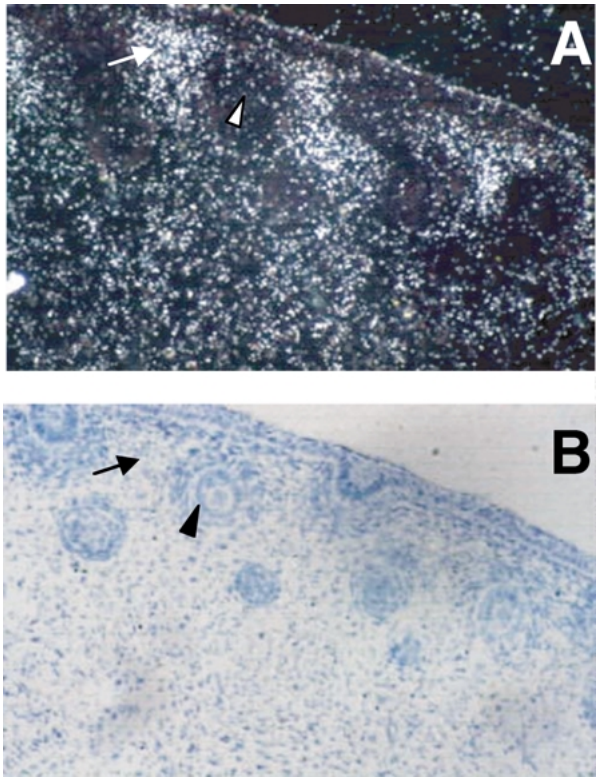

Figure 3. Dark field view (A) and a bright field view (B) of a parasagittal section through the snout of an E15.5 embryo. Lumican is expressed in focused areas in the mesenchyme close to the epidermis (arrow in A); Note that epidermal cells of the vibrissa-follicle is lumican-negative (arrowhead in A).

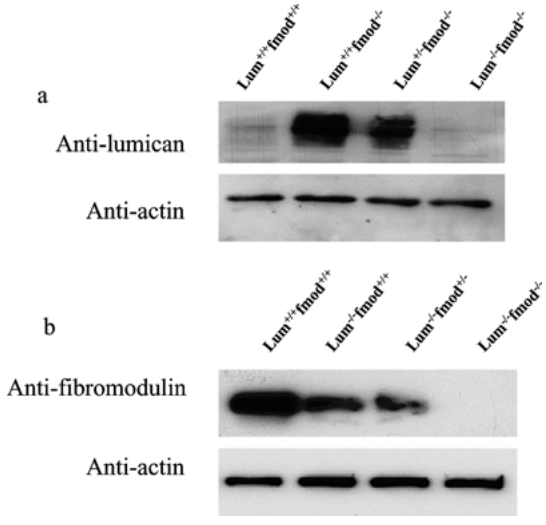

Figure 4. Analyses of lumican (a) and fibromodulin (b) levels in FDL tendons by semi quantitative immunoblotting [48]. Comparable staining for actin in all of the lanes indicates that similar amounts of total protein were loaded in each lane. Note that compared to the $Lum^{+/+}Fmod^{+/+}$ tendons that there is an apparent 8-fold over-expression of lumican in the $Lum^{+/+}Fmod^{-/-}$ tendons, and approximately half as much lumican in the $Lum^{+/-}Fmod^{-/-}$ as in the $Lum^{+/+}Fmod^{-/-}$.

(Figure 3). It is likely that lumican expression is closely regulated by epithelial-mesenchymal interactions. Fibromodulin is also reported to have a generally similar pattern of expression at sites of interstitial tissue deposition, cartilage and bone formation [39]. However, differences in tissue- and temporal-specific expression of these two proteoglycans speak of additional unique roles. Lumican is a major component of the cornea [11–13,40,41]. While fibromodulin message may be detectable by RT-PCR at the protein level, it is undectable in the normal cornea (Chakravarti, unpublished observations). In the sclera, both core proteins are present, but levels of fibromodulin may be higher than lumican. In the developing tendon, fibromodulin and lumican message is detectable from postnatal day 2 [42]. The lumican core protein, present in much lower amounts in the tendon, reaches maximal levels by postnatal day 4. On the other hand, higher levels of fibromodulin are maintained throughout, from postnatal day 4 to one month [42]. These differences and overlap in their expression patterns are important to our understanding of the $Lum^{-/-}$, $Fmod^{-/-}$ and $Lum^{-/-}Fmod^{-/-}$ phenotypes.

$Lum^{-/-}$ mice

$Lum^{-/-}$ mice are viable with total body weight reduced by ~15–20% as compared to the wild type. It also appears that $Lum^{-/-}$ females frequently have litters of smaller size. *In vitro* studies have shown that the lumican core protein inhibits collagen fibrillognesis and, in its presence, the collagen fibrils have smaller diameter [15,43], suggesting a similar role *in vivo*. Therefore, in the lumican-null mice we initially focused on the structure of collagen fibrils in the cornea. Indeed, transmission electron microscopy show that the collagen fibril structure is markedly altered in the corneal stroma of the lumican-null mice. In localized areas of the $Lum^{-/-}$ cornea, collagen fibrils range in diameter from 20–235 nm, as opposed to 10–42 nm seen in wild type animals. The $Lum^{-/-}$ corneas appear hazy in slit-lamp biomicroscopy examinations [12]. In a subsequent detailed study we found the collagen abnormality to be localized in the posterior stroma [40]. The collagen structural anomaly in the posterior stroma also coincides with increased backscattering of light from the posterior stroma, detected by *in vivo* confocal microscopy. X-ray diffraction studies further show that collagen fibril diameter and interfibrillar spacing is altered in the $Lum^{-/-}$ cornea [44]. It is known that corneal collagen fibril diameter across species is maintained between 25–35 nm and organized into a lattice structure to minimize diffraction of incident light for optimal vision [45,46]. The collagen abnormality and reduced corneal transparency phenotype of the $Lum^{-/-}$ confirms this connection between organized collagen matrix and corneal transparency. It further demonstrates a central role for lumican in the regulation of collagen structure and corneal transparency. Lumican is a component of the sclera as well, and the $Lum^{-/-}$ sclera shows specific changes in the collagen architecture and a thinning of the sclera (Chakravarti, unpublished observations). The sclera phenotype will be further discussed in the $Lum^{-/-}Fmod^{-/-}$ double-null phenotype section.

Lumican is widely expressed in various connective tissues and its absence has mild to severe effects in many organs and tissues. The collagen anomaly in the lumican-null mouse is also evident in the skin: a proportion of the fibrils have bigger diameter, irregular contour and some appear to be laterally fused. The functional consequence of the dermal collagen defect is loose and fragile skin, with an 86% reduction in tensile strength as assessed by biomechanical testing [12]. The long bones are stronger but brittle compared to wild types in biomechanical tests (Chakravarti and Jepsen, unpublished observations). A subtle cardiac phenotype includes histological changes in the myocardium and the valves (Chakravarti, unpublished observations).

$Fmod^{-/-}$ mice

Fibromodulin-deficient mice were developed and reported to be viable, fertile and without major functional defects [47]. By histology and light microscopy they were found to have normal structures of the heart, liver, lung, skin and cartilage. However histology and light microscopy of cross-sections of the tail and Achilles tendon showed abnormal tissue organization and fiber bundles. Transmission electron microscopy revealed abnormal collagen architecture. A fraction of the fibrils had irregular contours in cross section and there was an increase in the population of small diameter fibrils. These results suggest a role for fibromodulin in regulating maturation of collagen fibrils from small to large diameter fibrils, although functional consequences of these tendon collagen changes were not investigated.

$Lum^{-/-}Fmod^{-/-}$ mice

We have recently developed mice jointly deficient in lumican and fibromodulin by intercrossing the single-null mutants [42,48]. The double knock-out mice have helped to identify unique and overlapping functions of lumican and fibromodulin. Double-null mice are viable, fertile and often smaller (body weight reduced by as much as 24%) than their wild type littermates. The null mice have bowed legs, walk on the dorsal part of their hind legs and up to 40% of the double-nulls show varying degrees of gait abnormalities that are detectable from a very early age (<3 weeks). In biomechanical testing the knee joint of the double-null show two-fold increase in joint hypermobility. In histology of the knee joint, the distal condyle appears severely malformed, with a medial misalignment of the knee patella, a secondary patellar groove and compensatory overgrowth of the metaphyseal bone to support the misaligned patella. The double-null mice also suffer from age-related articular degeneration that may arise as a consequence of abnormal use of the knee joint. Tendon weakness may be a contributing factor in the gait abnormality. In biomechanical testing of the Flexor digitorum longus, lumican-deficiency alone has no effect on tendon

stiffness. However, in the fibromodulin-deficient background, tendon-stiffness is reduced in a lumican-gene dosage dependent way. Thus, compared to the wild type, tendon stiffness in the $Lum^{+/+}Fmod^{-/-}$, $Lum^{+/-}Fmod^{-/-}$ and $Lum^{-/-}Fmod^{-/-}$ is reduced by 25% ($p < 0.01$), 45% ($p < 0.01$) and 61% ($p < 0.001$), respectively. Measurements of tendon cross section shows a strict linear relationship with body weight; small body coincides with small tendon in the double-nulls. Therefore, some of the observed reduction in tendon strength could simply be a function of smaller tendons in the double-nulls. When tendon stiffness is normalized for cross sectional area (tendon modulus), as compared to wild type mice, the tendon modulus is reduced by 29% and 49% in the $Lum^{+/-}Fmod^{-/-}$ and the $Lum^{-/-}Fmod^{-/-}$, respectively. This is a significant reduction indicating that fibromodulin and lumican deficiencies alter the intrinsic property of tendons and thus demonstrated that their reduced strength is not just a function of smaller tendons.

The sclera is another tendon-like connective tissue that is affected by lumican and fibromodulin deficiencies [51]. Compared to the wild type mouse, the single-null and the lumican/fibromodulin double-null mice have thinner sclera. In double-null mice only, the whole eyes have increased axial length, possibly an outcome of reduced scleral strength. Transmission electron microscopy showed that all three null mutant sclera contain fewer flat sheets of lamellae than the wild type. The morphological changes in the collagen fibrils are similar in the sclera and tendon of the null mutants.

In vitro lumican and fibromodulin compete for the same binding site on collagen type I [16]. In tissues, lumican and fibromodulin deficiencies affect collagen architecture in very specific ways that suggest the *in vivo* regulation of fibrillogenesis by these two proteoglycans is different. In the $Fmod^{-/-}$ mice, the frequencies of small diameter fibrils increase. In the $Lum^{-/-}$ mice, both sclera and tendon show increased frequency of larger diameter fibrils, as noted before in the cornea and the skin [12]. The double-null tendon shows increases in small and very large diameter fibrils. Collagen fibrilllogenesis is a complex process, subject to regulation by multiple factors; fibromodulin may be required at the early step to stabilize small-diameter collagen fibril-intermediates and later for their maturation. In its absence, the small diameter intermediates accumulate but fail to mature. This may explain why fibromodulin-deficiency has such a marked effect on fibril structure and tissue strength where it is normally present at a high level. Lumican may be needed at a later stage, primarily to limit lateral growth of fibrils. In tendons at least, lumican functions become critical only in the absence of fibromodulin. The double-null tendon phenotype is far more severe than what one would expect based on the single-null phenotypes alone. This suggests that fibromodulin and lumican not only participate in a common pathway that regulates collagen structure, but that each, *in vivo,* has additional unique functions and implications. We still do not have a clear understanding, at the molecular level, of how collagen structure determines tissue strength. It is generally believed that proteoglycan bridges, that separate parallel arrays of collagen fibrils in the tendon, maintain hydration to resist and transmit tensile stresses [49]. It may be that small diameter collagen fibrils constitute more tensile younger tissues and larger diameter fibrils are characteristic of older tissues lacking tensile strength. Of course this is highly speculative and likely to be a simplistic view of the complex relationship between extracellular matrix ultrastructure and tissue strength.

Regulation of lumican and fibromodulin levels in tissues

Studies of knockout mice have unraveled an interesting regulation of these two proteoglycans in various tissues. In $Fmod^{-/-}$ mice, Svensson and coworkers showed increased immunostaining of lumican in the tendons of $Fmod^{-/-}$ mice [47]. By contrast RNAse protection assays indicated that the level of steady state lumican message is decreased. By immunoblotting of tendon protein extracts showed a dramatic increase in the lumican core protein (Figure 4) [48]. In the $Lum^{-/-}$ mice however, there is no increase in fibromodulin levels, but a slight decrease instead. We noted a similar phenomenon in the $Fmod^{-/-}$ sclera (Chakravarti et al., unpublished observations). By real time RT-PCR on RNA isolated from tendons, we did not detect an increase, but a slight decrease in lumican message in the $Fmod^{-/-}$ background [48]. These experiments confirm the finding that the increased lumican in the tissue occur by some feedback regulation at the posttranscriptional level. A possible explanation is that the cells may somehow sense increased accumulation of extracellular lumican and down regulate transcription. A comparison of transcript levels between $Lum^{+/+}$ and $Lum^{+/-}$ indicates that the amount of lumican message is dependent on the number of functioning lumican genes: $Lum^{+/+}Fmod^{+/-}$ mice show approximately twice as much relative expression of lumican as $Lum^{+/-}Fmod^{+/-}$ mice [48]. The results of real-time PCR analysis of fibromodulin are quite different from those of lumican. First, compared to the wild type mice, *Fmod* message is not over expressed in the $Lum^{-/-}$ genotypes. Second, there is no significant difference in the relative levels of fibromodulin message between the $Fmod^{+/+}$ and $Fmod^{+/-}$ genotypes, indicating an absence of gene-dosage effect [48].

The dramatic increase in lumican protein in the $Fmod^{-/-}$ mice is not due to increased synthesis of the lumican transcript as evidenced by the real time PCR data. Moreover, the increase in lumican protein may be due to reduced degradation and/or increased deposition of lumican on collagen fibrils, or it could also partly be the result of increased rate of protein synthesis. However, insights into mechanisms that regulate tissue levels of these proteoglycans will require further studies. These include estimation of the rate of lumican and fibromodulin synthesis by pulse-chase experiments as well as proteolytic processes involved in their degradation and turnover in the extracellular matrix.

The loss of fibromodulin leads to increased lumican in certain tissues, while the converse, an increase in fibromodulin, is not the case in the lumican-null; such differences in the regulation of lumican in the $Fmod^{-/-}$ mice and fibromodulin in the $Lum^{-/-}$ mice suggest the following. In a multistep regulatory process, fibromodulin precedes lumican. A highly speculative scenario is that some regulatory factor X regulates *Fmod* and another regulatory factor Y, and that Y regulates *Lum*. In the *Fmod*-null, more of the X product is free to act on Y, and higher levels of Y then up regulate lumican (Figure 5).

The lumican, fibromodulin and the double-null mice have provided unprecedented insight into the extent of functions they may have in a range of tissues and a complex mechanism for their co-regulation in tissues. We are at an exciting time in leucine-rich proteoglycan and keratan-sulfate biology. The field of heparan sulfate and cell surface proteoglycans, through the contributions of Merton Bernfield and others, has made significant progress in understanding their diverse role in cell-signaling and regulating cellular functions [50]. We can use a similar approach involving a combination of mouse models, classical biochemistry, cell-culture and gene expression and genomic methods to begin understanding the functions of keratan sulfates and leucine-rich proteoglycans in development, organogenesis and pathogenic changes in disease.

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