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Altered Expression of Genes in Experimentally Induced Myopic Chick Eyes

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Purpose: To identify a casual pathway between the alteration in visual experience, due to form deprivation and hyperopic defocus, and the increase in eye growth, we searched for candidate genes having regulatory effects on eye growth under myopic conditions.

Methods: The expression of the brain-derived neurotrophic factor, neurotrophin-3, sonic hedgehog, nerve growth factor, Six-3 and the Lh-2 group of genes in the transcriptional level after experimentally induced myopia (form-deprivation by goggles and by hyperopic defocus using negative spectacle lenses) were evaluated by semiquantitative reverse transcriptional polymerase chain reaction and Northern blot analysis.

Results: Results showed that only the sonic hedgehog gene was differentially expressed in the experimentally induced myopic retinal samples compared with controls.

Conclusions: The sonic hedgehog gene may have regulatory functions in the signaling of the cascade of events that leads to axial elongation and vitreous enlargement of the eye under myopic conditions. **Jpn J Ophthalmol 2001;45:137–143** © 2001 Japanese Ophthalmological Society

Key Words: Chick, form-deprivation myopia, negative lens-induced myopia, retina, sonic hedgehog.

Introduction

Form-deprivation myopia is a widely accepted method for induction of myopia in animals.[1–10] In chicks, occlusion of the eye during the early postnatal period impairs form vision and leads to myopia. The hyperopic defocus achieved with negative spectacle lenses is also known to induce myopia in animals.[10] The resulting eye enlargement stems from vitreous chamber elongation. Recently, the expression of transforming growth factor-β (TGF-β),[4] bone morphogenetic protein-2 (BMP-2),[11] neuroendocrine specific protein (NSP)-A and -C[3] and the delta-crystallin[5] group of genes in chick retinal tissues were reported to be altered by experimentally induced myopic treatment. Of the many transcriptional and growth factors expressed during the embryonic stages, the sonic hedgehog (Shh)[12,13] and nerve growth factor (NGF)[14] genes were reported to be involved in the regulation of the transcriptional levels of BMPs and TGF-βs, and NSP-A and -C,[14] respectively. The delta-crystallin gene was found to be dominantly expressed in the lens.[15] Six-3[15] and Lh-2[14] genes are presumed to be involved in lens differentiation and eye formation through the expression of the delta-crystallin gene.[15]

To identify a casual pathway between the alteration in visual experience (form deprivation and negative hyperopic defocus) and the increase in eye growth, we focused on candidate substances in the retina having possible regulatory functions in the cascade of signals that lead to axial elongation and vitreous enlargement under experimentally induced (both form-deprived and negative lens-induced) myopia because initiation and control of eye growth seem to be localized in specific regions of the eye.[16,17]

In the present study, retinal mRNA expression

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levels of Shh, NGF, Six-3, Lh-2, brain-derived neurotrophic factor (BDNF), and neurotrophin (NT)-3 after 3, 7, and 14 days of experimentally induced myopia (form-deprivation by goggles and hyperopic defocus with negative spectacle lenses) were evaluated by semiquantitative reverse transcriptional polymerase chain reaction (RT-PCR) and Northern blot analysis. The expression of Shh mRNA was significantly increased in form-deprived and negative lens-induced myopic retinal tissues after 3 and 7 days of treatment.

Materials and Methods

Induction of Myopia

Chicks (male white leghorn) were obtained from a local hatchery and maintained at 30°C under an alternating 12-hour light:dark cycle (6 AM–6 PM light; 6 PM–6 AM dark). All chicks were treated under the Guidelines for Animal Research at Kobe University School of Medicine, and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Form-deprivation myopia was induced in 1-day-old white leghorn chicks by fixing translucent plastic goggles over the right eyes, as described elsewhere.[4] Briefly, hemispheric goggles cut from the bottoms of 15-mL plastic test tubes were attached to the right eyes with a cyanoacrylate adhesive. The left eyes served as internal controls.

Lens-induced myopia was done as described elsewhere.10 Briefly, −15 diopter contact lenses made from PMMA (poly(methylmethacrylate)) material with a back optic radius of 7 mm and an optic zone diameter of 10 mm were used. The lenses were glued between rigid plastic, and Velcro support rings were attached to the right eyes with cyanoacrylate adhesives. Lenses were cleaned every 3 hours during the 12-hour light cycle. The left eyes served as internal controls.

RNA Isolation

Fifteen chicks were euthanized after 3, 7, or 14 days of experimentally induced myopic treatment. Eyes were enucleated and divided into anterior and posterior hemispheres. The vitreous and sclera were gently cleaned out. The retina, including the RPE (retinal pigment epithelium), was carefully separated from the choroid. Retinal tissues from control, form-deprived and lens-induced myopic eyes were subjected to RNA extraction.

Total RNA from the retinas was isolated by guanidine-isothiocyanate and cesium chloride centrifugation,[18] followed by phenol and chloroform:isoamyl alcohol purification and ethanol precipitation. The final RNA preparation was dissolved in diethyl pyrocarbonate-treated water. The amount of the purified RNA was determined by spectrophotometry (OD 260/280 ratio > 1.9).

Reverse Transcription-Polymerase Chain Reaction

With a commercially available kit (1st strand cDNA synthesis kit; Boehringer Mannheim, Indianapolis, IN, USA), 1.0 μL total RNA (1 μg/μL) was reversed transcribed. One microliter total RNA was added to a mixture of 2.0 μL of 10× reaction buffer, 5 mM $MgCl_2$, 3.5 mM dNTP mix, 2.5 μL oligo (dT) (20 pmol/μL), 50 units of RNase inhibitor, and 20 units AMV (avian myeloblastosis virus) reverse transcriptase, to a final volume of 20 μL. The mixture was first incubated at 30°C for 10 minutes, then at 42°C for 60 minutes. The mixture was further incubated at 99°C for 5 minutes to inactivate the AMV reverse transcriptase; then cooled on ice for another 5 minutes. The amount of RNA was controlled by monitoring the intensity of ribosomal RNA bands on a 1% agarose/formaldehyde-ethidium bromide gel. Furthermore, variation in the amount of RNA was minimized by monitoring the expression of β-actin mRNA, a housekeeping gene. The reverse transcription (RT) procedure was done as above. The following set of original primers designed from a published sequence were used for PCR amplification: (sense primer: TAAGGATCTGTATGCCAACACAGT, and antisense primer: GACAATGGAGGGTCCGGATTCATC).[19] The primers were synthesized by the Biologica Company (Nagoya) and purified by precipitation.

One microliter aliquots of the RT-mix were added to the PCR master mix consisting of 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.8 mM dNTP, 1.25 U of Ampli*Taq* Gold polymerase (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ, USA) and 0.2 μM of β-actin primer. The PCR cycle (Takara PCR Thermal Cycler MP; Takara Biochemicals, Kyoto) consisted of a preincubation step at 94°C for 10 minutes, followed by 17 to 35 cycles at 94°C and 1.5 minutes at 72°C each, and a final elongation step at 72°C for 10 minutes. The sequence of the amplified products was confirmed to correspond to the chick β-actin cDNA after force-cloning into pGEM-T (Promega, Madison, WI, USA). The nucleotide sequence of the cDNA inserts of isolated clones was verified with the ABI PRISM dye terminator cycle sequencing core kit (Perkin Elmer) with Ampli*Taq* DNA polymerase FS (Perkin Elmer) on

the 377 DNA Sequencing System (Applied Biosystems, Chiba).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Semiquantitative RT-PCR was used to detect changes in mRNA expression of selected genes. One microgram total RNA was reverse-transcribed to cDNA (as described above) and amplified by PCR. Polymerase chain reactions primers for chick NGF, NT-3, BDNF, Six-3, Lh-2 and Shh were originally designed, based on published sequences in the GeneBank report (Figure 1). The RT reaction was done as described above. The PCR cycle consisted of preincubation for 10 minutes at 94°C, then incubation for 1 minute at 94°C. A 1-minute incubation period at the following annealing temperatures was done: 50°C for Shh, 53°C for Six-3, 57°C for NGF and BDNF, and 58°C for NT-3 and Lh-2. A 1.5-minute elongation period followed at 72°C. Cycle numbers were determined empirically by sampling the PCR amplification after every three cycles between 17 and 41 cycles and selecting the approximate midpoint of linear amplification, in relation to the isolation of a single band. This was followed by a final elongation period of 10 minutes at 72°C, after which the products were separated by electrophoresis on a 1.0% agarose gel. The expected lengths of the PCR products were as follows: 668 base pairs (bps) for NGF, 427 bps for NT-3, 525 bps for BDNF, 443 bps for Six 3, 582 bps for Lh-2, and 555 bps for Shh. The products were separated by electrophoresis on a 1.0% agarose gel, visualized by ethidium bromide fluorescence, and photographed using high-resolution black and white polaroid film (Type 667, Polaroid film; Polaroid, Cambridge, MA, USA). To account for differences in the amounts of starting RNA between samples, the density of each band was divided by the density of the β-actin band for each sample. The bands of the expected lengths were extracted. The products were subcloned and subjected to sequence analysis, as mentioned above.

Northern Blot Analysis

For Northern blot analysis, 20 μg of each total RNA sample was size-separated on a 1% agarose/formaldehyde gel, transferred onto nylon filters (Boehringer Mannheim, Mannheim, Germany) by wicking, and fixed by ultraviolet-crosslinking (Stratagene, La Jolla, CA, USA). After pre-hybridization, the filters were hybridized with a digoxigenin-labeled probe (10 ng/mL) in 50% formamide hybridization buffer at 55°C overnight. The hybridized filters were washed twice with 2 × standard sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 minutes with each and twice with 0.1 × SSC and 0.1% SDS at 55°C for 15 minutes with each. The digoxigenin-labeled probes were detected with sheep anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) and an enhanced chemiluminescent detection system (Boehringer Mannheim). Probes were synthesized from clones of the RT-PCR products of Shh cDNA by PCR with PCR labeling mixture (Boehringer Mannheim). Primer sequences and cycle conditions were as previously described.

After drying, the filters were exposed to x-ray film (Amersham, Buckinghamshire, UK). To allow for the differences in the actual quantity of total RNA loaded on the gel, the filter was stripped for 10 minutes at 95°C using a solution containing 1 mM EDTA, 40 mM Na_2HPO_4, pH 7.2, and 5% SDS. Hybridization was also performed using the amplified product digoxigenin-labeled chick β-actin cDNAs as probes.

Image Analysis

Camera images from the semiquantitative RT-PCR data were captured by a Polaroid black-and-white camera (ACMEL CRT Camera, M-085-D; Tokyo) using a 337 Polaroid film. Images were saved and converted to Macintosh format TIFF files (360 and 720 pixels with 8 bits of gray level). The TIFF files of all the numerical densities (arbitrary units) were exported to the public domain NIH image 1.55 program for further analysis on a Macintosh computer.

Statistical Analysis

The data obtained from the densitometric readings of the semiquantitative RT-PCR were analyzed statistically with an unpaired Student *t*-test. A *P*-value of less than 5% was considered statistically significant.

Results

Equal amounts of the 240 base pair β-actin PCR products were detectable in the control and experimentally induced myopic retinal tissues. Semiquantitative RT-PCR confirmed that the expression of β-actin mRNA was not affected by experimentally induced myopia (data not shown).

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Gene		Primer Design	Expected length	Annealing Temperature	reference
NGF	sense primer	5'-GTCCATGCTGTACTACACTC-3'	668 bps	57°C	X04067
	antisense primer	5'-GGATAAATCTCCAGGCTGC-3'			
NT-3	sense primer	5'-ACAACTCTCCTCGAGTTCTC-3'	427 bps	58°C	M83378
	antisense primer	5'-CTTATCCATCTCCAGCCTAC-3'			
BDNF	sense primer	5'-CCTTCCTTTGTGTACCCCTT-3'	525 bps	57°C	*(Ref11)
	antisense primer	5'-CCCGATGAAAGAAGCTAGTG-3'			
Six-3	sense primer	5'-TCAACAAGCACGAGTCCATC-3'	443 bps	53°C	Y15106
	antisense primer	5'-GTGCTGGAGCCTGTTTTTAG-3'			
Lh-2	sense primer	5'-GCCTCTGA AATGGTCATGAG-3'	582bps	58°C	L35566
	antisense primer	5'- GTTCCKTCTGAATTTGGC TC-3'			
Shh	sense primer	5'-CAAGCTGGT GAAGGACCTG-3'	555 bps	50°C	L28099
	antisense primer	5'-CGTGAGTACCAATGGATGCC-3'			

Figure 1. Reverse transcriptional-polyermase chain reaction primer designs for nerve growth factor (NGF), neurotrophin (NT)-3, brain-derived neurotrophic factor (BDNF), Six 3, Lh-2, and sonic hedgehog (Shh). Expected sizes in base pairs (bps), and optimal annealing temperatures are indicated. Primers were designed from sequences published in GenBank report database.

The total RNA subjected to RT-PCR with NGF, BDNF, and the NT-3 primer sets was saturated after 35 rounds, while RT-PCR products targeting Six-3, LH-2, and Shh did not saturate even after 41 rounds of amplification. Consequently, comparisons were done after 30 cycles for NGF, BDNF, and NT-3, and after 35 cycles for Six-3, LH-2, and Shh, respectively. The expected sizes of all the RT-PCR products are listed in Figure 1. The amounts of RT-PCR products targeting NGF, BDNF, and NT-3 were not significantly different in the experimentally induced myopic retinal tissues in all treatment periods when compared with controls (Figure 2). The intensities of the single bands corresponding to the RT-PCR products of Six-3 and LH-2 were not significantly different in control and experimentally induced myopic retinal tissues in all treatment periods (Figure 3).

The expression of Shh mRNA was significantly increased in form-deprived and lens-induced myopic retinal tissues, respectively, after 3 and 7 days of treatment compared with controls by the semiquantitative RT-PCR method (Figure 4). Northern blot analysis followed using Shh cDNA probes. Results indicated overexpression of Shh mRNA (approximately 2.5 kb) in experimentally induced myopic retinal tissues at 7 days of treatment, consistent with data from the semiquantitative RT-PCR (Figure 5).

Discussion

In animal models, manipulation of retinal images to consistently produce retinal blur is known to result in myopia.[1–6, 8–10] Two experimental procedures have been extensively used in various studies dealing with myopia. One method involves form deprivation, which is achieved by fitting diffuser goggles over the test eye for a set period of time. A second model involves the use of negative spectacle lenses that cause displacement of normal "in-focus" retinal

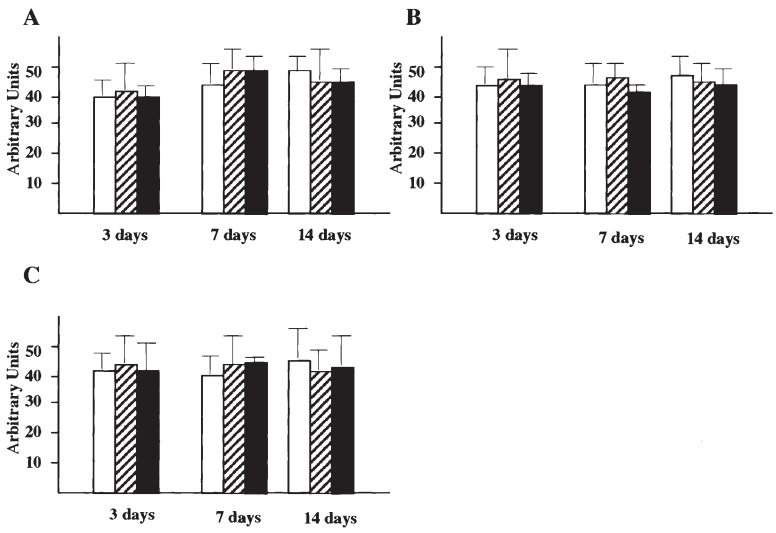

Figure 2. Results of digital image analysis of (**A**) nerve growth factor (NGF), (**B**) neurotrophin (NT)-3, and (**C**) brain-derived neurotrophic factor (BDNF) mRNAs in form-deprived myopic (FDM) ■ (n = 15 per time period), negative lens-induced myopic (−15 D). ▨(n = 15 per time period), and control □ (n = 15 per time period) chick retinas after 3, 7, and 14 days of myopic treatment by semiquantitative reverse transcriptional-polymerase chain reaction amplification. mRNA expression levels were not significantly different in control, form-deprived (FD), and negative lens-induced retinal samples after 3, 7, and 14 days of myopic treatment. Values are mean ± SD (arbitrary units).

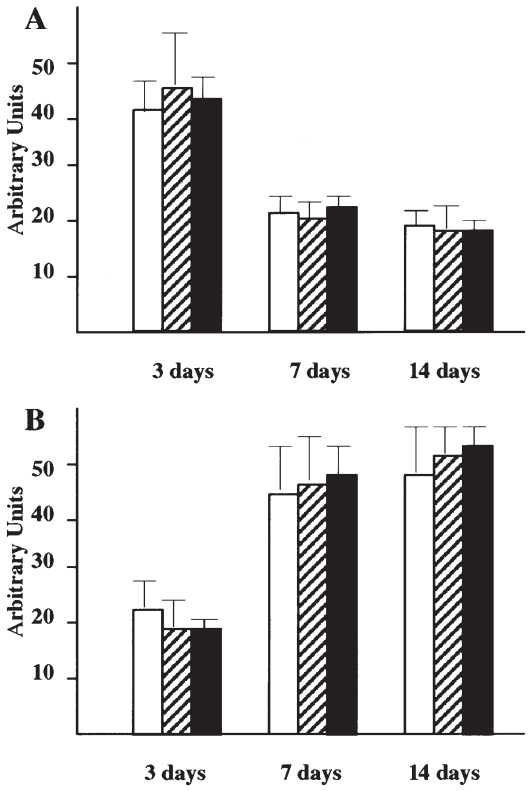

Figure 3. Digital imaging analysis results of Six-3 (A) and Lh-2 (B) mRNAs in form-deprived myopic (FDM) ■ (n = 15 per time period), negative lens-induced myopic (−15 D) ▨ (n = 15 per time period) and control □ (n = 15 per time period) chick retinas after 3, 7, and 14 days of myopic treatment by semiquantitative reverse transcriptional polymerase chain reaction amplification. mRNA expression levels were not significantly different in control and experimentally induced myopic retinal samples after 3, 7, and 14 days of myopic treatment. Values are mean ± SD (arbitrary units).

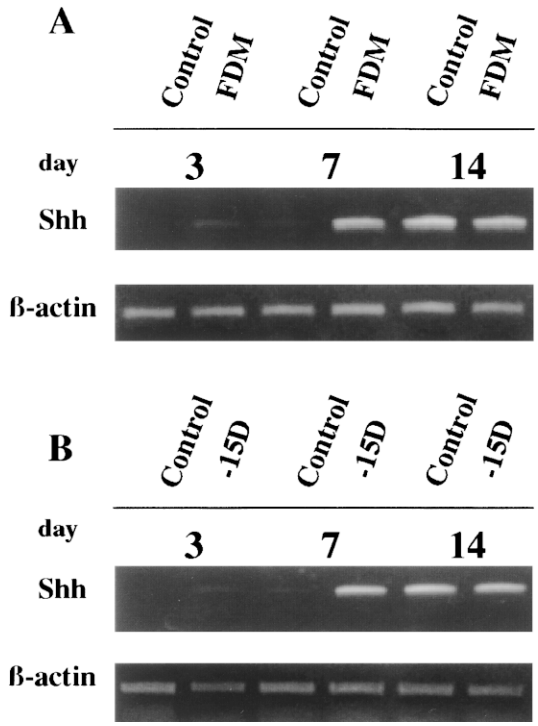

Figure 4. Sonic hedgehog (Shh) mRNA expression levels in form-deprived myopic (FDM) (n = 15 per time period) (**A**), negative lens-induced myopic (−15 D) (n = 15 per time period) (**B**), and control (n = 15 per time period) chick retinas after 3, 7, and 14 days of myopic treatment. Significant differences in expression were noted at 3 and 7 days of myopic treatment periods in both experimentally induced myopic groups.

images behind the retina. Spectacle lenses with a power of −10 D to −15 D have been reported to be more effective in inducing myopia.[10] Both form deprivation and negative spectacle lens manipulations lead to significantly increased ocular growth.[10]

We previously demonstrated that the expression of TGF-β,[4] BMP-2,[11] NSP-A, and C,[3] and delta crystallin[5] in post-hatch chick retinas were significantly different from controls after form deprivation and negative spectacle lens treatment. We then focused on other candidate substances that may have regulatory functions in the signaling system controlling eye growth. The expression of NGF,[20–22] NT-3,[23,24] BDNF,[25] Six-3,[15] Lh-2,[16] and Shh[26–28] were evaluated. These transcriptional and growth factors are expressed during the embryonic stages and are involved not only in the regulation of the TGF-β, BMP-2, NSP-A and -C, and delta crystallin group of genes, but also in the formation of the eye.[15,16,21,29–32] Based on evaluation of the expression of these genes by RT-PCR, only Shh showed significant mRNA upregulation in both form-deprived and negative lens-induced retinal tissues after 3 and 7 days of myopic treatment compared with controls.

Hedgehog is a secreted protein originally identified in Drosophila as having a role in segment polarity,[33] mutants being characterized as having loss of pattern and polarity of embryonic segments.[26,34] Seven hedgehog homologues have been isolated from different vertebrate species and have been shown to play important roles during development.[26,28] The best characterized of these homologues is Shh, which has been reported to act in the regulation of dorsoventral patterning of the neural tube and somites.[35–37] In the mouse and amphibian retina, Shh is localized in cells in the ganglion cell, amacrine cell and inner nuclear layers and is responsible for regulating mito-

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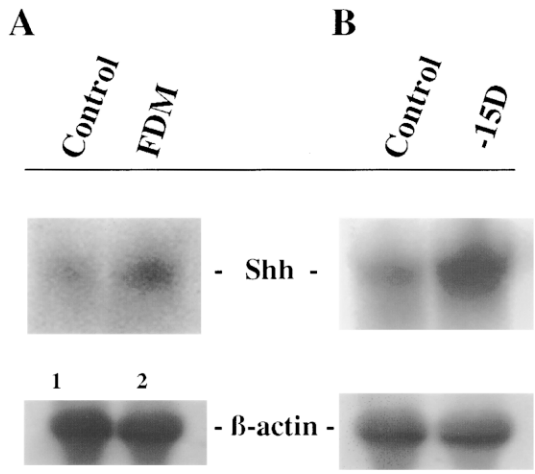

Figure 5. Northern blot analysis of sonic hedgehog (Shh) mRNA in chick retinas after 7 days of treatment. Significant differences in expression were noted at 7th day of myopic treatment periods in both experimentally induced (form deprivation (**A**) and negative hyperopic defocus (**B**) myopic groups.

genesis and photoreceptor differentiation.[27] Mutant Shh embryos throughout the stages of development have been noted to have absent bilateral eye structures.[29] This implicates the Shh gene in the normal formation and development of the eye, which includes the organization of the proximodistal axis of the eye.[29]

Myopia-inducing treatments (both form deprivation and minus lens-induced hyperopic defocus) were performed in 1-day post-hatched chicks. With reference to temporal studies dealing with induced myopia in chicks, myopic changes were evident within 2 days of treatment, and were significantly established after 5 days.[7] Based on our results, the timing of changes in Shh mRNA expression was most significant at day 7. This Shh mRNA change in expression is consistent with the development of myopia, hence implicating this gene in myopia genesis.

During the embryonic stages, Shh and BMP-2 were reported to exert opposing actions on the proliferation and differentiation of embryonic neural progenitor cells.[12,13] We have previously demonstrated that BMP-2 expression in chick retina was significantly decreased in both form-deprivation and lens-induced myopia, and that BMP-2 expression decreased gradually during 14 days of treatment.[11] The gradually increasing expression pattern of Shh and decreasing pattern of BMP-2 expression suggests that both genes may be involved in the regulation of eye growth in experimental myopia in the chick.

In an in vitro system, Shh proteins regulate mitogenesis and photoreceptor differentiation in the vertebrate retina.[38] In the developing mouse retina, treatment of cultures of perinatal mouse retinal cells with the amino-terminal fragment of Shh results in an increase in the numbers of rod photoreceptor cells, amacrine cells, and Müller cells.[27] These data indicate that the Shh gene product might not only have a role in axial length elongation and vitreous enlargement of the eye but also in the regulation of the expression of retinal-specific genes, such as the fully differentiated photoreceptor cells. Further studies are necessary to understand the exact role of the Shh gene and its possible interactions with other genes in the regulation of postnatal eye growth.

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