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Transcriptomic and proteomic analyses of sclera in lens-induced myopic guinea pigs

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Abstract

Background Myopia development is commonly assessed by an increase in axial length, which may lead to high myopia and visual impairment. This study aims to identify potential biomarkers and signaling pathways in the sclera during experimental axial elongation.

Methods A myopia guinea pig model was established using male guinea pigs aged 2–3 weeks, which underwent bilateral lens-induced myopization (LIM) (study group) or were left untreated (control group). An integrated analysis of transcriptomic and proteomic was performed to identify differentially expressed genes (DEGs) in the sclera. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted to explore the DEGs related signaling pathways. Promising candidate markers were further tested by Western blot analysis. Transmission electron microscopy was used to assess scleral fiber changes in myopic guinea pigs.

Results During the study period, axial elongation was significantly greater in the study group (0.59 ± 0.05 mm vs. 0.47 ± 0.02 mm; $P < 0.001$), accompanied by a reduction in the thickness of the retina (121.9 ± 2.50 μ m vs. 134.6 ± 0.48 μ m; $P < 0.001$), choroid (38 ± 1.0 μ m vs. 50 ± 0.8 μ m; $P < 0.001$), and sclera (100.8 ± 2.78 μ m vs. 155.6 ± 4.78 μ m; $P < 0.001$). Integrated transcriptomic and proteomic analyses identified 34 upregulated genes, with significant activation and enrichment of the circadian rhythm pathway. Among the top enriched pathways, key differentially expressed genes included retinoid-related orphan receptors ROR α and ROR β , which are recognized as critical signals modulating the scleral hypoxia response. Western blot analysis confirmed upregulation of ROR α , ROR β , melatonin receptor type 1 (MT1), and HIF-1 α in the sclera, while melatonin receptor type 2 (MT2) expression remained unchanged between the groups. Transmission electron microscopy revealed a significantly higher proportion of thin collagen fibers compared to thick fibers in the LIM group ($P < 0.05$).

Conclusions Axial elongation-related remodeling of scleral collagen is closely linked to circadian rhythm and hypoxia pathways, with ROR α , ROR β , melatonin receptors, and HIF-1 α identified as potential key regulators. Additionally, scleral fiber size decreases progressively with scleral remodeling in myopia development.

Keywords Lens-Induced myopia, Melatonin receptors, Retinoid-related orphan receptors (RORs), Transcriptomics, Proteomics

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Introduction

By 2050, an estimated 1.8 million people will suffer from vision loss due to refraction disorder, and axial myopia may become a leading cause of irreversible vision loss [1–3]. Despite extensive research, the mechanisms underlying myopic axial elongation remain incompletely understood [4]. Previous studies have demonstrated that axial elongation is accompanied by remodeling of the scleral extracellular matrix, involving continuous synthesis and degradation of its components [5, 6]. The structural changes occurring in the myopic sclera include tissue thinning, reduction in the content of glycosaminoglycans and collagen, and fibrosis [7]. The mechanisms responsible for or associated with the onset and progression of extracellular matrix remodeling and axial elongation have remained elusive so far, highlighting the urgent need for further investigation to develop effective strategies to prevent or halt axial elongation and myopization.

Scleral remodeling may serve as a target for retinal regulation during axial elongation, ultimately contributing to structural and morphological changes in myopic eyes [8]. Previous studies examining gene expression in animal models of myopia have reported alterations in the expression of mRNAs associated with circadian clock genes in both the retina and choroid [9, 10]. Melatonin, a key circadian-related hormone, is rhythmically synthesized by pinealocytes, retinal photoreceptors, and ciliary epithelial cells, following a diurnal cycle with peak levels during the night [11]. Melatonin exerts its effects by binding to G protein-coupled receptors, specifically melatonin receptor type 1 (MT1) and type 2 (MT2) [12]. Studies have shown that myopic eyes exhibit significantly higher serum melatonin concentrations compared to non-myopic eyes [13]. Additionally, increased expression levels of melatonin receptors have been reported in various animal models of myopia [14, 15]. Although the precise mechanism through which melatonin influences axial elongation, scleral remodeling, and thinning remains unclear, both melatonin and its receptors have been associated with retinal thinning in myopic eyes [16]. Therefore, it is essential to investigate whether melatonin and its receptors are involved in the process of scleral remodeling.

The retinoid-related orphan receptors (RORs), including ROR α (NR1F1), ROR β (NR1F2), and ROR γ (NR1F3), are a class of widely distributed nuclear melatonin receptors. Dysregulation of RORs has been implicated in various pathologies, including cancer, autoimmune disorders, eye development, and vascular eye diseases [17, 18]. Regulated by melatonin, ROR α and ROR β are expressed in the brain, pineal gland, and eye, where they mediate circadian rhythms and contribute to motor and visual functions in mice [19, 20]. In the eye, RORs play a role in regulating the normal development of the lens

and retina and may be associated with retinal vascular diseases. Among the RORs, ROR α has been reported to be involved in retinal vascular diseases, while ROR β has been described as a regulator of circadian rhythms and retinal neuronal differentiation [21].

In this study, we investigated myopia-related signaling pathways, as well as mRNA and protein expression levels, in the sclera of guinea pigs with lens-induced myopization (LIM). Our integrative approach aimed to enhance the understanding of the transcription-to-translation cascade in myopia, with a specific focus on scleral remodeling.

Methods

Experimental design and biological measurements

Three-week-old male guinea pigs were housed in the Department of Laboratory Animals at Beijing Tongren Hospital. They were provided with standard chow and an ample supply of water. The animal facility maintained an illumination cycle of approximately 450 to 500 lx during a 12-hour day and 0 lx during a 12-hour night. Room temperature was controlled at 24–26 °C with a humidity level of 60%. The study received approval from the Ethics Committee of Beijing Tongren Hospital, adhering to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were conducted in accordance with relevant guidelines and regulations. Additionally, the study was reported following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

The guinea pigs were randomly assigned to one of two groups: the normal control group ($n=10$) and the lens-induced myopia (LIM) group ($n=10$). The LIM group developed lens-induced myopia by wearing goggles with a refractive power of -10.0 diopters over both eyes. The goggles were secured to the orbital rims with tape, allowing the animals to fully open their eyes and blink. To ensure cleanliness and proper fit, the goggles were removed, cleaned, and reattached daily after inspection. During re-examinations, the goggles were temporarily removed and then reapplied. The entire study spanned 29 days, from baseline to conclusion.

Axial length measurements were obtained using ocular sonographic biometry (A/B-mode scan; oscillator frequency: 11 MHz; Quantel Co., Les Ulis, France) under topical anesthesia (oxybuprocaine hydrochloride eye drops; Santen Co., Osaka, Japan). Measurements were performed at baseline and weekly for a total of four weeks, specifically on day 1 (baseline), day 8, day 15, day 22, and day 29, all at 2:00 pm. For each measurement session, 10 readings were taken. If the standard deviation of these readings was less than 0.10 mm, the mean value was used for subsequent statistical analysis. At the conclusion

of the study, optical coherence tomography (OCT) was performed without anesthesia. Bilateral swept-source OCT (VG200D, SVision Imaging Co., Luoyang, Henan, China) was conducted using a laser beam with a wavelength of 1,050 nm. Only high-quality OCT images were recorded. A line scan pattern centered on the optic disc was employed. The thickness of the posterior retina and choroid was measured in both the horizontal and vertical sections. The horizontal measurement was taken from the 3 o'clock to the 9 o'clock position, 1,000 μm temporal to the optic disc. The vertical measurement was taken from the 6 o'clock to the 12 o'clock position, 1,000 μm superior and inferior to the optic disc. All OCT images were acquired on day 29 at 2:00 pm, immediately before the animals were sacrificed.

Tissue collection

All animals were sacrificed on day 29 at 4:00 pm. Prior to enucleation, OCT examinations were performed on the eyes of three randomly selected animals from each group. The cornea, lens, and vitreous were carefully removed, and scleral tissue was harvested under a microscope. All tissue samples were immediately preserved in liquid nitrogen and subsequently stored at -80°C . For histopathological examination, the enucleated globes were fixed in 10 mL of FAS Eyeball Fixative Solution (Wuhan Servicebio Technology Co. Ltd., Wuhan, China) for 24 h and then embedded in paraffin. Additional experiments were initiated within one week of tissue collection.

RNA extraction, cDNA library construction and RNA sequencing

Five eyes from both the control group and the LIM group were selected for RNA sequencing. Total RNA was extracted using the Trizol reagent kit (Invitrogen, Carlsbad, CA, USA), and its quality and quantity were assessed by spectrophotometry. For cDNA library construction, 1 μg of RNA per sample, with an optical density (OD) value between 1.8 and 2.0, was used. The NEBNext Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was employed to prepare the libraries. Single-stranded and double-stranded cDNA were synthesized using magnetic beads containing Oligo (dT) to enrich the mRNA. The purified double-stranded cDNA underwent end repair, A-tail addition, and adapter ligation, followed by fragment size selection and PCR amplification. Initial library quantification was performed using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). After dilution to 1.5 ng/ μL , the insert size of the library was measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA).

High-quality reads were aligned using Hisat2 software (version 2.1.0). Gene-level expression, in Fragments Per Kilobase per Million Reads (FPKM), was mapped, and significant changes in gene and transcript expression levels were determined using Ballgown software (version 2.10.0) [22]. Differentially expressed genes were identified using an absolute $\log_2$ (fold change) greater than 1 and a cut-off at a P -value of 0.05.

Protein extraction and proteomics

Five eyes from both the control group and the LIM group were used for proteomics analysis. In accordance with the Minimum Information about a Proteomics Experiment (MIAPE) guidelines from the Human Proteome Organization's Proteomics Standards Initiative (HUPO-PSI), protein samples were lysed using SDT solution (4% SDS, 100 mM) [23]. The homogenates were centrifuged at 14,000 g at 4°C for 20 min. Protein concentration was measured using a bicinchoninic acid (BCA) kit to ensure reproducibility. The total proteins were digested with trypsin in the presence of dithiothreitol (DTT) and trypsin buffer. The samples were desalted using SOLA™ SPE. After vacuum drying, the samples were resuspended and spiked with iRT peptides (1:10).

Proteomics analysis was performed using an HPLC system coupled to an Orbitrap Q Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA) via an EasySpray source. The tryptic peptides were dissolved in solvent A (2% acetonitrile in HPLC water) and directly loaded onto a homemade reversed-phase analytical column (25 cm length, 75/100 μm i.d.). After trap enrichment, the peptides were eluted using a linear gradient of solvent B (98% acetonitrile in HPLC water) at a constant flow rate of 250 nL/min over 50 min. The solvent gradient was as follows: 0–10 min, 98% A; 10–10.01 min, 98–95% A; 10.01–37 min, 95–80% A; 37–48 min, 80–60% A; 48–48.01 min, 60–10% A; 48.01–58 min, 10% A; 58–58.01 min, 10–98% A; 58.01–63 min, 98% A.

Proteomic data analysis was conducted by Shanghai Luming Biological Technology Co., Ltd. (Shanghai, China). Samples were loaded and separated using a C18 column (25 cm $\times$ 75 μm) on an EASY-nLCTM 120 system (Thermo, USA). Data-dependent acquisition (DDA) mass spectrometry (MS) was performed over an m/z range of 100 to 170. For data-independent acquisition (DIA), 56 DIA windows were collected, with the automatic gain control (AGC) target set to $3\text{e}6$ and auto for injection time. The collision energy was linearly ramped from 59 eV at $1/K_0 = 1.6\text{ Vs cm}^{-2}$ to 20 eV at $1/K_0 = 0.6\text{ Vs cm}^{-2}$. MS/MS spectra were recorded within the same m/z range of 100 to 170.

The raw data were processed using MaxQuant software (version 1.5.5.1) and searched against the Uniprot database (*Cavia porcellus*, release date: February 13, 2023).

The analysis parameters were as follows: the main search parts per million (ppm) was set to 5; mass tolerance for precursor ions in the first search was ± 20 ppm; and the maximum number of missed cleavages per peptide was set to 2. Carbamidomethylation of cysteine was designated as a fixed modification, while oxidation of methionine and acetylation of protein N-termini were set as variable modifications.

The false discovery rate (FDR) cutoff for peptide and protein identification was established at <0.01 . Protein abundance was determined using normalized spectral protein intensity. Differentially expressed proteins were identified using an absolute $\log_2$ (fold change) greater than 1 and a cut-off at a P -value of 0.05.

Western blot

After sacrificing the animals on day 29, the eyes of three randomly selected animals were harvested for western blot analysis. Scleral tissues were lysed in cold lysis buffer (Amresco 0754, Solon City, OH, USA) supplemented with protease inhibitors (Roche 11697498001, Roche Co., Basel, Switzerland) and phosphatase inhibitors (Roche 04906837001, Roche Co., Basel, Switzerland). The resulting tissue extracts were separated using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes according to standard protocols. Membranes were blocked with 5% skim milk in TBST (Tris-buffered saline with Tween 20) for 2 h, followed by overnight incubation with primary antibodies. On the following day, secondary antibodies were applied for an additional 2 h.

The primary antibodies used included: anti-ROR α (82390-1, 1:100, Proteintech, USA), anti-ROR β (17635-1-AP, 1:100, Proteintech, USA), anti-Melatonin Receptor 1 A (ab87639, 1:100, Abcam, USA), anti-Melatonin Receptor 1B (ab203346, 1:100, Abcam, USA), anti-HIF-1 α (YT2213, 1:100, Immunoway, USA), anti- β -tubulin (YM303, 1:100, Immunoway, USA), and anti-GAPDH (YM3029, 1:20,000, Immunoway, USA). Signals were detected using an ECL kit (Millipore Co., MA, USA), and images were captured with Total Lab Quant V11.5 (Clever Scientific LTD, Newcastle upon Tyne, UK).

The target bands were quantified and analyzed using ImageJ (National Institutes of Health, NIH, Bethesda, Maryland, USA), with β -tubulin and GAPDH serving as the internal control.

Transmission electron microscope

After sacrificing the animals, the eyes of three randomly selected animals were fixed in 2.5% Glutaraldehyde Fixative (Wuhan Servicebio Technology Co. Ltd., Wuhan, China) for 10 min. Each eyeball was pierced with a syringe through the equator, and the anterior segment of the eye,

along with the lens, was removed. The posterior eye wall was then cut into small pieces (approximately 1 mm³) and placed in fresh fixative, stored in the dark at room temperature. After approximately 2 h at room temperature, the samples were transferred to 4 °C for storage.

The tissues were embedded in epoxy resin and sectioned into 60 nm slices using a Leica UC7 Ultramicrotome (Leica Co. Ltd., Wetzlar, Germany). The sections were examined using a Hitachi transmission electron microscope (TEM) system (HT7800, HITACHI Co. Ltd., Tokyo, Japan), and images were captured [24].

Statistical analysis

The statistical analyses were performed using the R software (version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Differential expression and enriched pathways analysis were performed using the clusterProfiler R package (version 3.4.4) [25]. The two-tailed, unpaired Student's t -test and the Mann–Whitney test (used when sample sizes were less than 10) were employed for comparisons between two groups. For comparisons involving multiple groups, one-way ANOVA followed by Bonferroni correction was utilized. The Kruskal–Wallis test was applied for multiple comparisons when sample sizes were less than 10. P -values less than 0.05 were considered statistically significant.

Results

Longitudinal monitoring of myopia development in LIM guinea pigs

In both the control and LIM groups, axial length increased from 8.12 ± 0.01 mm and 8.13 ± 0.03 mm at baseline, respectively, to 8.59 ± 0.03 mm and 8.72 ± 0.02 mm at 4 weeks after baseline, at the end of the study. A significant difference in axial length between the two groups was observed at study end ($P < 0.001$) (Table 1). Correspondingly, axial elongation during the study period was significantly greater in the LIM group (0.59 ± 0.05 mm versus 0.47 ± 0.02 mm; $P < 0.001$) (Fig. 1A). At study end, retinal thickness at the posterior fundus pole was significantly thinner in the LIM group compared to the control group (121.9 ± 2.50 μ m versus 134.6 ± 0.48 μ m; $P < 0.001$) (Fig. 1B). Similarly, choroidal thickness at the posterior pole was significantly thinner in the LIM group (38 ± 1.0 μ m versus 50 ± 0.8 μ m; $P < 0.001$), as was scleral thickness (100.8 ± 2.78 μ m versus 155.6 ± 4.78 μ m; $P < 0.001$) (Fig. 1C, D–F).

Transcriptomic profiling of myopia in guinea pigs

In the transcriptomic profiling, we identified 14,588 quantifiable mRNAs, with 1,510 mRNAs upregulated and 563 mRNAs downregulated in the LIM group compared to the control group. The volcano plot illustrates

Table 1 Biometrics measurements (mean ± standard deviation) in guinea pigs

	Baseline	Follow-up 1 week	Follow-up 2 week	Follow-up 3 week	Follow-up 4 week	p-value
Anterior chamber depth (mm)						
Control	1.15±0.03	1.16±0.03	1.16±0.06	1.16±0.01	1.17±0.03	
LIM	1.14±0.03	1.14±0.07	1.15±0.04	1.16±0.03	1.15±0.02	> 0.05
Lens thickness (mm)						
Control	3.44±0.15	3.47±0.16	3.50±0.19	3.55±0.12	3.59±0.21	
LIM	3.43±0.16	3.51±0.19	3.62±0.15	3.69±0.17	3.73±0.17	< 0.05
Vitreous length (mm)						
Control	3.37±0.24	3.48±0.19	3.54±0.15	3.67±0.17	3.75±0.12	
LIM	3.36±0.16	3.44±0.21	3.56±0.18	3.65±0.11	3.75±0.14	> 0.05
Axial length (mm)						
Control	8.12±0.01	8.15±0.02	8.28±0.03	8.40±0.04	8.59±0.03	
LIM	8.13±0.03	8.30±0.04	8.41±0.04	8.56±0.04	8.72±0.02	< 0.001

LIM: lens-induced myopia

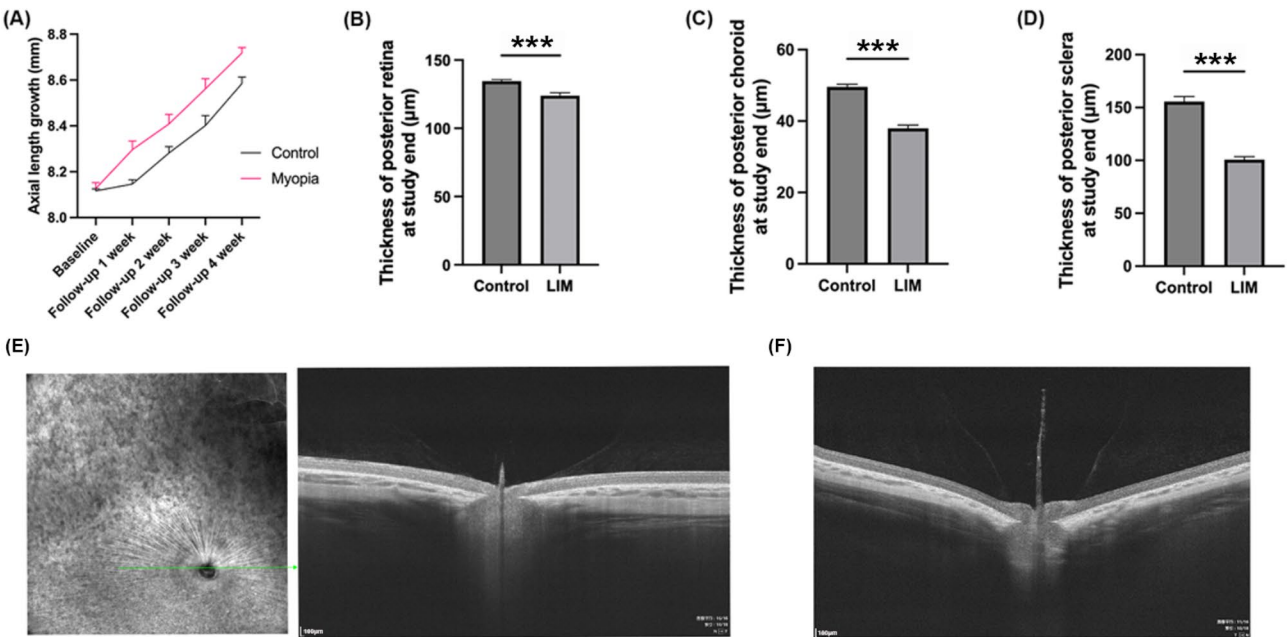


Fig. 1 **A:** Changes in axial length from study baseline to study end between the lens-induced myopia (LIM) study group and control group. **B:** Thickness of the posterior retina measured at 1000 μ m temporal to the optic disc at study end. **C:** Thickness of the posterior choroid measured at 1000 μ m temporal to the optic disc at study end. **D:** Thickness of the posterior sclera measured at 1000 μ m temporal to the optic disc at study end. **E, F:** Optical coherence tomographic images of the posterior pole of guinea pigs at the study end, in the control group (E) and the LIM study group (F). scale bar: 100 μ m. ***: $P < 0.001$

significant differences in mRNA expression levels between the two groups (Fig. 2A). Principal component analysis (PCA) was performed on the gene expression data, which were transformed to rank-normal scores to mitigate the influence of outliers. The first and second principal components explained 59.7% and 13.1% of the variance in the transcriptome, respectively (Fig. S1A). These results confirm the successful establishment of the LIM model, with a clear distinction in gene expression profiles between the control and LIM groups.

Functional enrichment analysis of Gene Ontology (GO) categories, including biological processes (BPs), cellular components (CCs), and molecular functions

(MFs), revealed that the top 10 significantly enriched pathways in each category were predominantly related to visual perception signaling pathways. Notably, bicarbonate transport and structural constituents of the eye lens emerged as the most enriched terms for biological and molecular functions, respectively (Fig. 2B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlighted increased expression of pathways related to p53 signaling, extracellular matrix (ECM)-receptor interaction, and circadian rhythms in myopic guinea pigs (Fig. 2C). Among the top enriched pathways, the circadian rhythm-associated *ROR α* and *ROR β* signaling pathways, along with related genes such as *HIF-1 α* , were

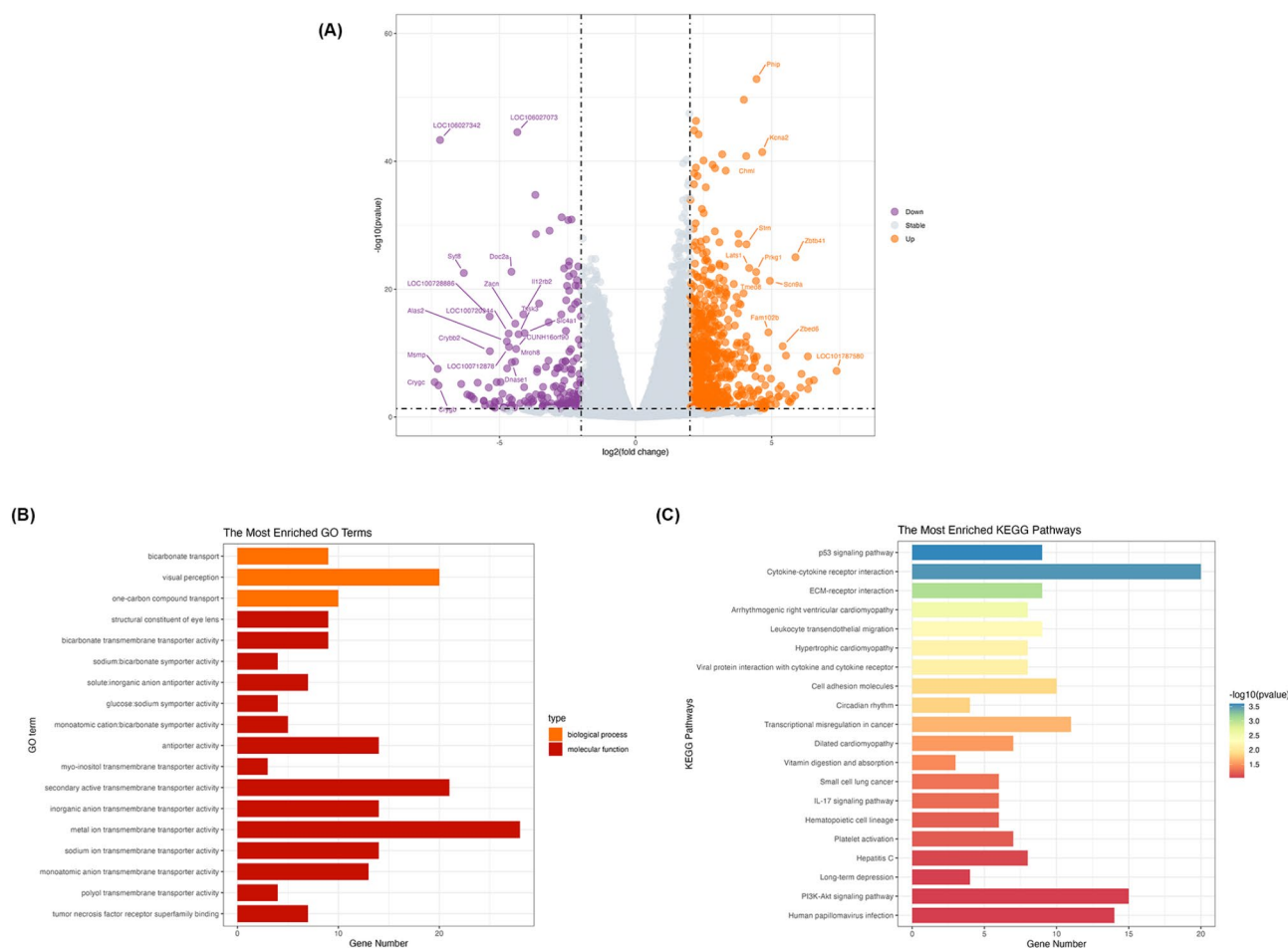


Fig. 2 A: Volcano plot showing differentially expressed genes in guinea pigs of the lens-induced myopia (LIM) study group and control group. **B, C:** Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses identifying the most significant affected pathways in the LIM study group ($P < 0.05$)

prominently featured. These findings suggest that circadian rhythms and hypoxia may play significant roles in scleral remodeling in myopic guinea pigs.

Proteomic profiling of myopia in guinea pigs

In the proteomic profiling, we identified 8,612 quantifiable proteins, with 111 upregulated and 84 downregulated in the LIM group compared to the control group. The volcano plot illustrates significant differences in protein expression levels between the two groups (Fig. 3A). Principal component analysis (PCA) revealed that the first and second principal components accounted for 31.8% and 23.0% of the variance, respectively (Fig. S1B), indicating distinct proteomic profiles between the LIM and control groups.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to characterize the functions of differentially expressed proteins. GO enrichment analysis indicated that the most significant biological process was myofibril assembly,

highlighting its involvement in scleral remodeling during myopia development. The most enriched cellular component term was contractile fiber, while actin binding emerged as the top molecular function, both contributing to structural alterations in the sclera (Fig. 3B). KEGG enrichment analysis identified the circadian rhythm signaling pathway as the most prominent pathway associated with differentially expressed proteins. Key genes driving this enrichment included *RORB*, *RORA*, and *BHLHE40*, underscoring the critical role of circadian rhythm regulation in scleral remodeling in myopic guinea pigs (Fig. 3C).

Integrated transcriptomic and proteomic analysis

In the integrated transcriptomic and proteomic analysis, a total of 8,450 genes were quantified at both the transcriptome and proteome levels. Four gene expression patterns emerged: 34 genes were upregulated in both transcriptomic and proteomic analyses (up-up group); 16 genes were downregulated in both (down-down group);

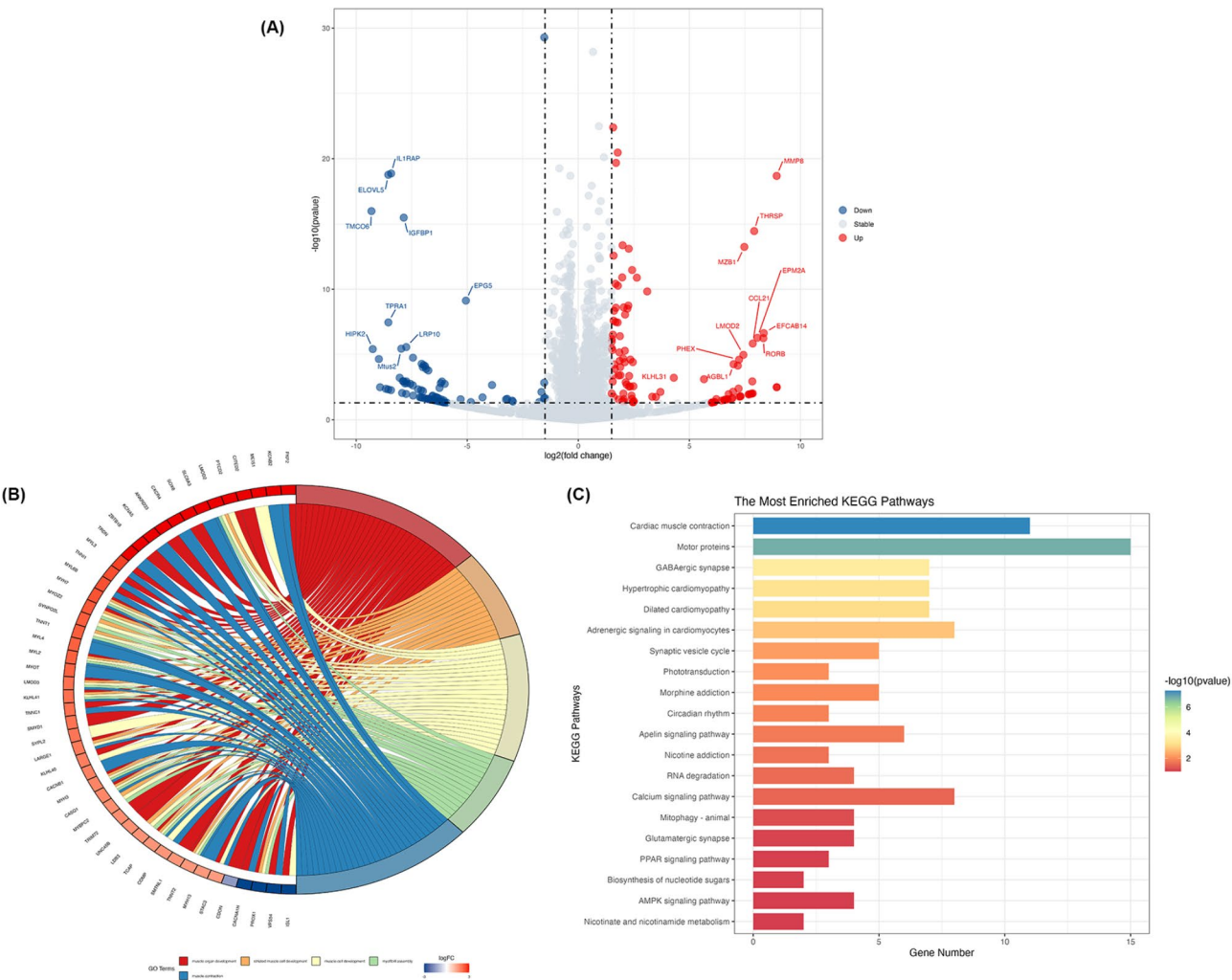


Fig. 3 **A:** Volcano plot shows differentially expressed proteins in guinea pigs of the lens-induced myopia (LIM) study group and control group. **B, C:** Gene Ontology (GO) of chordal graph and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses identifying the most significant affected pathways in the LIM study group, indicating the overexpression of genes involved in ROR α -related and ROR β -related circadian rhythms pathways ($P < 0.05$)

32 genes exhibited downregulated mRNA but upregulated protein levels (down-up group); and 45 genes showed upregulated mRNA but downregulated protein levels (up-down group). The overall correlation between mRNA and protein expression levels was 0.03 ($P < 0.05$) (Table S1; Fig. 4A). Notably, *RORA* and *RORB*, encoding melatonin receptors, were significantly upregulated at both the transcript and protein levels (up-up group). *RORB* ranked among the top 10 key regulators of axial elongation in myopia.

KEGG pathway analysis of the up-up group highlighted significant activation of the circadian rhythm pathway. Key contributors included *SOS2* and *SERPINB5*, which activated the p53 related signaling pathway; *RORA* and *RORB*, which mediated circadian rhythms; and *NEB* and *NEXN*, which were involved in cell–cell adhesion complexes (Fig. S2A). In contrast, GO analysis of the

down-down group revealed enrichment in pathways regulating *SUMO* ligase and *SUMO* transferase activities, with notable downregulation of genes such as *PIAS4*, *ZMIZ2*, and *PLPP2*, the latter also suppressing glycerolipid metabolism (Fig. S2B). Among the top enriched pathways, *RORA* and *RORB* emerged as critical regulators of scleral hypoxia responses, marking them as key alarm signals in myopia-associated scleral remodeling.

Network analysis using STRING (<https://string-db.org/>) indicated that the top 10 differentially expressed proteins (DEPs) were predominantly involved in circadian rhythm and hypoxia responses (Fig. 4B). In particular, *HMBOX1* and *CREBRF* (up-down group) were identified as activators of hypoxia and melatonin regulation pathways implicated in myopia development. Additionally, *ACTA1*, *COX6A2*, and *HIF-1 α* (down-up group) were highlighted, suggesting that post-transcriptional

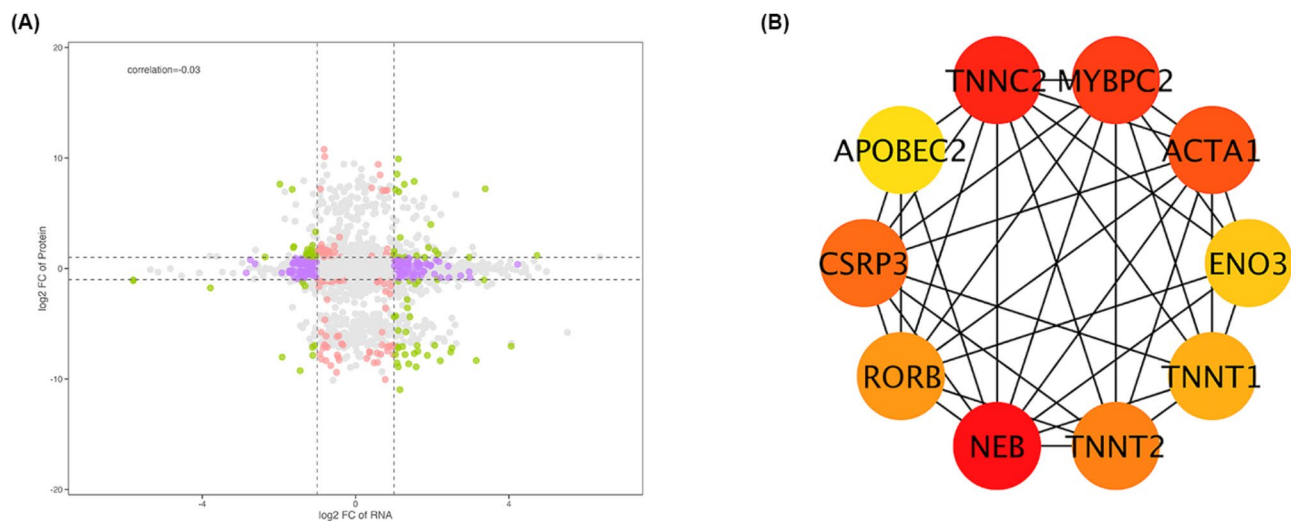


Fig. 4 Comparison of the changes in mRNA and protein in guinea pigs of the lens-induced myopia (LIM) study group and control group. **A:** A total of 34 genes were upregulated in both the transcriptomic and proteomic analyses, 16 genes downregulated in both comparisons, 32 downregulated mRNA that were upregulated at the protein level, and 45 gene upregulated at the mRNA level but downregulated at the proteins level with a correlation of 0.03 (green) ($P < 0.05$). We identified 79 significantly upregulated and 79 downregulated proteins with no mRNA expression changes (pink) ($P < 0.05$). A total of 229 significantly upregulated and 315 downregulated mRNA genes with no proteins expression changes (purple) ($P < 0.05$). **B:** The top 10 most significant proteins as revealed by String analysis mainly participated in inflammation activities. LIM: lens-induced myopia

regulation of the hypoxia signaling pathway may contribute to scleral remodeling during myopia progression.

Sclera biomarkers and fiber thinning

To identify scleral biomarkers for axial elongation, genes associated with myopia were filtered based on their expression levels in both transcriptomic and proteomic analyses. Notably, *RORA* and *RORB* were upregulated within the circadian rhythm pathway, a pathway previously demonstrated to be involved in myopia development in the retina [26]. Melatonin receptors type1 (*MT₁*) and melatonin receptors type2 (*MT₂*) were also the key genes that regulated retina thinning and myopia development [12, 13]. The post transcriptional of the hypoxia signal pathway represented by *HIF-1α* was also a prominent regulatory candidate for genetic interaction in human myopia that caused scleral hypoxia [27]. And as demonstrated by the transcriptomic analysis, KEGG pathway analysis indicated that *HIF-1α* plays a role in promoting myofibroblast transdifferentiation and extracellular matrix (ECM) remodeling in the sclera.

Based on these findings, we selected three candidate genes involved in myopia-related scleral remodeling—*RORα*, *RORβ*, and *HIF-1α*—from the up-up and down-up groups for further validation using Western blotting in scleral tissues. Western blot analysis demonstrated significant upregulation of *RORα*, *RORβ*, melatonin receptor type 1 (*MT₁*), and *HIF-1α* in the sclera (Fig. 5A–C, E). In contrast, the expression level of melatonin receptor type 2 (*MT₂*) showed no significant difference between the two groups (Fig. 5D). These results validate the

accuracy of our combined transcriptomic and proteomic analyses, as the expression patterns of candidate genes were consistent at both RNA and protein levels.

Additionally, transmission electron microscopy of the posterior sclera revealed a higher proportion of thin to thick collagen fibers in the LIM group compared to the control group. The fiber sizes were categorized into five groups based on their diameters, and the LIM contains lower fiber size compared to the control group. Significant differences were observed in the percentage of fibers across different diameter ranges: 800–1000 nm (22.8% vs. 45.3%), 1000–1200 nm (25.3% vs. 35.0%), 1200–1500 nm (12.3% vs. 16.0%), 1500–2000 nm (21.3% vs. 3.0%), and >2000 nm (18.3% vs. 0.7%) between the control and LIM groups (Fig. 5F–H).

Discussion

In this study on LIM-induced axial elongation in guinea pigs, proteomic and transcriptomic analyses revealed that circadian rhythm pathways in the sclera were upregulated during the scleral remodeling process (Fig. 5). Notably, melatonin receptors, particularly *RORα* and *RORβ*, were identified as key genes in the up-up regulation group. This finding sets our results apart from previous studies, which primarily focused on the role of melatonin in the retina [26]. Further evaluation of the downstream *HIF-1α* pathway suggests that *RORs* may play a role in the hypoxia-related mechanisms affecting scleral hypoxia. TEM analysis indicated that the fiber size of the sclera decreased in the LIM group. Overall, our findings indicate that scleral collagen remodeling during axial

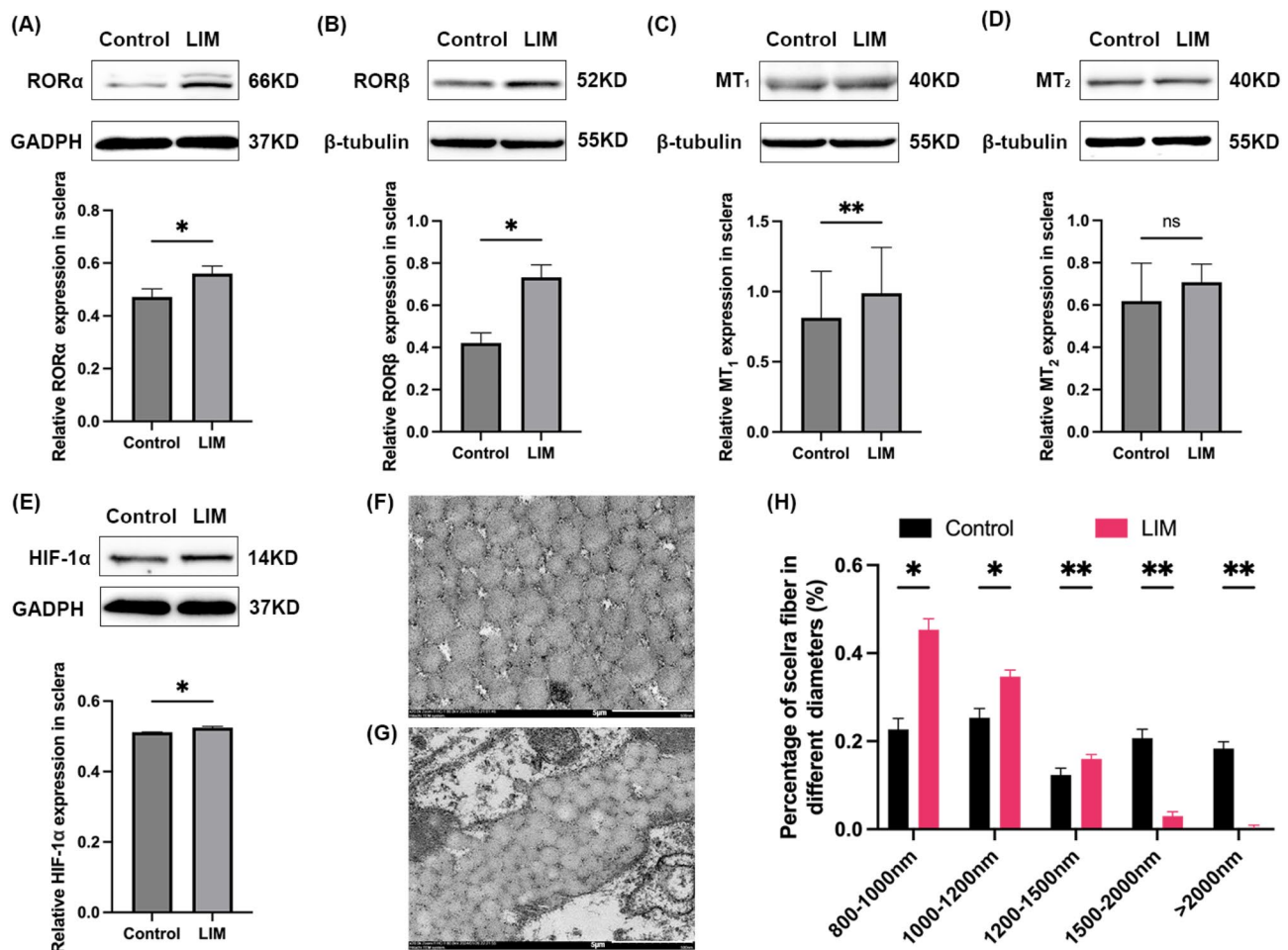


Fig. 5 Circadian rhythms pathway in the sclera of guinea pigs of the lens-induced myopia (LIM) study group and control group. **A-E**: Expression level of RORα, RORβ, melatonin receptors type1 (MT₁), melatonin receptors type2 (MT₂) and HIF-1α in the sclera. **F, G**: Transmission electron microscopical images of sclera collagen fibers in the control group (F) and the LIM (G) study group; scale bar, 5 μm. **H**: Percentage of sclera fiber in different diameters (n=3 biologically independent samples). Five images from one sclera. LIM: lens-induced myopia; MT₁: melatonin receptors type1; MT₂: melatonin receptors type2. *: $P < 0.05$; **: $P < 0.01$; ns: not statistically significant

elongation is closely linked to both circadian rhythm and hypoxia pathways, with RORα, RORβ, melatonin receptors, and HIF-1α emerging as potential key regulators.

The observations in our study align with findings from previous research, where lens-induced myopization resulted in retinal, choroidal, and scleral thinning in the experimental animals (Fig. 1) [28]. Melatonin has a reciprocal relationship with dopamine in the retina [29]. It has been suggested that light exposure and increased outdoor time stimulate dopamine release, which helps prevent myopia onset, raising the question of whether melatonin may also play a role in myopia control [26]. Prior studies have shown that exposure to regular light/dark cycles in eyes with induced myopia reduced axial length and increased choroidal thickness at night [29, 30]. Researcher have also found elevated melatonin concentrations in the serum and saliva in myopic, particularly in highly myopic individuals [31]. Two melatonin

membrane receptors, MT₁ and MT₂, have been identified and shown to form MT₁/MT₂ heteromers (MT_{1/2h}) in eyes [12]. Systemic circadian rhythm changes could influence local dopamine and melatonin concentrations and their endogenous rhythms, thereby affecting structural, metabolic, and neurochemical processes in the eye [29]. This is consistent with our observation that MT₁ was overexpressed in LIM guinea pigs compared to controls at a single time point during axial elongation. Since MT_{1/2h} activates signaling pathways similar to those of MT₁, and we observed no significant difference in MT₂ expression between groups, it suggests that MT₁ may play a predominant role in MT_{1/2h} activation in the sclera during axial elongation [32]. By evaluating melatonin expression in the sclera for the first time, our study provides further support for the involvement of circadian physiology in the mechanisms of axial elongation.

RORs may act as primary targets of nuclear melatonin receptors, with melatonin potentially binding directly to them to regulate gene expression [16]. As promising druggable targets, RORs have been implicated in mitigating several debilitating human diseases, including ocular pathologies. Previous studies have shown that interactions between ROR α and ROR β increase the risk for both wet and dry age-related macular degeneration (AMD) [33, 34]. Additionally, ROR α has been identified as a novel regulator of pathological retinal neovascularization by directly modulating inflammatory responses—a mechanism that may overlap with those involved in high myopia [19]. ROR β is widely expressed in the immature retina and plays dual roles in promoting rod and cone differentiation [35]. Recent studies indicate that ROR β may collaborate with ROR α to activate normal cone development and respond to eye diseases [36]. Although ROR α has been shown to influence the transcriptional activity of HIF-1 α in ischemic vascular diseases, its effects on hypoxia may vary depending on the specific disease model and tissue type [37]. Our study highlights a critical perspective: both ROR α and ROR β were overexpressed in the scleral tissue of myopic guinea pigs, activating the hypoxia pathway. This finding distinguishes our interpretation, suggesting that ROR-mediated circadian rhythm regulation could serve as a novel scleral target for myopia control.

Axial elongation is closely linked to the remodeling of the scleral extracellular matrix (ECM), characterized by alterations in ECM gene expression and collagen fiber thinning [4]. Previous studies have demonstrated that hypoxia disrupts choroidal vasculature, decreases choroidal blood flow, reduces choroidal thickness, and triggers scleral remodeling [38]. During this remodeling process, fibroblast-like cells undergo a phenotypic shift toward myofibroblast-like cells, accompanied by a reduction in collagen production [38]. Considering the spatiotemporal expression of ROR β and the altered circadian behavior observed in ROR β knockdown mice, it is plausible that ROR β plays a role in the transcriptional regulation of circadian clock effectors [39]. Specifically, ROR α and ROR β are implicated in the regulation of melatonin synthesis for two key reasons. First, the rhythmic expression of ROR α and ROR β is confined to the two primary melatonin-producing tissues in the central nervous system—the pineal gland and photoreceptors—and correlates with circadian melatonin biosynthesis [20, 40]. Second, the onset of rhythmic ROR β expression in the retina and pineal gland coincides with the initiation of melatonin production, a finding that was validated in our study [20]. Our findings further align with the observation that RORs upregulate the expression of HIF-1 α , contributing to scleral thinning and degeneration. Transmission electron microscopy (TEM) images revealed a decrease in the diameters of

scleral collagen fibers as myopia progressed. Collectively, these findings highlight scleral RORs as potential therapeutic targets for mitigating pathological axial elongation in myopia. Moreover, the association between scleral circadian rhythms and axial elongation offers a promising avenue for myopia prevention and treatment.

Several limitations of our study should be acknowledged. First, the process of axial elongation may be influenced by the type of goggles used to induce myopia. The −10 diopter goggles employed in our study could produce different effects compared to diffuse lenses, which have been used in other investigations. Second, the discrepancies observed between mRNA and protein levels could be attributed to several factors, including translation efficiency, post-transcriptional modifications, post-translational modifications, and protein stability. These regulatory mechanisms can significantly impact protein expression, resulting in a divergence from transcriptomic findings [41]. Third, our investigation focused on genes with up-up expression patterns in both transcriptomics and proteomics data. However, genes showing down-down expression patterns or inconsistent trends across both levels are also of research interest. Research is needed to understand the complex spatial and temporal dynamics of these molecular functions. Fourth, melatonin synthesis and release are significantly influenced by light and circadian rhythms, exhibiting considerable inter-individual variability in circulating levels. Consequently, reporting data from a single time point may lead to challenges in interpretation [42].

Conclusions

In conclusion, the remodeling of scleral collagen associated with axial elongation involves both circadian rhythm and hypoxia pathways, with ROR α , ROR β , melatonin receptors, and HIF-1 α emerging as potential key regulators. These findings provide new insights into the underlying mechanisms of myopia-related axial elongation and may contribute to the identification of novel therapeutic targets. Future studies should explore whether modulating circadian rhythms could serve as a promising strategy for controlling myopia progression.

Abbreviations

GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LIM	Lens-induced myopization
OCT	Optical coherence tomography
RORs	Retinoid-related orphan receptors
TEM	Transmission electron microscope

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-025-11422-2>.

Supplementary Material 1

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Not applicable.

Author contributions

W.B. Wei, H.Y. Li and X.H. Shi designed the study, H.Y. Li and X.H. Shi wrote the manuscript. H.Y. Li, L. Dong, C.Y. Yu, W.D. Zhou, H.T. Wu, Y.T. Li and R.H. Zhang collected the data and conducted the analyses, W.B. Wei and Jost. B. Jonas edited and revised the manuscript. All authors have approved the submitted version and agreed with the contribution's declarations.

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Data availability

The datasets generated and/or analysed during the current study are available in the Genomics Expression Omnibus Database (GEO) with the accession of GSE280609.

Declarations

Ethics approval and consent to participants

The study was approved by the Ethics Committee of Beijing Tongren Hospital, ensuring compliance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research. All methods were performed in accordance with the relevant guidelines and regulations. It is confirmed that the study is reported in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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