

# Upregulation of regulator of G-protein signaling 2 in the sclera of a form deprivation myopic animal model

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**Purpose:** Scleral remodeling is an important mechanism underlying the development of myopia. Atropine, an antagonist of G protein-coupled muscarinic receptors, is currently used as an off-label treatment for myopia. Regulator of G-protein signaling 2 (RGS2) functions as an intracellular selective inhibitor of muscarinic receptors. In this study we measured scleral RGS2 expression and scleral remodeling in an animal model of myopia in the presence or absence of atropine treatment.

**Methods:** Guinea pigs were assigned to four groups: normal (free of form deprivation), form deprivation myopia (FDM) for 4 weeks, FDM treated with saline, and FDM treated with atropine. Biometric measurements were then performed. RGS2 expression levels and scleral remodeling, including scleral thickness and collagen type I expression, were compared among the four groups.

**Results:** Compared with normal eyes and contralateral control eyes, the FDM eyes had the most prominent changes in refraction, axial length, and scleral remodeling, indicating myopia. There was no significant difference between control and normal eyes. Hematoxylin and eosin staining showed that the scleral thickness was significantly thinner in the posterior pole region of FDM eyes compared to normal eyes. Real-time PCR and western blot analysis showed a significant decrease in posterior scleral collagen type I mRNA and protein expression in the FDM eyes compared to the normal eyes. The FDM eyes also had increased levels of RGS2 mRNA and protein expression in the sclera. Atropine treatment attenuated the FDM-induced changes in refraction, axial length, and scleral remodeling. Interestingly, atropine treatment significantly increased collagen type I mRNA expression but decreased RGS2 mRNA and protein expression in the sclera of the FDM eyes.

**Conclusions:** We identified a significant RGS2 upregulation and collagen type I downregulation in the sclera of FDM eyes, which could be partially attenuated by atropine treatment. Our data suggest that targeting dysregulated RGS2 may provide a novel strategy for development of therapeutic agents to suppress myopia progression.

Myopia is the most common visual disorder and affects approximately half of the world's young adult population. East Asia has the highest prevalence of myopia, with more than 73% being myopic in Chinese, Singapore Chinese, and Taiwanese populations between the ages of 15 and 25 years [1]. The prevalence of myopia and the degree of severity are rapidly increasing [2]. Increased myopia is associated with an enhanced risk of pathological ocular complications and may lead to blinding disorders, such as premature cataracts, glaucoma, retinal detachment, and macular degeneration [3].

Studies have shown that both environmental factors and genetic factors contribute to myopia development [4-6]. However, despite years of intensive research, the precise mechanisms that control ocular growth and development of refractive errors are still not well known. Observations in

animal models strongly suggest that local factors within the eye play important roles in the regulation of ocular growth [7]. Many studies suggest that the eye is not dependent on the brain for visually guided growth regulation, rather it is dependent on a cascade of chemical events extending from the retina to the sclera that act to control vitreous chamber elongation [8-10]. Scleral remodeling occurs during axial elongation, which is one important mechanism for the development of myopia [11,12]. In mammals, the sclera is a fibrous connective tissue consisting of 90% heterologous collagen fibrils that comprise mainly collagen type I with small amounts of other fibrillar and fibril-associated collagens [13]. As an important component of the sclera, collagen type I is involved in pathological remodeling of myopia. The thinned posterior sclera in high myopia is associated with a general loss of collagen type I [12]. Understanding the nature of the signals that control scleral remodeling may identify new drug treatment options.

Atropine is currently used as an off-label treatment for myopia in children, especially in Southeast Asia [14-16].

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However, the mechanisms underlying the therapeutic effects of atropine in myopia are unknown. Previous studies have implicated the retina and choroid as potential sites of action for atropine [17]. Some *in vitro* studies that assessed the effects of atropine on muscarinic receptors (mAChRs) in scleral cells or tissue suggest that the sclera also is an important site of action for atropine [18,19]. mAChRs are G protein-coupled receptors (GPCRs) that play an important role in myopic progression [19]. An important component of GPCR signal coordination in human cells is the regulator of G-protein signaling (RGS) family of proteins that was discovered to have the ability to accelerate termination of GPCR signaling, thereby reducing the amplitude and duration of GPCR effects [20]. Over 20 different mammalian RGS proteins that share a conservative RGS domain have been identified. RGS2 is the one that selectively inhibits G<sub>q</sub>-coupled receptors, including mAChRs [21,22], and plays important roles in regulating functions of the immune, neurologic, and cardiovascular systems [23-25]. RGS2 is also a functionally important negative regulator of cardiac fibroblast responses in the myocardium [26]. Interestingly, RGS2 exhibits dynamic changes of expression in response to various stimuli, and dysregulation of RGS2 has been implicated in many human diseases, such as hypertension, cancer, and schizophrenia [27-29]. However, the expression profile and pathological importance of RGS2 in myopic eyes are unknown.

The goal of the present study was to determine whether there were changes in RGS2 mRNA and protein expression levels in the sclera of eyes during the development of form deprivation myopia (FDM) in guinea pigs. We found a significant increase in RGS2 expression associated with loss of collagen type I in the sclera from myopic eyes. Treatment with atropine attenuated upregulation of RGS2 expression and restored collagen expression in FDM sclera. This report is the first to demonstrate a strong association of RGS2 upregulation with myopia development in an animal study. Our data suggest that upregulated RGS2 may modulate scleral remodeling by downregulating collagen expression, thus contributing to the progression of myopia. This study also provides a basis for our future studies to determine whether targeting dysregulated RGS2 provides a novel strategy for development of therapeutic agents to suppress myopia progression.

## METHODS

**Animal model:** All procedures were approved by the Institutional Animal Care and Use Committee of Fudan University and were in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One hundred and twenty pigmented guinea pigs (2-weeks old)

from the Animal Experiments Laboratory (Fudan University, Shanghai, China) were randomly assigned to four groups. Biometric measurements were performed among the four groups before any intervention was done. In the normal group (n = 30), the guinea pigs were reared without any intervention. In the FDM group (n = 30), the guinea pigs were raised with a diffuser placed over the right eye for 4 weeks. In the FDM + atropine group (n = 30), the guinea pigs were raised with a diffuser placed over the right eye and an intravitreal injection of atropine solution was administered. In the FDM + saline group (n = 30), the guinea pigs were raised with a diffuser placed over the right eye and an intravitreal injection of sterilized saline was administered. The diffuser was then removed from the animals and biometric measurements were performed immediately.

**Intravitreal injection of atropine:** The animals were anesthetized by intramuscular injection of ketamine hydrochloride (37.5mg kg<sup>-1</sup>, Ketolar; Pfizer, New York, NY) and xylazine hydrochloride (5 mg kg<sup>-1</sup>, Rompun; Bayer AG, Germany). Additionally, 0.4% oxybuprocaine hydrochloride (Santen, Japan) was topically instilled. A monocular intravitreal injection (7 µl) of a freshly prepared atropine sulfate solution (10 mg/ml<sup>-1</sup>) or saline was administered every 3 days with a 0.03-mm needle (30G) for 4 weeks. The dosage of atropine was chosen on the basis of previous work in chicks [30], and our initial studies indicated that the atropine dosage used in this study was sufficient to inhibit the development of myopia in guinea pigs. After intravitreal injection, gentamicin ointment was applied to the injected area to prevent infection.

**Biologic measurements:** Biometric measures included refraction, anterior chamber depth (ACD), lens thickness (LT), and axial length (AL). The measurements were done as described previously [31]. In brief, refractive errors were measured using a hand-held streak retinoscope; all errors referred to spherical-component refractive error, defined as mean refractive error in the horizontal and vertical meridians. ACD, LT, and AL of eyes were measured by A-scan ultrasonography (11 MHz; Optikon Hiscan A/B) while animals were anesthetized with topical oxybuprocaine hydrochloride. AL of the eye was defined as the distance from the front of the cornea to the front of the retina. Ocular refraction and axial ocular dimensions were collected at the start and end of the experiment.

**Hematoxylin and eosin staining:** Eyes were collected and fixed in 10% phosphate-buffered formalin for 24 h (n = 10 eyes/group). Subsequently, eyes were embedded in paraffin. Marks were made at the 12 o'clock position on the corneal limbus. Sections (4 µm) were made from the marks in the anterior-posterior axis and stained with hematoxylin and

eosin (H&E). The sections were examined under a light microscope (BX41; Olympus, Japan), and photomicrographs were obtained with an Olympus DP20 digital camera (Olympus). The H&E staining results shown in the figures were generated from ten different guinea pigs, and the images shown in the figures are representative of 40 H&E staining pictures per group (four per guinea pig). We measured the scleral thickness in areas 0.5 mm from the optic nerve in the superior, inferior, nasal, and temporal planes in each sample. Measurements were obtained with Soft Cell<sup>A</sup> Imaging Software for Life Science Microscopy (Olympus).

**Tissue preparation for real-time PCR and western blot:** The animals were euthanized with a lethal dose of chloral hydrate. The eyes were enucleated and cut perpendicularly to the anterior–posterior axis, approximately 1 mm posterior to the ora serrate, using a razor blade under a surgical microscope (Topcon, Tokyo, Japan). The anterior segment of the eye was discarded. The posterior sclera was excised using a 7-mm diameter trephine, and the head of the optic nerve was discarded. The sclera was snap frozen in liquid nitrogen and stored at –80 °C.

**Real-time PCR:** The procedures for real-time-PCR were described previously [32]. In brief, total RNA was isolated using the phenol-chloroform extraction method of Chomczynski et al. [33].  $\beta$ -actin was used as the internal control. Sequences were obtained from the GenBank database. The primers are listed in Table 1. All experiments were performed at least three times, and the products were confirmed by sequencing using TaKaRa. The relative changes in gene expression were analyzed using the  $2^{-\Delta\Delta C_T}$  method described by Livak et al. [34] and normalized to the internal control  $\beta$ -actin. The fold change from the control group was calculated by the  $2^{-\Delta\Delta C_T}$ .  $\Delta\Delta C_T = (C_{T, RGS2} - C_{T, \beta\text{-actin}})_{\text{Treatment group}} - (C_{T, RGS2} - C_{T, \beta\text{-actin}})_{\text{Control Group}}$ . mRNA expression was normalized and expressed as the fold change from the control group. Specificity of real time-PCR was checked by analyzing melting curves.

**Western blot:** Protein was extracted from frozen sclera by using ice-cold extraction buffer (0.01 M Tris-HCl at pH 7.4, 0.15 M NaCl, 1% weight/volume Triton X-100, 0.1% sodium dodecyl sulfate, 1% deoxycholic acid, 1mM ethylene diamine tetraacetic acid) as well as protease inhibitors (1  $\mu$ M pepstatin, 1  $\mu$ g/ml leupeptin, and 0.2 mM phenylmethanesulfonyl fluoride). Samples (30  $\mu$ g) were electrophoresed and subjected to western blot using RGS2, collagen type I, and  $\beta$ -tubulin antibodies as described shortly [12]. The membranes were incubated overnight with primary antibodies at a 1:100 dilution (mouse anti RGS2; 100  $\mu$ g ml<sup>-1</sup>, Santa cruz, Dallas, TX) and 1:1000 dilution (rabbit and collagen type I; 1mg ml<sup>-1</sup>, Abcam, Cambridge, MA) at 4 °C in blocking solution. The membranes were then washed three times with TBST and incubated with a horseradish peroxidase- conjugated secondary antibody (goat anti-mouse for RGS2 and goat anti-rabbit for collagen type I at a 1:5000 dilution (1 mg ml<sup>-1</sup>, Abmart, Shanghai, China) for another 1 h at room temperature. The membranes were again washed three times with TBST. The reaction products were visualized with BeyoECL Plus western blotting detection reagents (Beyotime Institute of Biotechnology, Haimen, China). Images were captured with a Fuji Film LAS3000 imaging system and analyzed with MultiGauge software (FujiFilm, Tokyo, Japan).  $\beta$ -tubulin (Kang Chen, China) was used as a housekeeping protein to normalize protein loading. The results of protein expression were normalized and expressed as the fold change from the control group.

**Statistical analysis:** All values are shown as mean $\pm$ standard error of the mean (SEM). Differences between two groups were determined with paired or unpaired Student *t* tests. A difference at  $p < 0.05$  was considered statistically significant. Statistical analysis was performed using the SPSS 15.0 statistical software (IBM, Chicago, IL).

TABLE 1. THE SEQUENCES OF REAL-TIME PCR PRIMERS.

Gene	Primers sequence (5' to 3')	Annealing temperature (°C)	Product size (bp)
$\beta$ -actin	F: TTCTAGGCGGACTGTTACTAC	60	87
	R: CAATCTCATCTCGTTTTCTG		
RGS2	F: CAGTGGCTGCTTTACAAC	59	102
	R: TTACACAAGTCCTGGTAGAAT		
Col I	F: GGCGATGGTGGACGATACTAC	60	85
	R: GGCTCTGAGGGTGGTGTCTA		

**TABLE 2. BIOMETRIC MEASUREMENT BEFORE INTERVENTION.**

Groups	Eye	Refraction diopter (D)	Anterior chamber depth (mm)	Lens thickness (mm)	Axial length (mm)
Control	R	+3.42±1.13	1.27±0.03	3.04±0.10	7.61±0.12
	L	+3.32±0.93	1.29±0.02	3.08±0.13	7.65±0.11
FDM	R	+3.15±0.12	1.27±0.02	3.02±0.11	7.54±0.10
	L	+3.27±0.12	1.28±0.02	3.07±0.09	7.62±0.14
FDM+Saline	R	+3.25±1.22	1.30±0.02	3.02±0.08	7.58±0.13
	L	+3.13±1.15	1.29±0.02	3.04±0.12	7.60±0.10
FDM+Atropine	R	+3.33±0.14	1.28±0.03	3.08±0.12	7.64±0.11
	L	+3.27±0.11	1.28±0.02	3.05±0.07	7.60±0.13

FDM: Form Deprivation Myopia, R: Right, L: Left

**RESULTS**

*Confirmation of phenotypic changes induced by form deprivation:* There were no significant differences in refraction, ACD, LT, or AL among groups of guinea pigs before the start of the experiments (Table 2). The mean sphere refraction in FDM eyes (-1.28±0.09 D; n = 30,) was significantly more myopic than that in either the contralateral control eyes (+2.15±0.12; n = 30, p<0.05) or age-matched normal eyes (+2.10±0.73; n = 30, p<0.05; Table 3). There was no significant difference in refraction between control and normal eyes. The mean interocular difference in refraction for form deprivation animals (best sphere of deprived eye minus control eye) was -3.38 D, which was significantly more myopic than that of untreated animals (interocular difference +0.02 D). The AL of FDM eyes was elongated relative to that in the normal eyes by 0.26 mm (p<0.05; n = 30). Other parameters, such as AC and LT, showed no significant differences between the two eyes of the same animal after form deprivation and between

the same eyes of different animal, as previously reported [35] (Table 3).

*Scleral collagen type I downregulation induced by form deprivation:* H&E staining indicated posterior scleral thinning in treated eyes of the FDM group compared to those of the normal group (Figure 1). The scleral thickness was measured from photomicrographs, using a calibrated stage micrometer. The scleral thickness was significantly thinner in the posterior pole region of FDM eyes compared to normal eyes (Figure 1; 98±7.7 µm versus 136±8.5 µm, p<0.05, n = 10). Since the thinned sclera in high myopia is associated with loss of collagen type I, we compared expression levels of collagen type I mRNA and protein between FDM and normal eyes. Real time-PCR analysis showed a significant 50% decrease in posterior scleral collagen type I mRNA expression in the FDM eyes compared to the normal eyes (n = 10, p<0.05; Figure 2A). Western blot analysis indicated that the collagen type I protein level was also significantly decreased

**TABLE 3. BIOMETRIC CHANGES INDUCED BY FDM IN THE PRESENCE OR ABSENCE OF ATROPINE TREATMENT.**

Groups	Eye	Refraction Diopter (D)	Anterior Chamber Depth (mm)	Lens Thickness (mm)	Axial Length (mm)
Control	R(normal)	+2.10±0.73	1.28±0.02	3.26±0.09	8.01±0.04
	L(normal)	+2.13±0.90	1.29±0.01	3.27±0.08	8.02±0.06
FDM	R(FD)	-1.28±0.09*	1.29±0.01	3.28±0.09	8.27±0.08*
	L(normal)	+2.15±0.12	1.28±0.02	3.26±0.07	7.99±0.09
FDM + Saline	R(FD)	-1.37±0.08	1.32±0.03	3.26±0.09	8.30±0.14
	L(normal)	+2.15±0.13	1.33±0.04	3.21±0.10	8.03±0.12
FDM + Atropine	R(FD)	+1.32±0.19*	1.30±0.01	3.28±0.09	8.14±0.06*
	L(normal)	+2.14±0.27	1.33±0.03	3.35±0.11	8.04±0.09

FDM: Form Deprivation Myopia, FD: Form Deprivation, R: Right, L: Left



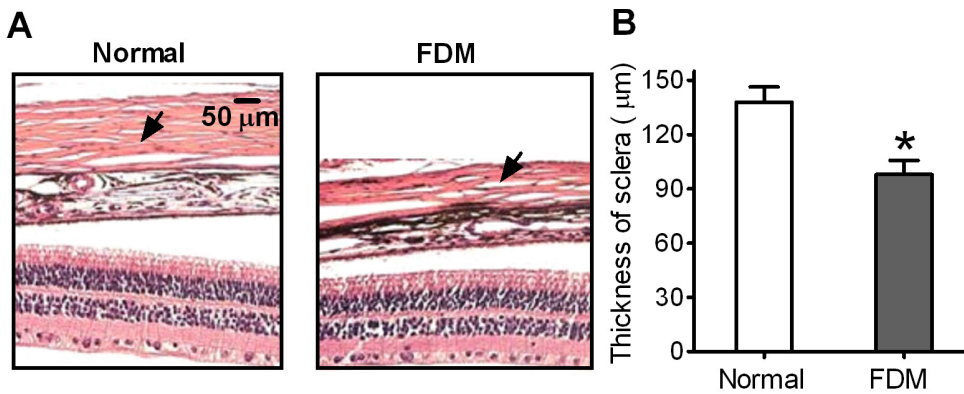


Figure 1. Form deprivation (FD) induced thinning in sclera. **A:** Representative photomicrographs of scleral sections stained with H&E in normal and form deprivation myopia (FDM) eyes. **B:** Quantitative analysis of the thickness of the sclera by Soft Cell<sup>^</sup> Imaging Software (n = 10 eyes in each group, six sections from each eye). Each point represents mean $\pm$ SEM.

Statistical analysis was performed on the mean data from each guinea pig using a Student *t* test, \**p*<0.05 compared to the control. Arrows indicate the sclera. Scale bar is 50  $\mu$ m.

by about 45% in treated eyes of the FDM group compared to that of the normal group (Figure 2B; n = 10, *p*<0.05).

**Scleral regulator of G-protein signaling 2 upregulation induced by form deprivation:** RGS2 has been reported to suppress collagen synthesis in fibroblasts [26]. Therefore, we analyzed effects of FDM on scleral RGS2 mRNA and protein expression. Real time-PCR showed that the scleral RGS2 mRNA level was significantly increased two-fold in the FDM eyes compared to normal eyes (Figure 3A; n = 10, *p*<0.05). Western blot also showed a significant 1.8-fold increase of RGS2 protein expression in the sclera of FDM eyes compared to normal eyes (Figure 3B; n = 10, *p*<0.05).

**Atropine treatment inhibits myopia progression:** As shown in Table 3, the mean of sphere refraction in FDM eyes treated with saline was  $-1.37\pm 0.08$  (n = 30), indicating development of myopia. Atropine treatment significantly attenuated the effect of form deprivation on the refraction of treated eyes ( $+1.32\pm 0.19$  in FDM eyes treated with atropine versus

$-1.37\pm 0.08$  in FDM eyes treated with saline; n = 30, *p*<0.05). Similarly, atropine treatment also partially attenuated FDM-induced elongation of ocular AL ( $8.14\pm 0.06$  mm versus  $8.30\pm 0.14$  mm; n = 30, *p*<0.05). These results indicate that atropine treatment significantly inhibits myopia progression in experimental animals. There were no significant differences in refraction and elongation of the contralateral control eyes between atropine- and saline-treated FDM animals (Table 3).

**Effects of atropine treatment on collagen production and regulator of G-protein signaling 2 gene expression in the sclera of form deprivation myopia eyes:** We next examined the effects of atropine treatment on posterior scleral thinning in eyes of the FDM animals. As shown in Figure 4, the sclera was significantly thicker in the posterior pole region in the atropine-treated group as compared to the saline control group ( $112\pm 6.5$   $\mu$ m versus  $87\pm 5.8$   $\mu$ m; n = 10, *p*<0.05). In contrast, no significant differences were observed in posterior

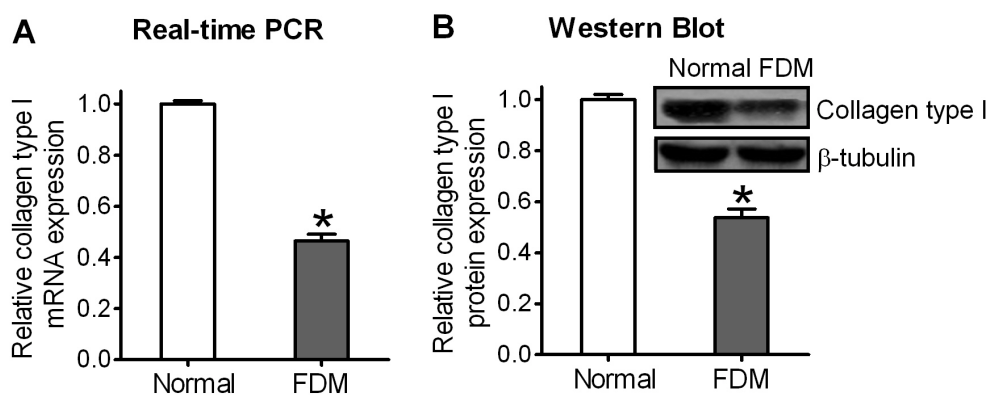


Figure 2. Scleral collagen type I downregulation induced by form deprivation (FD). **A:** Real-time-PCR analysis of collagen type I mRNA expression levels in the sclera of guinea pigs. Data were analyzed by the  $2^{-\Delta\Delta C_T}$  method and normalized to internal control  $\beta$ -actin as described in the Methods (n = 10 samples in each group). **B:** Western blot analysis of collagen type I protein expression level in

guinea pig sclera (inset), analyzed by measuring band density and normalized to  $\beta$ -tubulin (n = 10 samples in each group). mRNA and protein expression levels were normalized and expressed as fold change (ratio of treated group to normal eye group). Data shown are mean $\pm$ SEM. Statistical analysis was performed on the mean data using a Student *t* test, \**p*<0.05 compared to the control.

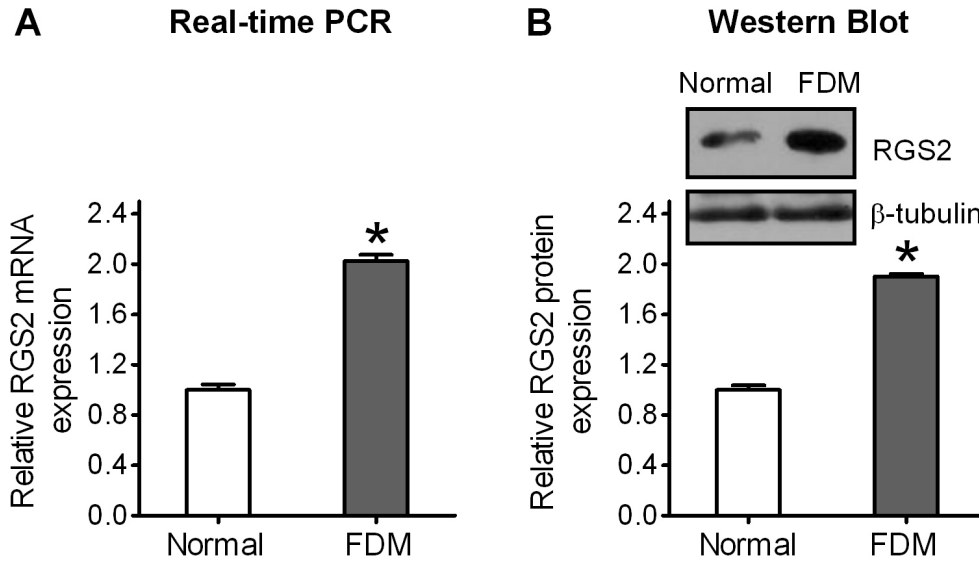


Figure 3. Regulator of G-protein signaling 2 (RGS2) upregulation in the sclera induced by form deprivation (FD). **A:** Real-time PCR analysis of RGS2 mRNA expression level in guinea pig sclera. Data were analyzed by the  $2^{-\Delta\Delta C_T}$  method and normalized to  $\beta$ -actin as described in the Methods (n = 10 samples in each group). **B:** Western blot analysis of RGS2 protein expression level in guinea pig sclera (inset), analyzed by measuring band density and normalized by  $\beta$ -tubulin (n = 10 samples in each group). mRNA and protein expression levels were normalized and expressed as fold change (ratio of

treated group to normal eye group). Data shown are mean $\pm$ SEM. Statistical analysis was performed on the mean data using a Student *t* test, \**p*<0.05 compared to the control.

scleral thickness of the contralateral control eyes between atropine- and saline-treated FDM animals (135 $\pm$ 5.5  $\mu$ m versus 134 $\pm$ 6.3  $\mu$ m; n = 10).

Compared to saline control, atropine treatment significantly increased collagen type I mRNA expression (Figure 5A; n = 10, *p*<0.05) and protein expression (Figure 5B; n = 10, *p*<0.05) in the sclera of FDM eyes by 2.3-fold and 1.4-fold, respectively. Interestingly, treatment with atropine also significantly decreased RGS2 mRNA expression (Figure 5C; n = 10, *p*<0.05) and protein expression (Figure 5D; n = 10, *p*<0.05) in the sclera of FDM eyes by about 30%.

### DISCUSSION

Scleral remodeling due to loss of collagen type I is an important mechanism underlying the development of myopia. Atropine, a nonselective muscarinic antagonist, is currently used as an off-label treatment for myopia, but the mechanism underlying this therapeutic effect remains unknown. This report is the first to demonstrate a strong association of upregulation of RGS2, a modulator of muscarinic receptors, with collagen downregulation and myopia development in FDM experimental animals. More importantly, our data demonstrated that RGS2 upregulation in the sclera induced by

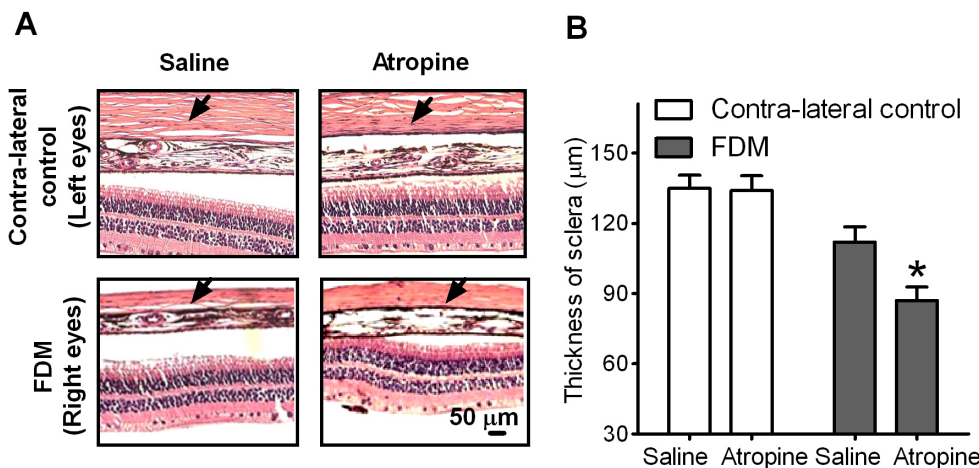


Figure 4. Effects of atropine treatment on scleral collagen production. **A:** Representative photomicrographs of scleral sections of H&E staining from the contralateral control and form deprivation myopia (FDM) eyes with or without atropine treatment. **B:** Quantitative analysis of the thickness of the sclera by Soft Cell<sup>A</sup> Imaging Software (n = 10 eyes in each group, six sections from each eye). Each point represents mean $\pm$ SEM. Statistical

analysis was performed on the mean data from each guinea pig using a Student *t* test, \**p*<0.05 compared to the saline control. Arrows indicate the sclera. Scale bar is 50  $\mu$ m.

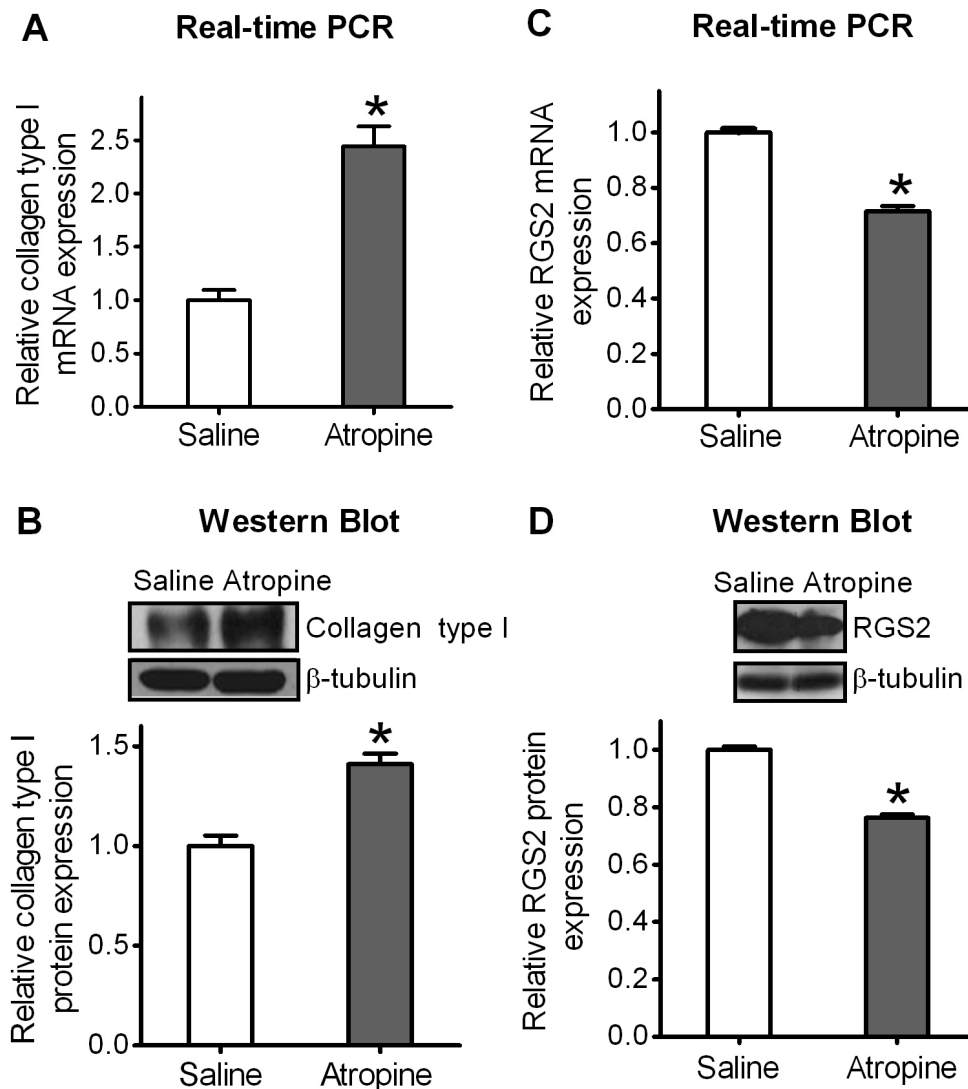


Figure 5. Effects of atropine treatment on collagen type I and Regulator of G-protein signaling 2 (RGS2) expression in the sclera of form deprivation myopia (FDM) eyes. Real-time-PCR analysis of collagen type I (A) and RGS2 (C) mRNA expression levels in the sclera of guinea pigs. Data were analyzed by the  $2^{-\Delta\Delta C_T}$  method and normalized to internal control  $\beta$ -actin as described in the Methods (n = 10 samples in each group). Western blots of collagen type I (B) and RGS2 (D) protein expression levels in the sclera of guinea pigs (top panels), analyzed by measuring band density and normalized by the internal control  $\beta$ -tubulin (n = 10 samples in each group). mRNA and protein expression levels were normalized and expressed as fold change (ratio of atropine group to saline group). Data shown are mean  $\pm$  SEM. Statistical analysis was performed on the mean data using a Student *t* test, \* $p < 0.05$  compared to the saline control.

form deprivation could be partially attenuated by treatment with atropine, which is consistent with the increase of scleral collagen expression and amelioration of myopia in FDM animals. These results suggest a new mechanism underlying atropine treatment of myopia.

RGS proteins belong to a diverse protein family that has the ability to accelerate signal termination in response to GPCR stimulation, thereby reducing the amplitude and duration of GPCR effects. RGS protein expression can be modulated by GPCR ligands and second messengers *in vivo* and *in vitro*. This seems especially pertinent for RGS2, a selective inhibitor of  $G_q$ -coupled receptors, including mAChRs [21,22], in as much as its expression is rapidly and substantially increased in response to neuronal activity [36], second messengers [37], and certain cellular stressors, such as heat shock, oxidative stress [36,38], and ischemia [39]. Therefore,

we used form deprivation, a well-established model for excessive eye elongation and myopia, to induce myopia in guinea pigs and compared the scleral RGS2 expression levels between the FDM eyes and normal control eyes. Under our experimental condition, myopic change and axial length growth were found in the FDM eyes of guinea pigs compared to both the contralateral eyes and normal eyes during an observation period of 4 weeks. These results are consistent with previous findings [40]. We also found that RGS2 mRNA and protein levels were significantly increased in the sclera of FDM eyes compared with normal eyes. Mammalian sclera is a fibrous connective tissue consisting of 90% heterologous collagen fibrils that comprise mainly collagen type I. Our data showed that upregulation of RGS2 was associated with downregulation of collagen type I expression in FDM eyes. Interestingly, a previous study showed that RGS2 inhibits

the production of collagen type I in the myocardium and functions as an important negative regulator of angiotensin II-induced fibroblast response [26]. Since the fibroblast is the only cell type in the sclera that produces scleral collagens [41], by analogy it seems likely that upregulation of RGS2 could inhibit collagen production in scleral fibroblasts. Thus, our results suggest that the upregulation of RGS2 expression may contribute to loss of collagen type I in the sclera during development of myopia.

There are many studies showing the localization of muscarinic cholinergic receptors within the eye, the effects of muscarinic agents on scleral remodeling, and the possible sites of action of such agents [19,42]. mAChRs are a group of neurotransmitter-activated GPCRs. Five distinct receptor genes (*CHRM1–CHRM5*) have been cloned, each encoding a muscarinic receptor protein 1–5 (M1–M5) with specific pharmacological properties [43]. Atropine has been found effective in clinical trials with children in preventing myopia progression [15,16]. This drug has also been tested in studies using animal models of myopia and was found to block axial elongation during the development of FDM [44]. Our data confirmed that atropine treatment attenuated the FDM-induced changes of refraction, AL, and scleral remodeling, suggesting that the progression of myopia in guinea pigs is inhibited by atropine treatment.

Previous studies showed that atropine may be able to change collagen structure and production of scleral extracellular proteins during myopia development in animal models [45]. Since RGS2 can inhibit the production of collagen [26], we investigated whether treatment with atropine could affect the upregulation of RGS2 expression induced by form deprivation, thus regulating the production of collagen in the sclera of myopic eyes. Indeed, RGS2 expression levels in the sclera of atropine-treated FDM eyes were 30% lower than that in saline-treated FDM eyes. This result is consistent with a 1.4-fold increase of scleral collagen type I expression seen in atropine-treated FDM eyes as compared to that in the saline-treated group. We did not directly compare the expression levels of scleral RGS2 and collagen type I between atropine-treated FDM eyes and normal eyes; thus, we could not exclude the possibility that there may be some yoking effects. However, since there was about a 1.8-fold increase of RGS2 and 45% reduction of collagen type I in FDM eyes as compared to normal eyes, it seems likely that atropine treatment, at least, partially attenuated alterations of scleral RGS2 and collagen type I in FDM eyes. As expected, atropine treatment increased the scleral thickness of FDM eyes from  $87\pm 5.8\ \mu\text{m}$  to  $112\pm 6.5\ \mu\text{m}$ , indicating atropine inhibition of scleral thinning in FDM eyes, whereas no significant

differences were observed in scleral thickness of the contralateral control eyes between atropine- and saline-treated FDM animals. We note that the sclera in the FDM eyes of the atropine-treated group was still thinner than their contralateral control eyes ( $135\pm 5.5\ \mu\text{m}$ ) or normal eyes ( $136\pm 8.5\ \mu\text{m}$ ). Interestingly, the mean sphere refraction of the FDM eyes in the atropine-treated group ( $+1.32\pm 0.19$ ) was improved as compared to that in saline-treated FDM animals ( $-1.37\pm 0.08$ ) but was still more myopic than their contralateral control eyes ( $+2.14\pm 0.39$ ). Thus, atropine treatment may only partially prevent form deprivation-induced scleral remodeling and the progression of myopia in guinea pigs, and altered regulation of RGS2 may be one of the underlying mechanisms. In fact, Song et al. [46] reported that muscarinic receptor activation with carbachol results in elevations in RGS2 mRNA levels and pretreatment with atropine blocked the carbachol-induced increase of RGS2 mRNA expression in human neuroblastoma. Therefore, it is possible that FDM induces excessive muscarinic receptor activation, which in turn causes RGS2 upregulation to trigger a feedback pathway to restrain the effects of muscarinic receptor stimulation in the sclera of animal eyes. Under physiologic conditions, endogenous RGS2 should be able to tightly control signaling mediated by muscarinic receptors. However, under pathological situations, such as FDM, it is possible that even upregulated RGS2 may not be sufficient to attenuate excessively activated muscarinic receptor signaling. As an antagonist, atropine should effectively block excessive muscarinic receptor signaling, thus preventing the progression of myopia. Since intravitreal injection of a relatively high dose of atropine (1%) was used to prevent myopia in guinea pigs in our studies, the sclera could be one site of action. Atropine treatment could attenuate muscarinic receptor activation, thus downregulating scleral RGS2 expression to prevent scleral remodeling. In the present study we focused on the sclera because scleral remodeling is an important mechanism for the development of myopia, and our initial study showed a significant upregulation of scleral RGS2 in myopic eyes. However, there is strong evidence from experimentally induced animal myopia that the myopia-generating signal begins at the retina [47]. It is possible that the inhibition of myopic changes in the sclera by atropine may simply reflect attenuation of the myopia-generating signal since atropine can act on various sites in the eye in addition to the sclera. In fact, previous studies have also implicated the retina and choroid as potential sites of action for atropine [17], and intravitreal injections of atropine should deliver the highest drug concentrations to the retina. Although changes in scleral expression levels of RGS2 and collagen type I were observed in atropine-treated eyes, we could not rule out the possibility of downstream effects involving other signaling



molecules in addition to an effect on scleral muscarinic receptors. Relatively high doses of atropine are widely used to prevent myopia in experimental animal studies [48], but a recent study showed that a dose of atropine of 0.01% seems to be a safe and effective regimen for slowing myopia progression in children [16]. It would be interesting to further examine the therapeutic effects of intravitreally applied atropine in our experimental model at doses below functional levels in sclera. Moreover, to fully understand the mechanisms underlying the therapeutic effects of atropine in myopia, further studies are necessary to determine the impact of atropine treatment on RGS2 expression in the retina and choroid of myopic eyes.

In summary, the expression of RGS2 was increased in the posterior sclera of guinea pigs in the FDM eyes; this was associated with a decrease of collagen in the FDM sclera. Treatment with atropine attenuated upregulation of RGS2 expression and restored collagen type I expression in FDM sclera. Since RGS2 plays an important role in cellular collagen homeostasis, our data suggest that upregulated RGS2 may modulate scleral remodeling by downregulating collagen type I expression, thus contributing to myopia progression. Atropine treatment may downregulate scleral RGS2 expression to prevent scleral remodeling. We and our collaborator are currently investigating whether RGS2 knockout will ameliorate pathological features of myopia in mice. This study will form a basis for our future studies to determine whether targeting dysregulated RGS2 provides a novel strategy for controlling myopia progression.

#### ACKNOWLEDGMENTS

This study was supported by a Research Grant Shanghai Pujiang Program (09PJ1402400), National Nature Science Foundation of China (81100689) and Shanghai Pudong Health Bureau Key Cooperation Projects (PW2012D-5). The authors also wish to thank Drs. Peter Abel, Yan Xie and Yaping Tu from Creighton University School of Medicine for helpful discussion and editing the manuscript.

#### REFERENCES

- Jones D, Luensmann D. The prevalence and impact of high myopia. *Eye Contact Lens* 2012; 38:188-96. [PMID: 22495679].
- Bloom RI, Friedman IB, Chuck RS. Increasing rates of myopia: the long view. *Curr Opin Ophthalmol* 2010; 21:247-8. [PMID: 20489624].
- Saw SM, Gazzard G, Shih-Yen EC, Chua WH. Myopia and associated pathological complications. *Ophthalmic Physiol Opt* 2005; 25:381-91. [PMID: 16101943].
- Feldkämper M, Schaefel F. Interactions of genes and environment in myopia. *Dev Ophthalmol* 2003; 37:34-49. [PMID: 12876828].
- Pacella R, McLellan J, Grice K, Del Bono EA, Wiggs JL, Gwiazda JE. Role of genetic factors in the etiology of juvenile-onset myopia based on a longitudinal study of refractive error. *Optom Vis Sci* 1999; 76:381-6. [PMID: 10416932].
- Lu B, Jiang D, Wang P, Gao Y, Sun W, Xiao X, Li S, Jia X, Guo X, Zhang Q. Replication study supports CTNND2 as a susceptibility gene for high myopia. *Invest Ophthalmol Vis Sci* 2011; 52:8258-61. [PMID: 21911587].
- Wallman J, Winawer J. Homeostasis of eye growth and the question of myopia. *Neuron* 2004; 43:447-68. [PMID: 15312645].
- Wallman J, Gottlieb MD, Rajaram V, Fugate-Wentzek LA. Local retinal regions control local eye growth and myopia. *Science* 1987; 237:73-7. [PMID: 3603011].
- Troilo D, Gottlieb MD, Wallman J. Visual deprivation causes myopia in chicks with optic nerve section. *Curr Eye Res* 1987; 6:993-9. [PMID: 3665562].
- Rada JA, Palmer L. Choroidal regulation of scleral glycosaminoglycan synthesis during recovery from induced myopia. *Invest Ophthalmol Vis Sci* 2007; 48:2957-66. [PMID: 17591860].
- McBrien NA, Lawlor P, Gentle A. Scleral remodeling during the development of and recovery from axial myopia in the tree shrew. *Invest Ophthalmol Vis Sci* 2000; 41:3713-9. [PMID: 11053267].
- Wang Q, Zhao G, Xing S, Zhang L, Yang X. Role of bone morphogenetic proteins in form-deprivation myopia sclera. *Mol Vis* 2011; 17:647-57. [PMID: 21403850].
- Marshall GE, Konstas AGP, Lee WR. Collagens in ocular tissues *Br J Ophthalmol* 1993; 77:515-24. [PMID: 8025051].
- Chua WH, Balakrishnan V, Chan YH, Tong L, Ling Y, Quah BL, Tan D. Atropine for the treatment of childhood myopia. *Ophthalmology* 2006; 113:2285-91. [PMID: 16996612].
- Fang YT, Chou YJ, Pu C, Lin PJ, Liu TL, Huang N, Chou P. Prescription of atropine eye drops among children diagnosed with myopia in Taiwan from 2000 to 2007: a nationwide study. *Eye (Lond)* 2013; 27:418-24. [PMID: 23288141].
- Chia A, Chua WH, Cheung YB, Wong WL, Lingham A, Fong A, Tan D. Atropine for the treatment of childhood myopia: safety and efficacy of 0.5%, 0.1%, and 0.01% doses (Atropine for the Treatment of Myopia 2). *Ophthalmology* 2012; 119:347-54. [PMID: 21963266].
- Schwahn HN, Kaymak H, Schaefel F. Effects of atropine on refractive development, dopamine release, and slow retinal potentials in the chick. *Vis Neurosci* 2000; 17:165-76. [PMID: 10824671].
- Qu J, Zhou X, Xie R, Zhang L, Hu D, Li H, Lu F. The presence of m1 to m5 receptors in human sclera: evidence of the sclera as a potential site of action for muscarinic receptor antagonists. *Curr Eye Res* 2006; 31:587-97. [PMID: 16877267].

19. Barathi VA, Beuerman RW. Molecular mechanisms of muscarinic receptors in mouse scleral fibroblasts: prior to and after induction of experimental myopia with atropine treatment. *Mol Vis* 2011; 17:680-92. [PMID: 21403852].
20. Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* 2000; 69:795-827. [PMID: 10966476].
21. Bernstein LS, Ramineni S, Hague C, Cladman W, Chidiac P, Levey AI, Hepler JR. RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate Gq/11alpha signaling. *J Biol Chem* 2004; 279:21248-56. [PMID: 14976183].
22. Clark MA, Sethi PR, Lambert NA. Active Galpha(q) subunits and M3 acetylcholine receptors promote distinct modes of association of RGS2 with the plasma membrane. *FEBS Lett* 2007; 581:764-70. [PMID: 17275815].
23. Oliveira-Dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, Mariathasan S, Sasaki T, Wakeham A, Ohashi PS, Roder JC, Barnes CA, Siderovski DP, Penninger JM. Regulation of T cell activation, anxiety, and male aggression by RGS2. *Proc Natl Acad Sci USA* 2000; 97:12272-7. [PMID: 11027316].
24. Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, Oliveira-dos-Santos A, Penninger JM, Muslin AJ, Steinberg TH, Wyss JM, Mecham RP, Blumer KJ. Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest* 2003; 111:445-52. [PMID: 12588882].
25. Tang KM, Wang GR, Lu P, Karas RH, Aronovitz M, Heximer SP, Kaltenbronn KM, Blumer KJ, Siderovski DP, Zhu Y, Mendelsohn ME. Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. *Nat Med* 2003; 9:1506-12. [PMID: 14608379].
26. Zhang P, Su J, King ME, Maldonado AE, Park C, Mende U. Regulator of G protein signaling 2 is a functionally important negative regulator of angiotensin II-induced cardiac fibroblast responses. *Am J Physiol Heart Circ Physiol* 2011; 301:H147-56. [PMID: 21498776].
27. Semplicini A, Lenzini L, Sartori M, Papparella I, Calo LA, Pagnin E, Strapazzon G, Benna C, Costa R, Avogaro A, Ceolotto G, Pessina AC. Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. *J Hypertens* 2006; 24:1115-24. [PMID: 16685212].
28. Cao X, Qin J, Xie Y, Khan O, Dowd F, Scofield M, Lin MF, Tu Y. Regulator of G-protein signaling 2 (RGS2) inhibits androgen-independent activation of androgen receptor in prostate cancer cells. *Oncogene* 2006; 25:3719-34. [PMID: 16449965].
29. Campbell DB, Lange LA, Skelly T, Lieberman J, Levitt P, Sullivan PF. Association of RGS2 and RGS5 variants with schizophrenia symptom severity. *Schizophr Res* 2008; 101:67-75. [PMID: 18262772].
30. McBrien NA, Moghaddam HO, Reeder AP. Atropine reduces experimental myopia and eye enlargement via a nonaccommodative mechanism. *Invest Ophthalmol Vis Sci* 1993; 34:205-15. [PMID: 8425826].
31. Liu R, Qian YF, He JC, Hu M, Zhou XT, Dai JH, Qu XM, Chu RY. Effects of different monochromatic lights on refractive development and eye growth in guinea pigs. *Exp Eye Res* 2011; 92:447-53. [PMID: 21396363].
32. Penha AM, Schaeffel F, Feldkaemper M. Insulin, insulin-like growth factor-1, insulin receptor, and insulin-like growth factor-1 receptor expression in the chick eye and their regulation with imposed myopic or hyperopic defocus. *Mol Vis* 2011; 17:1436-48. [PMID: 21655358].
33. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. *Anal Biochem* 1987; 162:156-9. [PMID: 2440339].
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001; 25:402-8. [PMID: 11846609].
35. Zhao HL, Wang RQ, Wu MQ, Jiang J. Dynamic changes of ocular biometric parameters: a modified form-deprivation myopia model of young guinea pigs. *Int J Ophthalmol* 2011; 4:484-8. [PMID: 22553707].
36. Salim S, Asghar M, Taneja M, Hovatta I, Wu YL, Saha K, Sarraj N, Hite B. Novel role of RGS2 in regulation of antioxidant homeostasis in neuronal cells. *FEBS Lett* 2011; 585:1375-81. [PMID: 21510939].
37. Osei-Owusu P, Sun X, Drenan RM, Steinberg TH, Blumer KJ. Regulation of RGS2 and second messenger signaling in vascular smooth muscle cells by cGMP-dependent protein kinase. *J Biol Chem* 2007; 282:31656-65. [PMID: 17681944].
38. Lin YR, Parikh H, Park Y. Loco signaling pathway in longevity. *Small GTPases* 2011; 2:158-61. [PMID: 21776417].
39. Endale M, Kim SD, Lee WM, Kim S, Suk K, Cho JY, Park HJ, Wagley Y, Kim S, Oh JW, Rhee MH. Ischemia induces regulator of G protein signaling 2 (RGS2) protein upregulation and enhances apoptosis in astrocytes. *Am J Physiol Cell Physiol* 2010; 298:C611-23. [PMID: 20032508].
40. Lu F, Zhou X, Zhao H, Wang R, Jia D, Jiang L, Xie R, Qu J. Axial myopia induced by a monocularly-deprived facemask in guinea pigs: A non-invasive and effective model. *Exp Eye Res* 2006; 82:628-36. [PMID: 16256987].
41. Barathi VA, Weon SR, Beuerman RW. Expression of muscarinic receptors in human and mouse sclera and their role in the regulation of scleral fibroblasts proliferation. *Mol Vis* 2009; 15:1277-93. [PMID: 19578554].
42. Arumugam B, McBrien NA. Muscarinic antagonist control of myopia: evidence for M4 and M1 receptor-based pathways in the inhibition of experimentally-induced axial myopia in the tree shrew. *Invest Ophthalmol Vis Sci* 2012; 53:5827-37. [PMID: 22836762].
43. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine

- receptors. *Pharmacol Rev* 1998; 50:279-90. [PMID: 9647869].
44. Diether S, Schaeffel F, Lambrou GN, Fritsch C, Trendelenburg AU. Effects of intravitreally and intraperitoneally injected atropine on two types of experimental myopia in chicken. *Exp Eye Res* 2007; 84:266-74. [PMID: 17101130].
  45. Luft WA, Ming Y, Stell WK. Variable effects of previously untested muscarinic receptor antagonists on experimental myopia. *Invest Ophthalmol Vis Sci* 2003; 44:1330-8. [PMID: 12601066].
  46. Song L, De Sarno P, Jope RS. Muscarinic receptor stimulation increases regulators of G-protein signaling 2 mRNA levels through a protein kinase C-dependent mechanism. *J Biol Chem* 1999; 274:29689-93. [PMID: 10514440].
  47. Fischer AJ, McGuire JJ, Schaeffel F, Stell WK. Light- and focus-dependent expression of the transcription factor ZENK in the chick retina. *Nat Neurosci* 1999; 2:706-12. [PMID: 10412059].
  48. McBrien NA, Stell WK, Carr B. How does atropine exert its anti-myopia effects? *Ophthalmic Physiol Opt* 2013; 33:373-8. [PMID: 23662969].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 2 July 2014. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.