KNOCKDOWN OF ZEBRAFISH LUMICAN GENE (ZLUM) CAUSES SCLERAL THINNING AND INCREASED SIZE OF SCLERAL COATS

Lung-Kun Yeh†§, Chia-Yang Liu¶, Winston W.-Y. Kao*, Chang-Jen Huang##, Fung-Rong Hu‡, Chung-Liang Chien†, and I-Jong Wang‡

From the †Department of Anatomy and Cell Biology College of Medicine, National Taiwan University, Taipei, Taiwan; §Department of Ophthalmology, Chang-Gung Memorial Hospital at Linko, Chang-Gung University College of Medicine, Taiwan, R.O.C.; ¶Department of Ophthalmology and * Cell Biology, Neuroscience and Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH, USA; ## Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan; and ‡Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan, R.O.C.

Running Title: Zebrafish model for myopia

Address correspondence to:

† Chung-Liang Chien., Ph.D., Department of Anatomy and Cell Biology, National Taiwan University, Taipei, Taiwan, R.O.C. address: 1, Sec. 1, Jen-Ai Road, Taipei, Taiwan 100; TEL: (886) 2-23123456 Ext. 88193, FAX: (886) 2-23915292, E-mail: chien@ntu.edu.tw;
‡I-Jong Wang, M.D., Ph.D., MBA., Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan, R.O.C. address: 7, Chug-Shan South Road, Taipei, Taiwan 100 TEL: (886) 2-23123456 Ext. 55192, FAX: (886) 2-23412875, e-mail: ijong@ms8.hinet.net

The lumican gene (Lum), which encodes one of the major keratan sulfate proteoglycans (KSPGs) in the vertebrate cornea and sclera, has been linked to axial myopia in humans. In this study, we chose zebrafish (Danio rerio) as an animal model to elucidate the role of lumican in the development of axial myopia. The zebrafish lumican gene (zLum) spans approximately 4.6 kilobases (kb) of the zebrafish genome. Like human (hLUM) and mouse (mLum), zebrafish Lum (zLum) consists of three exons, two introns, and a TATA boxless promoter at the 5′-flanking region of the transcription initiation site. Sequence analysis of the cDNA predicts that zLum encodes 344 amino acids. zLum shares 51% amino acid sequence identity with human lumican. Similar to hLUM and mLum, zLum mRNA is expressed in the eye and many other tissues, such as brain, muscle and liver as well. Transgenic zebrafish harboring an Enhanced Green Fluorescent Protein (EGFP) reporter gene construct downstream of a 1.7 kb zLum 5′-flanking region displayed EGFP expression in the cornea and sclera, as well as throughout the body. Down-regulation of zLum expression by antisense zLum morpholinos (MO) manifested ocular enlargement resembling axial myopia due to disruption of the collagen fibril arrangement in the sclera and resulted in scleral thinning. Administration of muscarinic receptor antagonists, e.g., atropine, pirenzepine, effectively subdued the ocular enlargement caused by morpholinos in in vivo zebrafish.
larvae assays. The observation suggests that zebrafish can be used as an in vivo model for screening compounds in treating myopia.

Myopia is a very common ocular disorder, which is characterized by excessive elongation of the eyeball. In Taiwan, the prevalence of myopia is about 84% of schoolchildren aged 16-18, and the prevalence of high myopia (< -6.0 D) at 18 years of age is 24% in girls and 18% in boys (1). In contrast, the prevalence of high myopia is much lower in Western countries, about 1% of the general population (2). These studies imply that genetic susceptibility of ethnic differences may account for high prevalence of myopia in Taiwanese.

The sclera contains a collagen-rich extracellular matrix that undergoes significant biochemical and biomechanical remodeling during the development of myopia (3). Linkage studies of high myopia have identified potential loci MYP1 (Xq28) and MYP3 (12q21-23); these loci are within and/or near the loci of human genome containing several genes that encode small leucine-rich proteoglycans (SLRP) i.e., biglycan (Xq27ter), decorin (12q21-22), lumican (12q21.3-22), and DSPG3 (12q21) (4-9). Our previous study showed that certain variations (rs3759223 (C->T)) of single nucleotide polymorphism (SNPs) in the lumican regulatory region may influence the promoter activities of lumican and affect fibrillogenesis in myopic eyes (10). Furthermore, Majava et al found that a novel single nucleotide polymorphism (SNP) (c.893-105G>A) of the lumican gene was associated with high myopia (11). Recently, our prospective case-control study also showed that genetic variation in the regulatory domains of the lumican gene (rs3759223 and rs3741834) were associated with high myopia susceptibility among the Han Chinese (12).

Lumican, a member of the small leucine-rich proteoglycan (SLRP) family, is one of the major extracellular components in interstitial collagenous matrices of corneal stroma, sclera, aorta, skin, skeletal muscle, lung, kidney, bone, cartilage, and intervertebral discs (13-20). In corneal stroma, lumican is a KSPG, whereas lumican presents as an under- or unglycanated glycoprotein in other tissues (13, 14, 21, 22). It has been proposed that the horseshoe-shaped lumican core protein binds collagen molecules to modulate collagen fibril diameter, while the N-linked GAG (glycosaminoglycan) chains regulate fibril spacing and stromal hydration for the formation and maintenance of transparent corneas (23-25). The wide distribution of lumican implies that lumican may have multiple functions in tissue morphogenesis and maintenance of tissue homeostasis, besides serving as a regulatory molecule of collagen fibrillogenesis (25). Indeed, lumican plays essential roles in wound healing by modulating epithelial cell migration (26) and in epithelium-mesenchyme transition of the injured lens (27), in addition to regulating collagen fibrillogenesis (20, 26). Lumican-null (Lum\(^{-/-}\)) mice and lumican- and fibromodulin-null (Lum\(^{-/-}\)Fmod\(^{-/-}\)) mice showed alterations of collagen fibril arrangement in interstitial connective tissues. Lum\(^{-/-}\)Fmod\(^{-/-}\) mice exhibited elongated axial lengths and thin sclera, which are characteristics of high myopia, and lumican-null mice (Lum\(^{-/-}\)) also had a slight elongation of the eyeball (28, 29). These findings indicated that these proteoglycans...
may be directly or indirectly involved in scleral development, resulting in the pathoetiology of high myopia.

The zebrafish is an excellent model to study vertebrate genetics and development (30-32). As a disease model, transgenic zebrafish provide several advantages, such as shorter duration of embryonic development and optically transparent embryos, its development also provides easy access for observation and treatment schemes during embryogenesis in comparison to transgenic mouse models. In this study, we examined the structure, expression pattern, promoter activity, and function of \textit{zLum}. Our data indicated that lumican is highly conserved between zebrafish and mammals (e.g., human and mouse) in respect to gene structure, expression patterns, and protein function. In particular, ocular enlargement and scleral thinning with changes in the ultrastructure of the sclera were noted when \textit{zLum} expression was down-regulated by antisense \textit{zLum} morpholinos (MO). Furthermore, \textit{in vivo} zebrafish larvae assays was performed to elucidate the potential application of several muscarinic receptor antagonists to attenuate increased scleral coats caused by \textit{zLum} knockdown with MO. Our results suggest that zebrafish can be used as an \textit{in vivo} model for screening compounds of treating myopia.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Zebrafish Husbandry-} Zebrafish were raised and maintained according to previously established protocols (33). Briefly, adult zebrafish and embryos were maintained at 28.5°C on a 14-h light and 10-h dark cycle. Embryos were sorted at the different stages required for each experiment and staged according to Kimmel et al. (34). Chorions were removed manually with Dumont Watchmaker’s Forceps No. 5. All procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University and performed in compliance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

\textit{Characterization of \textit{zLum}-} To identify the zebrafish expressed sequence tag (EST) clone encoding a putative protein sharing high sequence similarity with the human and mouse SLRP family proteins, we applied the Basic Local Alignment Search Tool (BLAST) analysis of the GenBank database using the full-length human lumican cDNA sequence. An approximately 4.6 kb \textit{Not I/Mlu I} zebrafish genomic DNA fragment containing the 5’ portion of the zebrafish lumican gene was amplified by polymerase chain reaction (PCR) and subcloned into the pBluescript SK vector (Stratagene, La Jolla, CA). The insert was sequenced, using T3, T7 and walk-in primers, by the DNA core of the Department of Molecular Genetics at the National Taiwan University.

The 5’- and 3’-ends of the \textit{zLum} mRNA were amplified using the 5’-Rapid Amplification of cDNA END (5’-RACE) and 3’-RACE Systems, respectively (Invitrogen, Carlsband, CA). For the 5’-RACE experiment, 1 μg of total RNA from zebrafish eyes was reverse transcribed with a lumican-specific primer (5’-AAGTAGAGGTATTTGATTCCGGTC-3’) corresponding to a sequence in exon 2 of the \textit{zLum} gene. The RNA templates were degraded by treatment with an RNase mix. A poly-dCTP tail was added to the 3’-end of the cDNAs with terminal deoxynucleotidyl transferase. The cDNA
was amplified with a second gene-specific primer (5'-GCACAAGAAGGTGATGAAACG-3') corresponding to a sequence from the junction between exon 1 and exon 2 in conjunction with the abridged anchor primer (5'-GGCCACGCGTCGACTAGTACGGGGGIGGG-3'). The resulting PCR products were diluted 100-fold and used as templates to be reamplified with a third gene-specific primer (5'-CAGACTTTAGAAGTCCAGCCAAC-3') in conjunction with the universal amplification primer (5'-CUACUACUACUAGGCCAGCGTCGACTAG TAC-3'). For 3'-RACE, PCRs were performed using a gene-specific primer (5'-GCCTCAGAGATCATTTGAATAG-3') corresponding to a sequence in exon 3 of the zLum gene. The cycling conditions were: 34 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min followed by a 10-min extension at 72°C at the end of the cycles. Finally, the 5'-RACE and 3'-RACE PCR products were gel purified, and the sequences were determined with a dideoxy sequencing protocol. The transcription initiation and termination sites of the zLum gene were determined by a sequence comparison between genomic DNA, the 5'-RACE product, and the 3'-RACE product, respectively.

**Sequence Alignment and Phylogenetic Analysis of zLum** - The amino acid sequences of the open reading frames (ORFs) were initially aligned using the ClustalW program, as previously described (35). The aligned amino acid sequences were used subsequently analyzed with the Neighbor-Joining distance analysis method to construct a phylogenetic tree by using Geneious Pro software (version 4.7; http://www.geneious.com). Bootstrap values were calculated from 100 replicates, and values >50% are indicated at each divergence point.

**RT-PCR** - Unless otherwise specified, RT-PCR reagents used in this procedure were purchased from Promega (Madison, WI). RevertAid™ H Minus First Strand cDNA synthesis kit was purchased from Fermentas (St-Leon-Rot, Germany). Zebrafish cDNA was synthesized using 40 μl of 5x reverse transcription buffer, 20 μl of 0.1 M dithiothreitol, 8 μl of 25 mM dNTPs, 10 μl of RNasin (40 units/ml), 10 μl of 50 mM random hexamers (Pharmacia, Piscataway, NJ), 10 μl of avian myeloblastosis virus reverse transcriptase (9.5 units/μl), and 1 μg of heat-denatured corneal poly(A)+ RNAs. Diethylpyrocarbonate-treated water was added to bring the final reaction volume to 200 μl, and the reaction was incubated at room temperature for 10 min, 42°C for 90 min, 100°C for 2 min, and 0°C for 5 min. Twenty microliters of each of the above RT reactions was added to 80 μl of a PCR mixture containing the following: 8 μl of 10x PCR buffer without MgCl₂, 8 μl of 25 mM MgCl₂, 10 μl of 20 ng/μl primers, 0.5 μl of Taq polymerase (5 units/μl), and 45.5 μl of H₂O. The cycling conditions were: 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min followed by a 15-min extension at 72°C at the end of these cycles. Primers were: CCGCTCGAGCGGATTTTGGCTGGATCC ATTC (Forward 5’- XhoI cut site) and TCCCCGCGGGGACTATTCAAAAGATGATCT CTGAGG (Reverse 3’- SacII cut site). The PCR product was confirmed by an appropriate restriction enzyme digestion and analyzed by electrophoresis on a 1.5% agarose gel.
Generation of an Epitope-Specific Anti-Zebrafish Lumican Antibody - To develop an anti-\textit{zLum} antibody, an oligopeptide deduced from \textit{zLum} cDNA was synthesized (N-terminal peptide, CNERNLKFIIVPTGIKY). The peptides were conjugated to keyhole limpet hemocyanin for antibody production in rabbits. The antibodies were purified through an immune absorbent column of the above zebrafish lumican oligopeptide conjugated to Sulfolink gel (Pierce, Rockford, IL) according to the manufacturer's instructions. Fractions containing purified anti-zebrafish lumican antibody were pooled and concentrated, and the protein concentration was measured by spectrophotometry at 280 nm.

In Situ Hybridization and Immunohistochemistry - Embryos were fixed in 4\% paraformaldehyde in 1x PBS overnight at 4°C, rinsed with PBS 3 times, transferred into 100\% methanol, and stored at -20°C until use. To prevent melanization, embryos raised to time points beyond the 24 hours post-fertilization (hpf) stage were treated with 0.003\% phenylthiourea (PTU). Whole mount RNA \textit{in situ} hybridization was carried out as described previously (35). Sense and antisense digoxigenin (DIG)-labeled oligonucleotide probes were obtained from Bio Basic Inc. (Ontario, Canada). The oligonucleotide sequence (5′-3′) was GTTTCCATCCAGCGCAAGGTCTCCAGTCTAGAGTAGTTGACCGGTAGCTAAATCTGCA. The hybridization signals were visualized with anti-digoxigenin (DIG) antibody-alkaline phosphatase conjugates using procedures recommended by Roche (Roche Applied Science, Indianapolis, IN). The sections were counterstained with 0.5\% neutral red and mounted.

Western Blot Analysis - Total proteins were extracted from adult fish eyes using lysis solution (10 ml containing 1 ml of 200 mM HEPES/KOH buffer (pH 7.5), 200 mM sucrose (0.86 g), 50 mM KCl (0.04 g), 2.5 mM MgCl$_2$ (0.005 g), and 100 µl of 100 mM DTT). To remove keratan sulfate chains, protein aliquots were incubated with 0.1 unit/ml endo-\(\beta\)-galactosidase (Sigma, St. Louis, MO) and 1 unit/ml keratanase (Sigma, St. Louis, MO) at 37°C overnight. The zebrafish corneal extracts were subjected to 10\% SDS-PAGE and then probed with the anti-zebrafish lumican N-terminal peptide antibody (0.1 µg/ml) as described.
above. The immuno-complex was visualized by incubation with goat anti-rabbit IgG alkaline phosphatase-conjugate (1:2,500, (Novagen, Madison, WI, USA)) and Western Blue® stabilized substrate (Promega).

**Analysis of zLum Promoter by Transgenic Zebrafish**- Genomic DNA, both 1.7 kb and 0.48 kb from the 5'-untranslated region of the zLum gene, were amplified with specific PCR primers and inserted into the multiple cloning sites of pBluescript II SK vectors (Stratagene, La Jolla, CA) containing an EGFP sequence. PCR primers are:

5’-ATAAAGATGCGGCCGCTCCATTAATTCGACAGACCAG-3’,
5’-ATAAAGATGCGGCCGAGGTAGACAACACGGTTATGT-3’ (forward primer) and 5’-CGACGCGTGGCTGCACAACTTAAATTACCTCCTAATTAAACGGTTATGT-3’ (reverse primer). The recombinant plasmids were propagated in *Escherichia coli* DH5α and purified with a QIAGEN Plasmid Purification Maxi kit. Purified plasmid DNA was adjusted to 50 ng/μl in distilled water and microinjected into one-cell-stage zebrafish embryos under a dissecting microscope. Embryos with GFP expression were observed and imaged under a fluorescence microscope.

**zLum Knockdown by Morpholino Injection**- A morpholino-antisense oligonucleotide (Gene Tools, Philomath, OR) were designed to target the 5’-untranslated and/or flanking regions, including the translation start codon of the respective genes. The MO sequence was designed as follows: zLum-MO,

5’-GATCCCAGAGCAAACATGGCGCTGAC-3’.

This oligonucleotide complemented the sequence from -8 through +17 with respect to the translation initiation codon. A search of the database did not identify any sequence similarity of known zebrafish genes to zLum-MO. A random sequence MO (RS-MO) serves as a control for zLum-MO: 5’-CCTCTTACCTCAGTACAATTATA-3’.

This RS-MO was obtained from Gene Tools as a standard control oligonucleotide with no target specificity. Solutions were prepared and injected at the 1-4-cell stage as described by Nasevicius et al. (36).

**Transmission Electron Microscopy**- Corneas of zLum-MO-injected, RS-MO-injected, and wild type zebrafish at 7 and 12 days post-fertilization (dpf) stage were fixed in 50 mM phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 24 h at room temperature. After re-fixation in 1% osmium tetroxide for 4 h at room temperature, the samples were washed in phosphate buffer, dehydrated, and embedded in Epon 812 epoxy resin. Semithick sections (100 nm) were stained with toluidine blue. Ultrathin 50-nm sections were collected on 75 mesh copper grids and stained with uranyl acetate and lead citrate, and images were photographed with a Hitachi 7100 Transmission Electron Microscope (TEM) (Hitachi, Tokyo, Japan) equipped with an AMT Digital camera.

**Image Analysis**- Collagen fibril diameters and scleral thickness were measured with Image-Pro Plus v 4.5. Corneal stroma, anterior sclera and posterior sclera from 6 zLum-MO and 6 wild type zebrafish at each 7 and 12 dpf stage were analyzed. Six to 12 areas of collagen fibrils were analyzed for each region in each group, generating 772 to 1678 measurements for each condition and 27 to 84 measurements were generated by analysis of scleral thickness for anterior and posterior sclera.
in each group at 12 dpf stage. Collagen fibers and scleral thickness are represented as the mean ± SD µm, all measurements were analyzed using Student’s t-tests assuming unequal variances.

**RESULTS**

**Primary Structure of zLum and Alignment Analysis of the Zebrafish Lumican Amino Acid Sequence**- We identified zLum by performing a BLAST search of the publicly available zebrafish databases with human LUM. The zLum gene is 11 kb upstream of zKera (keratocan) (Fig. 1). We have isolated clones representing the full open reading frames (ORF) of zebrafish lumican. A cDNA clone encoding the lumican ORF was subcloned into the pBluescript SK vector (Stratagene, La Jolla, CA). The ORF of the zLum gene was 1,032 bp long and encoded 344 amino acid residues. The proved nucleotide sequence of the zebrafish lumican gene (zLum) gene, complete cds was submitted to the GenBank database under the GenBank Accession Number GQ376197. This sequence has been scanned against the database and is significantly related to the sequence (NM_001002059.1.), which is derived from BC071347.1 and has not yet been subject to final NCBI review (40).

The entire genomic DNA sequence of zLum was shown in supplementary data (supp Fig.1). The zLum gene spans approximately 4.6 kb (4610 bp) and contains three exons and two introns (Fig. 1). Sequence analysis revealed that exon 1 contains 26 untranslated nucleotides, exon 2 contains 24 noncoding and 880 coding nucleotides, and exon 3 contains 152 bases of coding sequence and 1,106 bases of 3’-untranslated sequence. The transcription initiation site marked +1 was determined by 5’-RACE, as described in Methods. The first translation initiation ATG codon is located at the 844th base downstream of the beginning of exon 1. There was no TATA consensus sequence found in the approximately 2.58 kb of proximal 5’-flanking region. The full-length zLum cDNA clone (approximately 1.9 kb) contains a 1,032-bp ORF of 344 amino acids of zLum core protein. Like other SLRP core proteins
such as bovine lumican (41), bovine mimecan (42) and mouse keratocan (43), zebrafish lumican consists of three distinct domains, a highly conserved central leucine-rich repeat region flanked by hypervariable N- and C-terminal regions. As shown in supp Fig. 1, after the signal peptide (M-Y, the first 20 aa), the negatively charged N-terminal domain contains a possibly sulfated tyrosine and four possibly conserved cysteine residues that form intra-molecular disulfide bonds, followed by nine tandem leucine-rich repeats (LXXLXLXXNXL/I) which are similar to lumican of other species and might mediate binding to other extracellular matrix components and C-terminal globular domains containing two conserved cysteine residues.

Sequence Alignment and Phylogenetic Analysis: For comparison, multiple alignment analysis of the predicted amino acid sequences of zLum with those of other species is shown in supp Fig. 2. Using the sequence of zLum, a search for homologous sequences was made with BLAST. The predicted amino acid sequences showed a high homology among lumican core proteins of different species. Zebrafish lumican shared 51% amino acid identity to human, 50% amino acid identity to mouse and 53% amino acid identity to chicken lumican, respectively. Human lumican shared 86% amino acid identity to mouse and 68% amino acid identity to chicken lumican.

To analyze the evolutionary relationships, a bootstrapped neighbor-joining tree using Jukes-Cantor model was used to illustrate the relationship between different 18 species (Fig. 2). The numbers at the nodes represent the statistical confidence estimated by the bootstrap procedure. Bootstrap values were calculated from 100 replicate, and values >50% are indicated at each divergence point. The bootstrap values allow inspection of the relationships among clades with low or no ambiguity. The mouse lumican appears to be more closely related to the lumican of human and primates. The zebrafish and chicken lumican are closer to those of Salmo salar and Taeniopygia guttata as compared to human and other mammalian lumican, whereas they displayed similar structure and >50% homology with those of other species.

Spatial Distribution of Zebrafish Lumican mRNA: To further confirm the expression patterns of zLum, RT-PCR analysis was carried out using template cDNAs that were reverse transcribed from total RNAs from various tissues. The results of RT-PCR revealed that an approximately 1-kb RT-PCR product was abundant in most tissues examined, e.g., eye, brain, liver, muscle, and fin (Fig. 3). Whole-mount in situ hybridization was used to analyze the expression of zLum mRNA during development. The results revealed that zLum mRNA was widely expressed in many tissues throughout the fish body, and it is highly expressed in the embryonic fore-, mid-, and hindbrain, anterior spinal cord, and eyes at 1 dpf and 3 dpf (Fig. 4A-C). The corresponding control sense riboprobes showed negative staining in samples (Fig. 4D). Lumican could be found in the corneal stromal layer of adult human and mouse corneas (44). In the zebrafish eye, zLum mRNA was also expressed mainly in the corneal stromal layer (Fig. 4E-F) and sclera (Fig. 4G-H), which was similar to other species (45, 46).
negligible hybridization signals in samples (Fig. 41-L).

**Characterization and Spatial Distribution of Zebrafish Lumican Protein-** An affinity-purified anti-zLum antibody against a synthetic peptide N-terminal peptide, (CNERNLKFIPIVPTGIKY) corresponding to the 18 N-terminal amino acid residues deduced from the zLum cDNA was generated to detect zebrafish lumican. Immunohistochemistry with anti-zLum antibodies showed that similar to human and mouse lumican which were both found in the corneal stromal layer (26), zLum was also present mainly in corneal stromal layers (Fig. 5A-B) and scleral tissue (Fig. 5C-D), and weak immunostaining signal was also detected in the corneal epithelium and surrounding tissues, such as the iris and ciliary body. No immunoreactivity could be detected in samples incubated with pre-immune rabbit IgG (Fig. 5E-H). Tissue sections probed with anti-keratan sulfate (KS) antibody demonstrated that KS-GAG (keratan sulfate - glycosaminoglycan) was primarily present in corneal stroma, little or no immune reactivity could be seen in the corneal epithelium (Fig. 5I-J). Keratanase treatment abolished the immune reactivity seen in the stroma by the anti-KS antibody (Fig. 5M-N). It was of interest to note that no immune reactivity was seen in the scleral tissue by the anti-KS antibody (Fig. 5I, 5K-L). This observation indicates that KS-GAG is not present in the scleral tissue, consistent with the notion that zLum exists as KSPG in corneal stroma and under-glycanated glycoprotein in other tissues, such as the corneal epithelium and sclera.

To further demonstrate that the lumican in zebrafish cornea is a proteoglycan, total lysate from zebrafish eyes were treated with or without keratanase or endo-β-galactosidase digestion and were then subjected to western blotting analysis. Fig. 6 showed that a major band of 50 kD and multiple high molecular weight bands ranging from 60 to 170 kD were labeled by the antibodies (lane 1) in specimens prepared from eyes without enzyme digestion. By contrast, in specimens treated with keratanase (lane 2) or endo-β-galactosidase digestion (lane 3), the high molecular weight bands diminished, but the major band at 50 kD remained. These data indicated that the zLum protein isolated from eyes exists in two forms, as a KSPG and an under-glycanated core protein.

**zLum Promoter Activity in Transgenic Zebrafish-** To confirm that the genomic DNA clone of zLum isolated was indeed derived from a functional allele, we examined promoter activity of the 5’ flanking sequence of the zLum genomic DNA, using transgenic zebrafish microinjected with zLumpr1.7-EGFP-bpA and zLumpr0.5-EGFP-bpA, respectively (Fig. 7B-C). Live transgenic EGFP-positive embryos were screened and selected under a fluorescent microscope. In transgenic zebrafish, 86% (212/246) of the 1.7-kb zLum promoter fragment-injected embryos expressed the EGFP transgene throughout the fish body, including the eyes, at 3 and 7 dpf (Fig. 7D-F). However, the 0.5-kb promoter fragment of zLum failed to drive EGFP expression in transgenic zebrafish (0 /113) (Fig. 7G). These results indicated that the 1.7-kb zLum promoter is able to drive EGFP expression in the transgenic fish, whereas deletion of the 5’ 1.2 kb of the sequence completely eliminates EGFP expression, suggesting that the 1.2-kb region between the 1.7-
kb and 0.5-kb 5’ flanking sequence consists of the necessary regulatory elements controlling lumican promoter activity for zLum expression in vivo.

Effects of zLum Knockdown on Embryonic Development- To investigate zLum function during zebrafish development, MO was microinjected into fertilized zebrafish eggs. Western blot showed that zLum protein decreased after zLum-MO was injected. (Fig. 8A) Retarded development was seen in the zLum-MO embryos as compared to wild type embryos at the 22 hpf stages (Fig. 8C-D). Significant morphological variations could be readily identified between zLum-MO embryos and control embryos beginning at 6 dpf stage, whereas there were no identifiable differences in phenotypes between the control RS-MO and wild type embryos. About 58.6% of zLum-MO-injected 7-dpf larvae (277/473) exhibited severe major defects, whereas less than 1% of the RS-MO negative control group (1/105) and 2.1% of the wild type group with mock injection of PBS alone (9/438) had minor morphological changes. A higher dose of zLum-MO (20 ng/2 µl) produced more severe abnormalities in embryos and led to a higher mortality rate (187 dead embryos out of 211 total injected). The most common abnormalities of the zLum MO-injected group were enlarged eyes, enlarged pericardium, and deformed body shape (Fig. 8E-F). In particular, significantly enlarged ocular eyeballs were noticed in the zLum-MO-injected group compared to the RS-MO-injected group and wild type group from around the 6 dpf stage. Significant increases in axial lengths were noted in the zLum-MO-injected group compared to those of RS-MO-injected group and wild type group (276.83 ± 27.4 µm (MO) vs. 237.04 ± 12.8 µm (RS control) and 276.83 ± 27.4 µm (MO) vs. 243.32 ± 10.69 µm (WT), respectively, all p<0.05) (Fig. 8G). Moreover, the zLum-MO-injected group had larger eyeball diameters in comparison to those of the RS-MO-injected group and wild type group (443.12 ± 58.1 µm (MO) vs. 337.58 ± 16.6 µm (control) and 443.12 ± 58.1 µm (MO) vs. 342.57 ± 14.31 µm (WT), respectively, all p<0.05) (Fig. 8H). The control group injected with RS-MO had normal eye morphologies like the mock-injected control group.

Decreased Lumican Synthesis Results in Abnormal Collagen Architecture of the Corneal Stroma and Scleral Tissue- To elucidate the function of lumican in regulating collagen fibrillogenesis in the fish eye, eyes of zLum-MO fish were subjected to morphological analysis with TEM at 7 and 12 dpf (n = 6, in each group). Fig. 9 shows the ultrastructural changes of collagenous matrix at corneal stroma (CS), anterior sclera (AS) and posterior sclera (PS) area (as shown in Fig. 9A) between the zLum-MO and wild type group at 12 dpf stage. The fibril architecture of corneal stroma and anterior sclera collagen of MO-treated group differed from that of control group and appeared irregularly, with large variations in fibril diameter and fibril spacing in comparison to WT control (compare Fig. 9C to 9D; Fig. 9E to 9F). However, there was no significant difference in the diameter of collagen fibrils at the posterior sclera area between both group (compare Fig. 9G to 9H). Fig. 9B, summary of the data of fibril diameter measurement, showed that there was significant difference in the collagen fibril diameter of corneal stroma and anterior sclera matrix between the wild type and zLum-MO group at 12 dpf stage.
([CS]): 13.80 ± 2.84nm (WT) vs. 22.71 ± 4.46nm (MO), p< 0.05, and (AS): 14.91 ± 3.32nm (WT) vs. 20.42 ± 3.63nm (MO), respectively, p< 0.05), whereas there was no significant difference in the collagen fibril diameter of posterior sclera (PS) matrix between the wild type and zLum-MO group at 12 dpf stage ([PS]: 16.34 ± 4.0nm (WT) vs. 16.01 ± 3.71nm (MO), p> 0.05).

Interestingly, a larger size of eyeball accompanied by thinner sclera was noted in the zLum MO-injected larva (Fig. 8F, 9, Fig. 10). Lower magnification of electron microscopy showed that posterior scleral tissue from the wild type group consisted of approximately 3-4 layers of fibroblastic cells with newly formed collagen fibrils in relatively regular rearrangements at 7 dpf stage in the posterior sclera (Fig. 10A). However, there was only about 1 layer of fibroblastic cells in the posterior sclera of MO-treated larva at 7 dpf stage (Fig. 10B), which consisted of very few and irregular collagenous matrix with large variegations in fibril diameters and fibril spacing (Fig. 9H). Fig. 10C showed a significant decrease in the posterior scleral thickness found in zLum-MO treated group (MO) in comparison to that of control group (WT) at 7 dpf stage ([PS]: 5.93 ± 0.81µm (WT) vs. 1.49 ± 0.68µm (MO), p< 0.05); in contrast there was no significant decrease in the thickness of anterior sclera thickness between both MO and WT groups([AS]: 5.64 ± 1.01µm (WT) vs. 6.19 ± 0.60µm (MO), p> 0.05)). At 12 dpf stage, the scleral thickness is significantly decreased at both anterior and posterior regions of sclera of MO-treated group ([AS]: 5.44 ± 0.94µm (WT) vs. 3.36 ± 0.37µm (MO); and (PS): 4.87 ± 1.34µm (WT) vs. 1.36 ± 1.12µm (MO), p<0.05) (Fig. 10C).

In conclusion, our results demonstrated that the ultrastructural changes of scleral tissue caused by decreased lumican synthesis with morpholinos were similar to the features found in sclera of mammalian model of high myopia. Taking the advantage that zebrafish eye development can be easily manipulated, e.g., administration of morpholinos and analyzed, we have attempted to develop a protocol of using zebrafish as an experimental model to test the efficacy of compounds, e.g., antagonist of muscarinic receptors, which can potentially modulate the pathoetiology of myopia as described below.

**Administration of Muscarinic Receptor Antagonists Reverts The Enlargement of Scleral Coats Caused by zLum Knockdown with Morpholinos**– To evaluate if the zebrafish could be an experimental drug screening model for treatment of induced myopia, we tested several different muscarinic receptor antagonists that have been used in treating myopia. As shown in Fig. 11, two drugs out of three muscarinic receptor antagonists tested, atropine (0.5%) and pirenzepine (0.25%), produced promising outcomes in that there were significantly reduction in excessive axial elongation and enlarged scleral coats observed in the treated zLum-MO group as compared to those of untreated zLum-MO group (Fig. 11D, E vs. C). Whereas, no obvious improvement was noted in the eye of fish treated with 0.01% methoctramine (M2 receptor antagonist) and injected by zLum-MO at 7 dpf stage (Fig. 11F). To better evaluate the effects of drugs, we measured the diameters of retina and sclera of the experimental fish (as shown in Fig. 12A, B). Fig. 12C (lane 1-5) showed a significant reduction in the excessive...
axial elongation found in the atropine-treated and pirenzepine-treated groups as compared with those of the untreated zLum-MO group at 7 dpf stage ((205.01 ± 21.10 µm (atropine-treated) and 208.27 ± 23.51 µm (pirenzepine-treated) vs. 248.71 ± 20.41µm (untreated), both have p <0.001, n=55, 64, and 70, respectively)), whereas there was no significant change in the axial length in methoctramine-treated group (242.05 ± 21.68 µm , p> 0.05, n=56). Fig. 12C (lane 6-10) showed a significant decrease in scleral diameters in the atropine-treated and pirenzepine-treated groups as compared with those of the untreated zLum-MO group at 7 dpf stage ((323.51 ± 46.83 µm (atropine-treated) and 326.18 ± 32.88 µm (pirenzepine-treated) vs. 399.07 ± 59.68 µm (untreated), both have p values<0.001, n=55, 64, and 70, respectively)), whereas there was no significant reduction of scleral diameter in methoctramine-treated group at 7 dpf stage (381.47 ± 43.74 um , p > 0.05, n=56).

Another index of eye enlargement is the ratio of retina diameter to sclera diameter (the smaller the ratio the larger the eye). Fig. 12D showed that there was a significant decrease in the ratio of RPE/scleral coat was noted during ocular enlargement caused by the knockdown of zLum ((97.22%±3.86% (WT) vs.56.11%±4.64% (MO), p<0.001)). The administration of muscarinic receptor antagonists, 0.5% atropine and 0.25% pirenzepine, effectively attenuate the decrease in the ratio of RPE/scleral coat caused by zLum knockdown group ((71.29%±8.52% (atropine-treated) and 71.33%±9.37% (pirenzepine-treated) vs.56.11% ± 4.64% (MO), both have p values< 0.001, respectively)), whereas there was no obvious reversion of the decreased ratio of RPE/scleral coat in the 0.01% methoctramine-treated (58.19%±6.37% (methoctramine-treated) vs.56.11%±4.64% (MO), p > 0.05)).

**DISCUSSION**

In the present study, we have demonstrated that zebrafish lumican has all structural features of SLRPs, i.e., a central domain of nine leucine-rich repeats flanked by N- and C-terminal domains with conserved cysteine residues (47). Interestingly, zLum does not have a TATA box in its promoter. Unlike the TATA-less promoters of housekeeping genes, the zebrafish lumican promter does not have a GC-rich sequence. As shown in Fig. 7, our results implied that zLum exists as a KSPG in corneal stroma but as unglycanated glycoprotein in sclera.

The sclera, a fibrous extracellular matrix, contains irregularly arranged lamellae of collagen fibrils, proteoglycans, elastin, and matrix secreting fibroblasts (48). The changes of collagen matrix in the sclera caused by increased or decreased proteoglycans may lead to changes in the biomechanical properties of the sclera and may ultimately lead to the alteration of the eyeball shape seen in myopic patients (48-50). Therefore, the sclera is a dynamic tissue rather than a static container of the eye. Moring et al. found that biglycan and lumican mRNA levels were lowered in the sclera during experimentally induced myopia and increased during recovery in the tree shrew model (51). Lum(-/-) mice and Lum(-/-)Fmod(-/-) mice presented altered collagen fibril diameter in sclera (52-53), suggesting that these proteoglycans play an important role in the biomechanical properties of the sclera. Our zebrafish model indicated that altered collagen
fibril diameter in corneal stroma and anterior sclera by \( zLum \) knockdown with morpholinos is similar to some features of the \( Lum^{(-/-)} \) mice (Fig. 10). Although the collagen fibril diameter was altered in lumican-null mice, there was no ocular enlargement found in \( Lum^{(-/-)} \) mice. Young TL et al proposed that the phenotypes of \( Lum^{(-/-)} \) mice and \( Lum^{(-/-)}Fmod^{(-/-)} \) mice may be the possibility of false positive results due to “hitchhiker” effect (54-55). However, ocular enlargement and scleral thinning resembling characteristics of high myopia could be readily recognized in our zebrafish model of decreased synthesis of lumican caused by \( zLum \) knockdown with morpholinos. This difference can be in part explained by the fact that in mice there are more layers of lamellar organization in anterior and posterior sclera tissue than that of zebrafish, thus mouse sclera may be more resilient to the loss of lumican and maintain a stable sclera structure seen in \( Lum^{(-/-)} \) mice.

The changes in refractive status in developing myopia are greatest from the elongated axial length of the eyeball rather than from the changes of corneal and anterior lens curvature. In humans, thinning of the scleral tissue, particularly at the posterior pole following the elongation of eyeball, is a characteristic of high myopia which is compatible with our observations in this zebrafish model in that the most immature collagen fibrils are located in the posterior sclera (48). Therefore, decrease and/or disruption of the arrangement of collagen fibrils in this region are highly probable to weaken sclera strength and integrity and result in ocular enlargement seen in our zebrafish model.

In this study, we also found that the number of scleral fibroblasts are significantly reduced at the posterior sclera of \( zLum \)-MO injected fish (Fig. 10B). Thus, the lumican protein may also affect the functions of scleral fibroblasts during early development. Kao et al has suggested that lumican is a matrikine and can modulate fibroblast activities in addition to serving as a regulator of collagen fibrillogenesis (25, 56).

In present study, we have also established a brand new platform of using the \( zLum \)-MO zebrafish as a screen tool for identifying drugs that can affect myopic progression. Recently, Barathi et al confirmed the presence of all five (M1~M5) mAChRs in human and mouse scleral fibroblasts (57). Muscarinic acetylcholine receptors have also been found in the brain of the zebrafish by radioligand binding techniques (38). Liu et al demonstrated that muscarinic antagonists acted directly on the sclera to prevent myopia development induced by form deprivation in guinea pigs in which myopia progression was accompanied by over-expression of muscarinic acetylcholine receptors (M1 & M4) (58).

Furthermore, atropine, a nonselective muscarinic antagonist, and pirenzepine, an antagonist specific for M1, have been found to be effective in clinical trials to prevent myopia progression (59, 60, 61, 62). In experimentally induced myopia including chicks and tree shrews, several muscarinic antagonists have been applied to control ocular growth in different species, although the mechanism of drug effects against myopia remain unclear (63-65). However, variable effects on experimental myopia were shown by applying these muscarinic antagonists when administered intravitreously or subconjunctival injection (37, 63). In current study, we chose these muscarinic antagonists, i.e. 0.5% atropine, 0.25% pirenzepine
and 0.01% methoctramine, according to previous study with minor modifications (37). In our result, we showed that the effect of ocular enlargement due to the reduction of \( z\text{Lum} \) protein was blocked by atropine and pirenzepine, but not by the M2-selective receptor antagonist, methoctramine. Thereby, the observations are similar to the effects of muscarinic receptor antagonists in other myopic animal models and clinical trials. With our model, one can quickly screen drugs that may have therapeutic effects on controlling myopia progression within a week. Another advantage of our model is it can be easily and conveniently carried out by adding the drugs to the embryos kept in 96-well tissue culture plates without any injection procedures to the experimental animals.

In conclusion, we have identified and characterized the zebrafish lumican gene. Lumican is highly conserved during evolution. \( z\text{Lum} \) shares high homology with human and mouse lumican with respect to gene structure, expression pattern, and promoter function. Moreover, we present evidence of ultrastructural changes of ocular enlargement, which are similar to the features of high myopia in humans. We have also established a protocol of drug screening test for the identification of compounds that are of potential in modulating scleral growth. Therefore, the zebrafish can serve as a potential vertebrate model for studying the early development of corneal and scleral diseases.

FOOTNOTES

We would like to thank Ms. Chun-Wen Chen, Ms. Yu-Ching Wu, and Ms. Ting-Shuan Chiang for their excellent technical assistances throughout the course of this study and Dr. Bei-En Chang and Dr. Huey-Jen Tsai for their critical comments on the manuscript. We also would like to thank Ms. Hui-Chun Kung and Ya-Ling Chen for preparation of TEM studies at the Microscope Center of Chang-Gung Memorial Hospital. The studies were supported in part by NSC grants: 923112B002004, 933112B002031, 933112B002007, 932314B002090, 942314B002043, 952314B002149MY3, 972314B182A059, 982314B182A029MY3, 983112B002040 and grants from NIH/NEI EY12486(CYL) and EY011845(WWYK), Research to Prevent Blindness, and Ohio Lions Eye Research Foundation. CYL was a recipient of Olga Weiss Scholarship from RPB. The nucleotide sequence for the zebrafish lumican gene has been deposited in the GenBank database under the GenBank Accession Number GQ376197 (zebrafish lumican gene).
REFERENCES


**Figure Legends**

**Fig. 1. Schematic diagram showing the organization of the zLum gene.** The zebrafish lumican gene is 11-kb upstream from the keratocan gene. It contains three exons and two introns. The figure shows the zebrafish lumican gene drawn to scale. Blank boxes indicate the coding region of the mRNA. The translation start and stop codons are indicated by ATG and TAA, respectively.

**Fig. 2. Neighbor-joining phylogenic tree of the lumican protein.** Phylogenetic tree of lumican protein from 18 different species. The numbers at the nodes represent the statistical confidence estimates computed by the bootstrap procedure. Bootstrap values were calculated from 100 replicate, and values >50% are indicated at each divergence point. Canis familiaris (dog); Equus caballus (horse); Sus scrofa (pig); Bos taurus (cow); Mus musculus (mouse); Rattus norvegicus (rat); Pan troglodytes (chimpanzee); Macaca fascicularis (macaque); Macaca mulatta (rhesus mulatta); Homo sapiens (human); Pongo abelii (Sumatran orangutan); Ornithorhynchus anatinus (platypus); Salmo salar (Atlantic salmon); Danio rerio (zebrafish); Silurana tropicalis (xenopus); Taeniopygia guttata (Zebra Finch); Coturnix japonica (Japanese Quail); Gallus gallus (chicken). The tree was constructed with Geneious Pro software (version 4.7; http://www.geneious.com).

**Fig. 3. zLum expression by RT-PCR analyses.** RT-PCR was carried out using total RNAs from adult fish eye (lane 1), brain (lane 2), heart (lane 3), liver (lane 4), gut (lane 5), muscle (lane 6), and fin (lane 7). Note that amplified zLum PCR product could be found not only in the eye but also in other tissues (bottom panel).

**Fig. 4. In situ hybridization analyses of zLum mRNA in early zebrafish larvae (whole mount) and adult fish cornea (sections).** Hybridization signals were demonstrated with anti-digoxigenin antibody-alkaline phosphatase conjugates. (A) zLum mRNA was detected in zebrafish larvae (24 hpf). (B) In older zebrafish larvae (48 hpf), the zLum mRNA was detected in the eyes and all the major subdivisions of the embryonic central nervous system, including the fore-, mid-, and hindbrain and the spinal cord. (C) Strong hybridization signals could be also found in corneal tissue at the 48 hpf. (D) No hybridization signals could be detected in sense probe hybridization group at the 48 hpf larval stage. (E) In the zebrafish eye, z lum mRNA was predominantly detected in the corneal stromal layer. (F) Higher magnification showed that hybridization positive signals are observed in the corneal stromal layer. (G) zLum mRNA was also detected in the periocular extracellular matrix. (H) Higher magnification showed that hybridization-positive signals are observed in the scleral layer (arrow): (I, J, K, L) No hybridization signals could be detected in the adult eye (I), cornea (J), sclera (K, L) using a sense probe in hybridization. Sections were counterstained with fast red. (scale bars = 100 μm, E, G, I, K; scale bars = 50 μm, F, H, J, L)
Fig. 5. Immunohistochemistry staining pattern of zebrafish eye using the epitope-specific anti-zlum antibody. (A) The lumican protein was found in zebrafish eye tissue. (B) Note that lumican protein was found mainly in the corneal stromal layer. (C, D) The lumican protein was also found in the scleral tissue (arrow). (E-H) No immunoreactivity was detected in the negative control group. (E: whole eye; F: corneal tissue; G, H: scleral tissue) (I-P) Tissue sections treated with (M-P) and without (I-L) keratanase and stained with an anti-keratan sulfate antibody. (I, J) Tissue sections showed that keratan sulfate chains existed mainly in the corneal stromal layer. (K, L) Keratan sulfate chains did not exist in the scleral layer. (M-P) There was nearly no immunoreactive reaction in tissue sections treated with keratanase and stained with anti-keratan sulfate antibody. (A, E, I, M: scale bars = 200 μm; C, G, K, O: scale bars = 100 μm; B, D, F, H, J, L, N, P: scale bars = 50 μm; arrow indicates scleral tissue: D, H, L, P).

Fig. 6. Western blot analysis of zebrafish lumican. Total proteins extracted from adult zebrafish eyes by lysis buffer were subjected to 10% SDS-PAGE followed by western blotting. Samples without treatment (lane 1) and with either keratanase (lane 2) or endo-β-galactosidase treatment (lane 3) were probed with a rabbit anti-zebrafish lumican antibody. The result showed multiple bands with a similar smearing pattern without enzyme digestion (lane 1), whereas one approximately 50-kDa band was visualized after treatment with keratanase (lane 2) and endo-β-galactosidase (lane 3). These data demonstrate that the zebrafish lumican protein contains keratan sulfate chains.

Fig. 7. Generation of transgenic fish harboring zLumpr1.7-EGFP SV40 and zLumpr0.5-EGFPSV40. (A) Schematic representation of the zebrafish lumican gene. (B) Structure of zLumpr1.7-EGFP SV40 (3.3 kb). It contains a 1.7-kb 5’-regulatory region of the zlum gene, the untranslated region of exon 1 (844 bp), an SV40 polyadenylation signal, and the pEGFP vector sequence. (C) Structure of zLumpr0.5-EGFPSV40 (2.0 kb). It contains a 0.5-kb 5’-regulatory region of the zlum gene, the untranslated region of exon 1 (844 bp), an SV40 polyadenylation signal, and the pEGFP vector sequence. (D) EGFP expression was observed in the 3 dpf zebrafish after injecting linearized zLumpr1.7-EGFP SV40 DNA fragment. (E) Under different observation view at the same fish (3 dpf), strong EGFP expression was detected in corneal tissue. (F) EGFP was expressed at the 7 dpf stage. (G) As a control, no EGFP was detected after injecting the linearized zLumpr0.5-EGFP SV40 DNA fragment.

Fig. 8. Ocular enlargement developed due to the reduction of zLum protein by morpholino microinjection. (A) Western blot was carried out using embryo lysates from wild type embryos (lane 1), and zLum-MO-injected 3-dpf embryos (lane 2). It showed that zLum protein decreased after zLum-MO was injected. (B) This figure showed the definition of axial length (AL) and diameter (D) of eye in zebrafish. (C) RS-MO-injected embryos showed normal phenotypes as a control group at 22 hpf stages. (D) Developmental delay was found in the zLum-MO-injected group at 22 hpf stages. (E) Normal phenotype was noted in the RS-MO-injected group at the 7 dpf stage. (F) Significant ocular enlargement was noted in the zLum-MO-injected group at the 7 dpf stage. (G) Significantly increased axial length was noted in the zLum-MO-injected group as compared to the RS-MO-injected embryos and wild type embryos at 7 dpf (Respectively, p<0.05). (H) Significantly increased eyeball diameter was also noted in
the zLum-MO-injected group compared to the RS-MO-injected embryos and wild type embryos at 7 dpf (Respectively, \(p<0.05\)).

**Fig. 9.** \(z\text{Lum}\)-MO knock-down induces ultrastructural changes in the corneal stroma (CS), anterior sclera (AS) and posterior sclera (PS). (A) WT fish at 12 dpf stage in toludine blue staining. The figure indicates corneal stroma (CS), anterior sclera (AS), and posterior sclera (PS). (B) The diameters of collagen fibril were analyzed in the corneal stroma, anterior and posterior sclera of 12 dpf-old-wild type and \(z\text{Lum}\)-MO-injected group. Significant increasing in collagen fibril diameter of corneal stroma and anterior sclera are noted in the \(z\text{Lum}\)-MO group, whereas the diameter of collagen fibril in posterior sclera is not significantly different in both groups. (C-H) Morphological comparison of collagen fibril architecture in the corneal stroma (C-D), anterior scleral tissue (E-F) and posterior scleral tissue (G-H) between the control group (C, E, G) and \(z\text{Lum}\)-MO-injected group (D, F, H) at the 12 dpf stage. (C) TEM micrograph showing regular and smaller fibril architecture of collagen localized in the corneal stroma of the wild type group. (D) Irregular arrangement and increasing collagen fibril diameter was found in the corneal stroma of the \(z\text{Lum}\)-MO-injected group. (E) TEM micrograph showing relatively regular fibril architecture of collagen localized in the anterior sclera of the wild type group. (F) Irregular collagen fibrils with increasing fibril diameter were noted in the anterior sclera of the \(z\text{Lum}\)-MO-injected group. (G) Top is adjacent to the retina. TEM micrograph showing fibril architecture of collagen localized in the posterior sclera of the wild type group. (H) Top is adjacent to the retina. TEM micrograph showing irregular and little collagen fibril architecture was noted in the posterior sclera of the \(z\text{Lum}\)-MO-injected group. (C-H, scale bar: 100 nm)

**Fig. 10.** Ultrastructure changes in scleral thinning in \(z\text{Lum}\)-MO group. (A) Top is adjacent to the retina. Two to three layers of scleral fibroblastic cells with collagen fibril formation between the layers found at the posterior sclera of the WT fish at 7 dpf stage. (B) Top is adjacent to the retina. Only one to two layers of fibroblastic cells at the posterior sclera of the \(z\text{Lum}\)-MO-injected fish at 7 dpf stage. (C) Scleral thinning was observed obviously in the \(z\text{Lum}\)-MO-injected fish at 7 dpf stage. The phenomenon was much more prominent in the \(z\text{Lum}\)-MO-injected fish at 12 dpf stage. In particular, significant scleral thinning was observed in the posterior sclera of the \(z\text{Lum}\)-MO-injected fish at 7 and 12 dpf stage as compared with wild type group. (A, B: scale bar: 1.5 um).

**Fig. 11.** Zebrafish larvae assay for drug screen. (A) Normal phenotype of WT fish at 7 dpf stage. (B) Normal phenotype of RS-MO-injected embryos at 7 dpf stage. (C) Significantly enlarged eyeball of \(z\text{Lum}\)-MO-injected fish at 7 dpf stage. (D) Significantly decreasing in ocular enlargement was noted in the \(zlum\)-MO-injected larvae at 7 dpf stage after treating with 0.5% atropine for 2 days. (E) Decreasing in ocular enlargement was also found in the \(z\text{Lum}\)-MO-injected larvae at 7 dpf stage after treating with 0.25% pirenzepine for 2 days. (F) No obvious changes in the phenotypes of \(z\text{Lum}\)-MO-injected fish at 7 dpf stage after treating with 0.01% methoctramine.

**Fig. 12.** Zebrafish drug screen assay. (A-B) This two figures showed the definition of outer margin of retinal pigmented epithelium layer (**RPE(red color)**) and diameter of scleral coat (**D(green color)**) in
zebrafish. (C) Significantly decreasing in excessive axial elongation in the zLum-MO-injected fish at the 7 dpf stage after treating with 0.5% atropine (A) and 0.25% pirenzepine (P), whereas no obvious changes in excessive axial elongation after treating with 0.01% methoctramine (M). (lane1: WT; lane2: MO+ 0.5%A; lane3: MO+ 0.25%P; lane4: MO+ 0.01%M; lane5: MO). Significantly decreasing in the diameter of scleral coat of the zLum-MO-injected fish at the 7 dpf stage after treating with 0.5% atropine and 0.25% pirenzepine, whereas no obvious changes in the zLum-MO-injected group treated with 0.01% methoctramine. (lane 6: WT; lane 7: MO+ 0.5%A; lane 8: MO+ 0.25%P; lane 9: MO+ 0.01%M; lane 10: MO) (D) Significantly decreasing in the ratio of RPE/scleral coat (%) was noted during ocular enlargement developed due to the reduction of zLum protein. Some muscarinic receptor antagonists (atropine and pirenzepine) attenuate the decreasing ratio of RPE/scleral coat due to the reduction of zLum protein, whereas there was no obvious changes in the decreased ratio of RPE/scleral coat in the methoctramine-treated group (lane1: WT; lane 2: MO+ 0.5%A; lane 3: MO+ 0.25%P; lane 4: MO+ 0.01%M; lane 5: MO).
Figure 1.

Figure 2.
Figure 7.

A. Zebrafish Lumican Gene

B. zLumpr1.7-EGFP SV40

C. zLumpr0.5-EGFP SV40

D, E, F, G. Images showing expression of EGFP in different stages.
Figure 8.
Figure 9A

![Diagram](image)

Figure 9B

![Bar chart](image)
Figure 9C-H.
Figure 10.

Figure 10C.
Figure 11.

WT  Control  MO

MO+0.5% A  MO+0.25% P  MO+0.01% M

Figure 12. A-B.
Figure 12. C

Figure 12. D.
Knockdown of zebrafish lumican gene (ZLUM) causes scleral thinning and increased size of scleral coats
Lung-Kun Yeh, Chia-Yang Liu, Winston W.-Y. Kao, Chang-Jen Huang, Fung-Rong Hu, Chung-Liang Chien and I-Jong Wang

J. Biol. Chem. published online June 15, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M109.043679

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/06/14/M109.043679.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2010/06/15/jbc.M109.043679.full.html#ref-list-1