Identification of Apolipoprotein A-I as a “STOP” Signal for Myopia*

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Axial eye growth is naturally controlled from infancy to early adulthood by a vision-dependent mechanism (emmetropization) with the primary aim of matching the ocular axial length to the optical power (1). A failure of the emmetropization process can lead to myopia or to hyperopia. An excessive longitudinal growth of the ocular globe will place the focal plane of the eye in front of the retina; the eye is myopic or short sighted. Conversely an insufficient axial growth will place the focal plane of the eye behind the retina: the eye is hyperopic or farsighted (1). Studies on experimental animal models have demonstrated that emmetropization is driven by visual inputs (2–4) and primarily controlled by the retina (2, 4). The control of ocular axial growth by visual factors has been extensively investigated in chicken experimental models (3, 4). Applying negative lenses over the eye of the chicken places the focal plane behind the retina (hyperopic defocus), accelerates axial eye growth, and leads to a myopic condition (lens induced myopia (LIM))1. Conversely applying positive lenses places the focal plane before the retina (myopic defocus), slows down axial eye growth, and leads to a hyperopic condition (lens induced hyperopia (LIH)). In both cases, the size and shape of the eye are adjusted to compensate for the defocus and move the retina toward the focal plane. Moreover applying light diffusers over the eye of the chicken induces a very robust axial growth referred to as form deprivation myopia (FDM).

According to a popular working hypothesis, originally formulated by Wallman (2), these signals can be divided in two categories: “GO/GROW” signals that accelerate axial growth and “STOP” signals that prevent the growth. GO/GROW signals are induced by negative lenses (hyperopic defocus) or form deprivation, whereas STOP signals are induced by positive lenses (myopic defocus). An excess of GO/GROW signals or a defect of STOP signals is thought to underlie the excessive longitudinal growth of the ocular globe observed in myopic subjects (1). The molecular substrate of GO/GROW and STOP signals has not been fully elucidated yet, but changes in gene and protein expression have been correlated to these signals.

Good visual acuity requires that the axial length of the ocular globe is matched to the refractive power of the cornea and lens to focus the images of distant objects onto the retina. During the growth of the juvenile eye, this is achieved through the emmetropization process that adjusts the ocular axial length to compensate for the refractive changes that occur in the anterior segment. A failure of the emmetropization process can result in either excessive or insufficient axial growth, leading to myopia or hyperopia, respectively. Emmetropization is mainly regulated by the retina, which generates two opposite signals: “GO/GROW” signals to increase axial growth and “STOP” signals to block it. The presence of GO/GROW and STOP signals was investigated by a proteomics analysis of the retinas from chicken with experimental myopia and hyperopia. Of 18 differentially expressed proteins that were identified, five displayed an expression profile corresponding to GO/GROW signals, and two corresponded to STOP signals. Western blotting confirmed that apolipoprotein A-I (apoA-I) has the characteristics of a STOP signal both in the retina as well as in the fibrous sclera. In accordance with this, intraocular application of the peroxisome proliferator-activated receptor α agonist GW7647 resulted in up-regulation of apoA-I levels and in a significant reduction of experimental myopia. In conclusion, using a comprehensive functional proteomics analysis of chicken ocular growth models we identified targets for ocular growth control. The correlation of elevated apoA-I levels with reduced ocular axial growth points toward a functional relationship with the observed morphological changes of the eye. Molecular & Cellular Proteomics 5:2158–2166, 2006.

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1 The abbreviations used are: LIM, lens induced myopia; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; FDM, form deprivation myopia; HDL, high density lipoprotein; LDLR, low density lipoprotein receptor; LIH, lens induced hyperopia; LRP, low density lipoprotein-related receptor protein; PPAR, peroxisome proliferator-activated receptor; TGF, transforming growth factor; 2D, two-dimensional; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EST, expressed sequence tag; AA, amino acid; D, diopter.
with these signals (5). Phenotypically the expression levels of a STOP signal should be increased in response to myopic defocus (e.g. positive lenses), and an induction of this signal should reduce the excessive axial growth induced by experimental myopia. Conversely the levels of a GO/GROW signal should be increased in response to hyperopic defocus (e.g. negative lenses) or form deprivation, and an induction of this signal should exacerbate the axial growth induced by experimental myopia. For instance, according to these criteria, the transcription factors Pax6 (6) and ZENK (7) display GO/GROW and STOP profiles, respectively. Furthermore peptides like fibroblast growth factor-2 (8) or glucagon (9, 10) have been shown to function as STOP signals, whereas TGF-β acts as a GO/GROW signal (8, 11). In the case of glucagon both its activity and expression profile suggest it might be a STOP signal as its mRNA levels in the retina are increased by myopic defocus (10). In many cases reported so far, the expression profiles of putative STOP and GO/GROW signals have been assessed at the mRNA level (1, 5, 10), which is not always correlated with protein expression due to posttranscriptional regulatory mechanisms (12). In the current study, we looked systematically for proteins up-regulated in response to myopic defocus and demonstrated that apoA-I functions as a STOP signal by its capability to inhibit excessive ocular axial growth.

EXPERIMENTAL PROCEDURES

This study was comprised of two parts: (i) a proteomics analysis (using 2D PAGE, MALDI-TOF-MS, and -MSMS) of the retina and fibrous sclera of chicken that had been exposed to hyperopic or myopic defocus for either 1 or 7 days and (ii) an investigation of the effect on eye growth of injecting a peroxisome proliferator-activated receptor α agonist into the vitreous. Animals for use in part i were visually treated in Brisbane, Australia, and ocular tissues were sent to Basel, Switzerland for analysis. Part ii experiments were carried out in Tübingen, Germany. Experiments were conducted in accordance with the “Australian code of practice for the care and use of animals for scientific purposes” of the National Health and Medical Research Council, and approval for this project was obtained from the University Animal Ethics Committees of the Queensland University of Technology and the University of Tübingen.

Sample Preparation—10-day-old male chickens (Rhode Island Red/Rhode Island White cross) were subjected to one of three different visual treatments: form deprivation with translucent goggles (n = 30), lens induced myopia (n = 30) with negative lenses (-15 dipters), and lens induced hyperopia (n = 30) with positive lenses (+15 dipters). These treatments were applied monocularly for either 1 or 7 days. Animals not given a specific visual treatment were included as controls (CTL group, n = 15). At the end of the treatment period the eyes were obtained, the retinas and fibrous scleras were isolated in PBS and snap frozen in liquid nitrogen. For 2D electrophoretic separation, the frozen tissue was pooled and resuspended in Rabiloid buffer (13) at seven retinas or 10 fibrous scleras/ml. After 20 min at room temperature, samples were centrifuged at 16,000 × g for 20 min. Subsequently the supernatant was withdrawn and stored at −80 °C. About 50 μl of this protein extract (approximately 10 mg/ml) was diluted 2-fold in 2× SDS-PAGE sample loading buffer (Invitrogen) to perform later Western blot analysis. For an independent set of samples, the tissues were not pooled but collected and analyzed individually by Western blot.

2D PAGE—Two-dimensional electrophoresis was carried out on narrow range immobilized pH gradients (ultrazoom gels) according to Hoving et al. (13). For the first dimension, samples of ~0.5–1 mg of protein were loaded onto the IPG strips (GE Healthcare) by reswelling the strips in sample solution (14). Isoelectric focusing was performed on the Multiphor II apparatus (GE Healthcare) for ~70 kV-h at 20 °C using the following voltage gradient: (i) 3 h at 300 V, (ii) 5-h linear gradient from 300 to 3500 V, and (iii) continue at 3500 V until target kV-h. Five replicates of each sample were run on each of the four gradients. After focusing, IPG strips were equilibrated in equilibration buffer (6 M urea, 2% SDS, 50 mm Tris, pH 8.8, 30% (v/v) glycerol) with 2% DTT for 12 min in the first step and 5% iodoacetamide for 6 min in the second step (15). For the second dimension, IPG strips were applied to 20 × 25 cm (GE Healthcare) SDS-PAGE gels (12% T, 2.6% C), which were run overnight at 20 mA/gel at 15 °C. The gels were stained overnight in SYPRO Ruby (Molecular Probes, Eugene, OR).

Image and Data Analysis—The gel images were digitized as im gel files with the FLA-3000 fluorescence imager (Fuj) provided by Raytest, Straubenhart, Germany) using 473 nm excitation and a 520-nm high pass emission filter. Where necessary, img files were converted to 16-bit TIFF files using the “Img2tif” program provided with the scanner (Raytest). Image analysis was carried out using the Progenesis Discovery package (Non-Linear Dynamics, Newcastle, UK). For each gradient, the analysis included three replicates for each of the eight groups: the controls and the three treatments (FDM, LIM, and LIH) at two time points (1 and 7 days of treatment). Spot volumes were normalized in each gel according to the “Match Ratio” method. Spots were considered as differentially expressed if there was at least a 2-fold difference of normalized expression level (normalized spot volume) with a p < 0.05 level (t test) significance between control and any of the treatments. Because the digitized images sometimes contain artifacts due to incomplete resolution of spots or background staining, differences were always checked and corrected, if necessary, by visual analysis of the original gels. The criterion to classify an expression profile as a STOP profile was that the normalized expression level should be 2-fold higher in the hyperopic group (treated with +15 D lenses) than in the control group and also higher than in the myopic groups (treated with −15 D lenses). An expression profile was classified as a GO/GROW profile if the normalized expression level of one of the myopic groups was 2-fold higher than that in the control group; also the expression levels for both myopic groups should be higher than for the control and hyperopic groups.

In-gel Trypsin Digestion—For spot picking, gels were prepared as above but stained with colloidal Coomassie Blue G-250 using the method of Anderson (16). Spots of interest were excised and in-gel digested with modified porcine trypsin (Promega, Madison, WI) as described previously (17) using a microtitre plate format (CB080, Proxeon, Odense, Denmark). Spots were finally extracted with 5% formic acid.

MALDI-MS on PerSeptive Voyager Elite Instrument—α-Cyano-4-hydroxycinnamic acid/nitrocellulose matrices were prepared by a modified version of the fast evaporation technique of Jensen et al. (18). A solution of nitrocellulose in acetone (10 mg in 0.5 ml; Trans-Blot transfer medium, Bio-Rad) was admixed to a suspension of α-cyano-4-hydroxycinnamic acid in isopropanol (20 mg in 0.5 ml; Sigma). 0.5 μl of this solution was applied to the sample plate followed by 0.8 μl of sample solution in 15% CH₃CN, H₂O, 2% HCOOH corresponding to 5% of total sample. Reflectron-positive MALDI mass spectra were recorded on a PerSeptive Voyager Elite mass spectrometer (Framingham, MA) at 20-kV accelerating potential in the delayed extraction mode using standard settings for delay times and grid voltages. Samples were irradiated by a nitrogen laser pulse at 337 nm, and 256 laser shots were summed. Spectra were in most cases calibrated internally on known background signals.
MALDI-MS and -MSMS on 4700 Proteomics Analyzer—Selected samples were analyzed using the Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems Inc., Framingham, MA) in MS and MSMS mode. Tryptic hydrolysates were collected in a second microtitre plate and were purified on ZipTips (Millipore Corp., Bedford, MA) using a Tecan Genesis ProTeam 150 system (Tecan, Maennedorf, Switzerland). After washing with 2 × 5 μl of 80% ACN, 0.1% TFA the tips were equilibrated with 2 × 5 μl of 0.1% TFA, and the hydrolysate was applied. After washing with 4 × 5 μl of 0.1% TFA, peptides were directly eluted onto ABI 4700 MALDI targets (100-well plates) with 2 μl of a solution of α-cyano-4-hydroxycinnamic acid matrix (3 mg/ml in 50% ACN, 0.1% TFA) applied to the back end of the ZipTips. Both MS and MSMS data were acquired with a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser with a 200-Hz repetition rate; 2000 shots were accumulated for spectra in MS mode, and 4000 shots were accumulated for spectra in MSMS mode. MSMS mode was operated with 1 keV, and products of metastable decomposition at elevated laser power were detected. MS data were acquired with close external calibration and calibrated internally on trypsin background. MSMS data were acquired using default instrument calibration.

Database Search—Initial searches were performed against release August 19, 2002 of the nonredundant National Center for Biotechnology Information (NCBI) database using the program PepSea (Proxeon): a mass accuracy better than 80 ppm was expected for masses of tryptic peptides (one missed cleavage allowed), and fitting signals were manually checked for adequate signal to noise. Generally observed errors were less than 50 ppm. The minimum number of peptides required for a match was individually adjusted dependent on protein size and extent of background contributions. For identical proteins identified in different spots, less intense spectral patterns were verified by comparison with the most intense peptide mass fingerprint as reference. Recently MS data searches were repeated against the UniRef_100 database (UniProt Reference Clusters, European Bioinformatics Institute, August 24, 2005), resulting in the identification of the hypothetical protein Q5ZML3_CHICK (UniProt ID), which had not been retrieved in the original search. In this case the Mascot program was used with an expected mass accuracy better than 80 ppm and with the possibility of one missed tryptic cleavage.

Nanoelectrospray MSMS—The sample left over from the MALDI mass fingerprint analysis was concentrated/desalted on a needle (borosilicate capillary, Proxeon) that was packed with ~100 nl of POROS R3 sorbent (Applied Biosystems Inc.). In short, after loading the sample, a washing step with 12 μl of 0.1% formic acid in aqueous solution followed, and then the peptide mixture was directly eluted into the spraying needle (gold-coated borosilicate capillary, Proxeon) by the addition of 1–2 μl of 2:50:48 (v/v/v) HCOOH/CH3OH/H2O. The spraying process was started by applying a voltage difference (900 V) between the needle tip and the orifice (1.5-mm distance) of the ABI-Sciex QSTAR mass spectrometer (Toronto, Canada). A full mass spectrum was acquired over a wide mass range (m/z 350–2000), and parent ions (mass window of 2 Da) of interest were mass-selected and fragmented by collision-induced dissociation with nitrogen (collision energy was adjusted manually; collision gas pressure was set to 5 arbitrary units).

Interpretation of MS and MS/MS spectra was performed manually to obtain a peptide sequence tag. The following database searches were performed with the program PepSea (Proxeon): a mass accuracy better than 100 ppm was set for parent masses of tryptic peptides (one missed cleavage allowed) and 0.1 Da for the fragment masses. The searches were performed against an EST database (August 8, 2002, 5,024,497 entries). Once the protein sequence was identified (translated EST or combinations of ESTs), a combined retrospective MALDI and nano-ES-MSMS analysis was performed to calculate the sequence coverage.

Immunodetection of ApoA-I—Standard Western blot procedure (19) was performed with an anti-chicken apoA-I polyclonal antibody (kindly provided by Prof. W. Schneider, University Departments at the Vienna Biocenter) at 1:10,000. Afterward the membranes were probed again either with a monoclonal anti-GAPDH antibody (Clone 6C5, Biodesign, Sacco, ME) or a polyclonal anti-Actin antibody (A2066, Sigma) at 1:10,000 to obtain a loading control.

Immunohistochemistry—Immunohistochemistry was performed on paraffin sections to study the expression level of apoA-I on tissue sections within the eye. Primary anti-chicken apoA-I antibodies were used at 1:1000 and revealed with an alkaline phosphatase-coupled secondary antibody using fast red.

Intravitreal Injections—The experiments were started on day 8 posthatching on three groups containing seven to eight animals. Daily injections were performed for 4 consecutive days in the vitreous of male White Leghorn chicken (Gallus domesticus) under ether anesthesia. Immediately after the first injection one eye was subjected to LIM treatment (~7 diopter lenses) through the 4 days of the experiment. Animals of the LIM reference group received vehicle solution (70% saline + 30% DMSO) both in the LIM eye and its fellow contralateral eye. The two other groups were treated with the peroxisome proliferator-activated receptor α (PPAR-α) agonist GW7647 (Sigma). For each group, the LIM eye was injected, respectively, with doses of 63 or 189 μg of GW7647 in 12.5 μl of vehicle solution, whereas the fellow contralateral eye received 12.5 μl of vehicle solution. The injections were performed with a 0.3-mm (30 gauge) × 8-mm syringe cannula into the vitreous through skin, sclera, choroid, and retina close to the margin of the upper orbit. 70% ethanol was used to disinfect feathers and skin surrounding the injection site and to sterilize needles between injections.

Ocular Axial Length Measurements by A-scan Ultrasonography—To measure axial ocular dimension, a modified low frequency A-scan ultrasound biometer (Echourle, Phako Systems Inc., MN, St. Paul, MN) with a transducer probe was used. The probe was designed for the human eye (20). Therefore its best spatial resolution was at about 2 cm from the transducer tip. To be able to measure chicken eyes the probe was elongated using a rubber tube (length, 12 mm; inner diameter, 4 mm; outer diameter, 6 mm) (9). The tube was filled with tap water, and its tip was covered with Parafilm. Care was taken to avoid air bubbles being trapped in the tube. Measurements were performed in an alert hand-held chicken. The cornea was locally anesthetized by one drop of a 2% Xylocaine solution (AstraZeneca GmbH, Wedel, Germany). For measurements the tip of the rubber tube was moistened with the Xylocaine solution, and the cornea was touched for about 1 s. To determine the anterior chamber depth, lens thickness, vitreous chamber depth, and axial length of the eye ultrasound traces were temporarily frozen. Axial length was defined as the distance from the corneal surface to the retinointerstitial interface, that is the sum of anterior chamber depth, lens thickness, and vitreous chamber depth. Measurements of axial length were performed before the first injection (base-line measurements) and after the 4-day treatment period. Three to four measurements were performed to determine the axial length of each eye.

Data Analysis—After the 4-day treatment period, the differences in axial length between the LIM treated eyes and their fellow contralateral control eyes (interocular differences) were calculated. Subsequently the interocular differences were expressed in percentage values relative to the average intraocular difference value of the LIM reference group. The first step of the statistical analysis was to examine whether the interocular values of the three groups were homogeneous using an analysis of variance test. Afterward the differences between the LIM reference group and the groups treated with GW7647 were examined posthoc using Student’s t test.
RESULTS

List of Proteins with GO/GROW and STOP Profiles in the Retina—Our 2D gel analysis of retina samples revealed a limited number of consistent differences between the treatment groups: 37 of ~5000 spots that were analyzed across the four pH gradients. These 37 protein species were analyzed by mass spectrometry, and 30 were identified. Because some of the identified protein species were variants of the same protein, the 30 identified spots corresponded to a total of 18 different proteins. Five of these displayed GO/GROW profiles, and two had STOP profiles (Table I) as illustrated with two examples of differentially expressed proteins (Fig. 1). ApoA-I displays a STOP profile after 1 and 7 days of treatment (Figs. 1A and 2B) as it was up-regulated in the LIH group at both time points. In contrast to this, vimentin displays a GO/GROW profile after 1 and 7 days of treatment (Figs. 1B and 2B) as it was up-regulated in the FDM and LIM groups at both time points. Because our primary focus was on STOP signals (see the Introduction), apoA-I was selected for confirmation studies by antibody-based methods (in the absence of such tools for Q5ZML3_CHICK).

Western Blot Confirmation of the Expression Profile of ApoA-I—Up-regulation of apoA-I in the chicken eye under conditions of myopic defocus could be confirmed on several sets of independent samples. The Western blot expression profile of apoA-I in the retina after 1 and 7 days of treatment (Figs. 3A and 3B) corresponds to the 2D gel analysis of the same samples (Fig. 1A) confirming the up-regulation apoA-I in the LIH groups after 1 and 7 days of treatment. Moreover apoA-I was also up-regulated in the sclera of the 1-day LIH group (Fig. 3B). Because these data were derived from pooled samples, individual retinas of three control and three LIH animals were processed and analyzed separately after 1 day of treatment. Again apoA-I levels were clearly increased in the LIH animals compared with the untreated controls (Fig. 3C). The three experiments confirmed that apoA-I is up-regulated in the hyperopic group. Our Western blot experiments consistently showed that two apoA-I bands are detected in retina and fibrous sclera tissues at about 25 kDa; both bands were increased in hyperopic animals. Further examination of the isoform distribution of apoA-I by a 2D Western blot of a control retina sample revealed two isoforms of apoA-I (Fig. 3D). Chicken apoA-I is known to be transcribed as a 264-aa

| Protein name and accession number | Number of isoforms | Observed molecular mass | Calculated molecular mass | pI | Type of expression profile |
|---------------------------------|-------------------|------------------------|---------------------------|----|---------------------------|
| Apolipoprotein A-I P08250       | 1                 | 24                     | 28,808                    | 5.2 | STOP                      |
| Hypothetical protein Q5ZML3     | 1                 | 33                     | 28,157                    | 5.15| STOP                      |
| CRMP-62 O99635                  | 2                 | 55, 55                 | 62,730                    | 5.8, 5.85| GO/GROW                  |
| B-creatine kinase P05122        | 3                 | 42, 42, 41             | 42,553                    | 5.8, 5.9, 6.2| GO/GROW                  |
| γ-enolase O57391                | 1                 | 38                     | 47,651                    | 4.6 | GO/GROW                  |
| Tubulin α-1 chain, fragment P02552| 1              | 40                     | 46,414                    | 5.1 | GO/GROW                  |
| Vimentin P09654                 | 6                 | 40, 45, 40, 42, 42, 20 | 53,200                    | 4.4, 4.4, 4.5, 4.6, 4.7, 4.35| GO/GROW                  |

A  Example of STOP profile: Apo A1

B  Example of GROW profile: Vimentin

FIG. 1. Examples of protein that display profiles corresponding to GO/GROW and STOP signals. A, example of STOP profile: normalized spot volume profile of apoA-I in the retina across the eight indicated groups; error bars represent the standard deviation of the groups. B, example of GO/GROW profile: normalized spot volume profile of Vimentin in the retina across the eight indicated groups; error bars represent the standard deviation of the groups. The y axis depicts normalized intensities. CTL, control. * indicates significant differences as described under “Experimental Procedures.”
preproprotein that is subsequently processed in two steps through a 246-AA proform into the 240-AA mature protein (21) with a concomitant shift to a more acidic pI. The apoA-I protein identified by mass spectrometry corresponds to the 240-AA mature form as evidenced by the presence of a minor peak in the peptide mass fingerprint of this spot matching the predicted N-terminal peptide of the mature form DEPQTPLDR (observed mass, 1069.485 Da; calculated monoisotopic mass, 1069.5040 Da).

**Validation of ApoA-I as a STOP Signal**—To validate apoA-I as a functional STOP signal, we used a PPAR-α agonist, GW7647. Compounds of this class are known to increase the expression levels of apoA-I in a variety of tissues and cell types (22, 23). To ascertain whether GW7647 is effective in our experimental model, it was injected intravitreally in myopic animals, and the levels of apoA-I in the eye were examined by immunohistochemistry using polyclonal anti-apoA-I antibodies as described by Tarugi et al. (24). Representative eye sections from vehicle-injected control (Fig. 4A) and LIM animals (Fig. 4B) showed very similar apoA-I expression patterns and levels, confirming our previous finding that the LIM treatment did not alter the levels of apoA-I. However, when animals subjected to LIM treatment (4 days of −7 diopter lenses) were injected intravitreally with GW7647 (Fig. 4C), the protein levels of apoA-I were specifically increased in the fibrous sclera and the ganglion cell layer of vehicle injected animals (Fig. 4, A and B).

**Axial Length Measurements of Control and GW7647-treated Eyes**—Afterward we examined whether GW7647 could prevent the development of experimental myopia (LIM, 4 days of −7 diopter lenses). Three groups of LIM animals were injected with vehicle (n = 7), 63 μg of GW7647 (n = 8), or 189 μg of GW7647 (n = 8). The interocular differences of axial length were calculated for the three treatment groups.
and expressed as percentages with the vehicle-injected LIM animals axial length data considered as the base-line degree of myopia. The average values of the three groups are represented as a bar graph in Fig. 5: myopia was reduced by about 30% in the GW7647-treated animals. A statistical analysis with the analysis of variance test applied to the three groups revealed that the GW7647 had a significant effect ($p < 0.05$).

Posthoc analysis, using Student’s $t$ test, showed that both doses of GW7647 significantly reduced the development of experimental myopia ($p < 0.05$ in both cases).

**DISCUSSION**

The goal of the present study was to correlate the expression patterns of differentially expressed proteins with their potential functional role in the development of myopia. Using that concept, we identified five proteins (CRMP-62, B-creatine kinase, $\gamma$ enolase, Tubulin $\alpha$-1 chain, and Vimentin) that display GO/GROW profiles and two (Q5ZML3_CHICK and apoA-I) that display STOP profiles. Interestingly our findings do not overlap with previous reports on GO/GROW or STOP signals in myopia. A likely explanation is that the genes/protein reported previously (1) are classes of molecules (peptides, receptors, and transcription factors) that are less accessible by 2D PAGE. Conversely the soluble proteins represented in our 2D analysis had not been extensively investigated for their relation to myopia. We found that three of the five proteins that display a GO/GROW profile are related to cellular morphology: Vimentin and Tubulin $\alpha$-1 chain are structural proteins, whereas CRMP-62 is involved in neuronal polarity (25). This is consistent with the morphological changes in the retina of myopic animals observed by Liang et al. (26). In the following subsections, we will first address a few points regarding the consistency of our results on apoA-I followed by a discussion on the relevance of our findings and how they relate to a possible metabolic anomaly in myopia.

Because apoA-I is an abundant plasma protein, there is the possibility that its differential expression might be due to a plasma contamination of our samples. However, several observations argue against this explanation. First in the event of a contamination, other abundant plasma proteins, such as albumin, should display the same profile in the retina as apoA-I; this was not the case according to our 2D gel analysis. Moreover apoA-I consistently displayed a STOP profile in several independent observations. In pooled retina samples, apoA-I was up-regulated in the hyperopic group after 1 and 7 days of treatment. In pooled fibrous sclera samples, apoA-I was also up-regulated in the hyperopic group after 1 day of treatment. Finally in a set of individual retina samples, apoA-I was clearly and consistently up-regulated in the hyperopic eyes after 1 day of treatment. Taken together, these findings prove that the differential expression of apoA-I is a reproducible and relevant observation. Western blot confirmations showed that two bands of apoA-I are up-regulated in the hyperopic animals. These two bands correspond to the two protein species that were resolved by 2D PAGE Western blot, indicating that the two main apoA-I species present in the
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retina are differentially expressed. Besides a detection problem, masking by a stronger spot could also be the reason why only one of the two apoA-I species was identified in the 2D gel-based differential analysis.

We have shown that apoA-I displays a STOP profile both in the retina and the fibrous sclera and that an up-regulation of apoA-I inhibits ocular axial elongation, which suggests that apoA-I plays a role in the transmission of the STOP signal. To our knowledge this is the first time that a candidate STOP signal has been identified with a protein expression profile that is consistent with its function. This further supports the notion that GO/GROW and STOP signals are mediated at the protein level. Elucidation of the molecular signaling events upstream and downstream of apoA-I will now be crucial to learn more about the etiology of myopia and to ultimately develop new therapeutic approaches (1).

In the present study, we have shown that myopia can be reduced by a PPAR-\(\alpha\) agonist, a class of compounds generally used to treat metabolic disorders (22) and known to act upstream of apoA-I (23). PPAR-\(\alpha\) agonists are in wide clinical use to manage several types of dyslipidemias and prevent the associated risk of atherosclerosis and coronary artery disease (22) by raising HDL and lowering triglyceride levels (22). The effects of the PPAR-\(\alpha\) agonists on HDL metabolism are thought to occur through transcriptional mechanisms that increase the expression of apoA-I, the major protein constituent of the HDL lipoproteins (23). Interestingly myopia has been associated (27, 28) with a condition that is treated with PPAR-\(\alpha\) agonists (22): the pre-insulin-resistant state known as the metabolic syndrome or syndrome X (28). PPAR-\(\alpha\) agonists have been used in several clinical studies to improve the dyslipidemic profile observed in the metabolic syndrome (22): low HDL and hypertriglyceridemia (29). As we have shown that myopia can be reduced by a compound that acts on lipid and lipoprotein metabolism (22, 23), it is tempting to speculate that myopia might be associated with a metabolic disturbance as suggested by Cordain et al. (27, 28). Several mediators in pathways involved in metabolic disorders have also been associated with myopia: insulin (1, 27), glucagon (9, 10, 30), nitric oxide (1, 31, 32), TGF-\(\beta\) (11, 33), plasminogen (11, 32), and retinoic acid (1, 27). A systemic disturbance in the regulation of these pathways could impair the emmetropization process and promote the development of myopia. According to Cordain et al. (27, 28), the increased incidence of myopia in many countries is associated to high glycemic load Western diets and hyperinsulinemia. In this respect, apoA-I seems to be a good candidate to link myopia to environmental conditions and metabolic disorders. Remarkably insulin is known to regulate the expression of two transcription factors involved in myopia: Pax6 (34) and ZENK (35). Furthermore the expression of ZENK is regulated both by insulin and glucose levels (35), and in turn, ZENK acts upstream of apoA-I (36). It would be interesting to investigate whether some components of this cascade are active within the retina.

Other signaling cascades that could play a role in modulating ocular axial growth downstream of apoA-I include the plasminogen (11) and Wnt (37) pathways. Chicken apoA-I is found in several types of lipoprotein particles (24) that have been reported to inhibit the plasminogen pathway: HDL, very low density lipoprotein, and low density lipoprotein (38, 39). Plasminogen activation acts as a GO/GROW signal by processing latent TGF-\(\beta\) to its active form (11). Plasminogen also activates the matrix metalloproteinases (40), which play an important role in experimental myopia (41). ApoA-I could act as a STOP signal by inhibiting the plasminogen pathway, thereby preventing the activation of TGF-\(\beta\) and matrix metalloproteinases. In the chicken, apoA-I could also modulate plasminogen activation through the low density lipoprotein receptor (LDLR) family (42). An important point to mention is that chicken apoA-I is considered the functional equivalent of both mammalian apoA-I and apoE (43) as apoE is not expressed in birds (44). Therefore, in the chicken apoA-I takes over the function of mammalian apoE as a ligand for the receptors of the LDLR family, like LRP that regulates plasminogen and matrix metalloproteinase activation (45). Several members of the LDLR family are involved in the development of the central nervous system: megalin, very low density lipoprotein receptor, and apoER2 (46). Furthermore LRP5 and LRP6 function as coreceptors for Wnt signal transduction (36) and are important for the development of the eye (47, 48). For instance, mouse embryos homozygous for an insertion mutation in the Lrp6 gene exhibit microphthalmia (47). A recent genetic study has shown that MFRP, membrane-type Frizzled-related protein, is important for the control of ocular axial length (49), further suggesting an involvement of the Wnt pathway.

Based on our observations and on the discussed literature data, the role of apoA-I in the regulation of emmetropia seems to be worthy of further investigation. More specifically, we propose that the signaling position of apoA-I downstream of metabolic events and upstream of apolipoprotein receptors involved in signaling pathways might be relevant to myopia. To test this hypothesis we will need to learn more about the function, the receptors, and the metabolism of apolipoproteins in the eye. Moreover as the metabolism of apoA-I significantly differs in birds and mammals, it is important to find out how our discovery in chicken translates to human refractive disorders.

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