

Role of Cyclic Adenosine Monophosphate in Myopic Scleral Remodeling in Guinea Pigs: A Microarray Analysis

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PURPOSE. Myopia induction accompanies increased scleral cyclic adenosine phosphate (cAMP) levels and collagen degradation in mammalian models. We compared the scleral gene expression changes following monocular form deprivation (FD) with those induced by adenylate cyclase activation with forskolin (FSK) in guinea pigs.

METHODS. Guinea pigs were assigned to FD, FSK-treated, and age-matched (AM) control groups. FSK was injected monocularly into the inferior palpebral subconjunctiva daily for 4 days. After scleral RNA extraction, a gene microarray scanner and software were used to evaluate the gene expression patterns, followed by pathway analysis using Gene Ontology tools. Quantitative PCR (qPCR) was used to analyze the expression of 10 candidate genes in separate sets of form-deprived, vehicle-injected, and AM animals.

RESULTS. FSK injections differentially regulated 13 collagen subtypes compared to AM and FD groups. FSK also downregulated *Acta2* and *Tgf-β2* compared to the AM eyes. Collagen subtypes and *Acta2* underwent larger downregulation in the FSK group than during FD. FSK differentially regulated *Rarb*, *Rxrg*, *Fzd5*, *Ctnnd2*, *Dkk2*, and *Dkk3*, which have been linked to ocular growth. Only a few genes were differentially expressed between the FD and AM groups. There was 80% agreement in the direction of gene regulation between microarray and qPCR results. No significant differences were identified between vehicle-injected and AM eyes.

CONCLUSIONS. Collagen, a major scleral extracellular matrix component, is degraded during myopia. Given that FSK and FD both promote myopia through increased collagen degradation, targeting cAMP signaling pathway genes could suppress myopia development.

Keywords: cAMP, forskolin, myopia, sclera, microarray

Myopia is a refractive error characterized by an abnormal increase in eye size, resulting from an alteration of the emmetropization process. High myopia (over -6 diopters [D]) is of particular concern, as it is often associated with sight-threatening pathologies such as myopic retinopathy, posterior staphyloma, and retinal thinning and detachment.^{1,2} The prevalence of myopia, especially of high myopia, has been increasing worldwide over the past few decades.^{3,4} Because the development of myopia is influenced by environmental and genetic factors,^{5,6} animal models are being used to clarify gene-environment interactions.

Studies on animal models of myopia have shown that the sclera undergoes extensive structural and biochemical remodeling, leading to a weaker and thinner tissue along with abnormal ocular elongation.^{7,8} Such scleral changes accompany reduced collagen synthesis (particularly collagen type I), a major scleral extracellular matrix (ECM) component. Inhibition of transforming growth factor-beta (TGF- β) isoforms could account for these effects as they promote collagen synthesis and mediate myofibroblast transdifferentiation. Also, the mRNA expression of *Tgf-β2* showed the largest decline during myopia development.^{9,10} Nevertheless, increased scleral myofibroblast transdifferentiation has been reported in myopic tree shrews¹⁰ and guinea pigs,¹¹ with the latter study suggesting myopia

induction and biomechanical stress as factors mediating this phenotypic change. While various cellular and structural changes are associated with myopia development, the signaling events leading to inhibited collagen synthesis along with increased ECM turnover are still unclear. Accordingly, studies have focused on identifying the signaling pathways/messengers that affect collagen levels. There are indications that pathways regulating cyclic adenosine 3',5'-monophosphate (cAMP) levels may be involved because changes in scleral cAMP content resulting from either stimulating or inhibiting adenylate cyclase (AC) either promoted or inhibited myopia development in guinea pigs, respectively.¹²

Activation of AC inhibited collagen synthesis and reduced fibroblast to myofibroblast transdifferentiation in various tissues.^{13–15} In addition, stimulating AC activity with forskolin (FSK) inhibited both the proliferation of pulmonary fibroblasts¹⁶ and TGF- β -mediated collagen synthesis in cardiac fibroblasts.¹⁷ Form deprivation (FD) also elevated scleral cAMP levels in guinea pigs, which declined toward baseline values after the FD treatment was terminated. Furthermore, subconjunctival FSK injections for 2 weeks resulted in a myopic shift in refraction when these animals were exposed to a normal visual environment, whereas inhibition of AC by subconjunctival SQ22536 injections inhibited myopia progression in eyes



subjected to FD for 2 weeks.¹² Other key findings from the above study were that the FSK-treated eyes developed significant amounts of myopia compared to the vehicle-injected eyes. FSK treatment also inhibited collagen gene expression in both cultured human scleral fibroblasts (HSFs) and the guinea pig sclera. These findings suggest that stimulating AC promoted myopia development by inhibiting collagen synthesis. While these results are informative, the effects of daily FSK treatment on scleral gene expression levels have not been studied in guinea pigs. Such a characterization may pinpoint novel candidate genes and pathway targets for myopia control. To address this deficiency, we performed a gene microarray analysis that simultaneously identified numerous differentially expressed genes,^{18,19} which were then grouped into significant biological pathways using appropriate analytic tools

METHODS

Animal Model

Three-week-old pigmented guinea pigs were ordered from Bi Kai Experimental Animal Farm (Danyang, Jiangsu) and reared in 12-hour light/dark cycles in standard cages with food and water available ad libitum. Their right eyes were either treated with FSK or covered with latex facemasks for 4 days to induce myopia.^{20,21} The treatment of animals was conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol for handling animals was approved by the animal care and ethics committee at Wenzhou Medical University (Wenzhou, China).

Myopia Induction and Drug Treatment Groups

FSK (Tocris Bioscience, Bristol, UK) was dissolved in 3% dimethyl sulfoxide (DMSO) and diluted with 0.9% saline. Guinea pigs ($n = 12$) were randomly assigned to form-deprived ($n = 4$), FSK-treated ($n = 4$), and age-matched (AM) normal groups ($n = 4$). FSK (10 μM , 100 μL) was injected daily for 4 days into the inferior palpebral subconjunctiva of the right eye of anaesthetized and restrained animals. In our previous study, after 2 weeks of such treatment, scleral cAMP levels increased, along with a myopic shift in refraction, while retinal function remained unaffected.¹² Hence, this dosage was used in the current study.

Sclera Isolation

After 4 days of FD or FSK treatments, the refractive error of all animals was measured in minimal lighting using an eccentric infrared photoretinoscope.²² The animals were administered an overdose of chloral hydrate, euthanized via cervical dislocation, and their eyes were enucleated. The isolation of scleral tissue was carried out on ice immediately after enucleation. The cornea, iris, lens, and vitreous body were discarded. The sclera was scraped free of the retina and choroid, snap frozen in liquid nitrogen, and stored at -80°C . Total RNA was extracted with a purification kit (RNeasy Fibrous Tissue Mini Kit; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA samples with an A260/A280 ratio of at least 1.8 as measured with a spectrophotometer (NanoDrop; ThermoFisher Scientific, Waltham, MA, USA) were used for gene expression analysis.

Microarray Analysis

The extracted RNA was shipped on dry ice to a commercial provider (Hangzhou Patrick Biotech, Hangzhou, China) for

microarray processing and data analysis. Total RNA (200 ng) was converted to cDNA, and microarray analysis was carried out on an scanner using custom gene expression arrays (Agilent G2505C; Agilent Technologies, Shanghai, China) according to the manufacturer's protocol (Agilent one-color custom gene expression array, design ID 050980; Agilent Technologies). The probe signal in the 75th percentile of each microarray assay was shown to be representative of the entire microarray signals. Hence, the raw data were normalized against the values generated in the 75th percentile as recommended by the manufacturer (Agilent Technologies). Differentially expressed genes were identified using software (GeneSpring GX; Agilent Technologies) with the statistical significance estimated using 2-way ANOVA. Microarray analysis identified the differentially expressed genes between the following groups: (1) FSK and AM, (2) FSK and fellow eyes, (3) FD and FSK, (4) FD and AM, and (5) FD and fellow eyes. Pathway analysis was performed on genes that were differentially regulated by at least 1.5-fold (and $P < 0.05$) using the Gene Ontology (GO) enrichment tool.²³⁻²⁵

Quantitative Real-Time PCR (qPCR)

The expression pattern of 10 differentially regulated genes (see Table 4) whose fold change (FC) was at least 1.5 ($P < 0.05$) from microarray analysis was validated using qPCR in the sclera of form-deprived, fellow, and untreated AM eyes ($n = 4$ each). These genes were chosen based on their possible roles in ocular growth and scleral ECM remodeling. We also determined the expression of three scleral ECM genes in a separate set of guinea pigs ($n = 4$) injected with a vehicle control for 4 days (100 μL 0.1% DMSO in saline) compared to the untreated AM eyes.

Total RNA (0.5 μg) from the sclera was converted to cDNA using reverse transcriptase according to the manufacturer's protocol (M-MLV; Promega Corp., Madison, WI, USA). Guinea pig-specific primers were designed using primer3 software,²⁶ and qPCR was performed in duplicate using a master mix (SYBR Green; Applied Biosystems, Carlsbad, CA, USA) on a PCR system (ViiATM 7; Applied Biosystems). Differential gene expression was estimated using the $2^{-\Delta\Delta\text{CT}}$ method^{27,28} and normalized to the reference gene β -actin.

Western Blot

The scleral samples from animals exposed to FD and untreated AM controls, stored at -80°C , were homogenized in 200 μL of lysis mixture containing radio-immunoprecipitation assay buffer (Beyotime, Shanghai, China), 1 mM phenylmethylsulfonyl fluoride (Beyotime), and a protease inhibitor cocktail (Roche, Germany). Homogenization was carried out in a ball mill, and the samples were subsequently sonicated for 30 to 60 seconds, followed by centrifugation at 13,000g for 10 minutes at 4°C . Supernatants were aspirated and their protein concentration estimated using an assay (BCA Protein Assay; Beyotime). Aliquots of each sample containing 30 μg of protein were separated by electrophoresis in a 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA), and blocked with 5% nonfat milk for 2 hours at room temperature. The membranes were incubated overnight at 4°C with primary antibodies against α -SMA (43 kDa, 1:500) (ab5694; Abcam, Cambridge, MA, USA) and α -tubulin (55 kDa, 1:1000) (ab52866; Abcam). The membranes were then washed three times with a mixture of Tris-buffered saline and Tween (TBST: 10 mM Tris-HCl [pH 8.0]; 150 mM NaCl; and 0.1% Tween-20) and incubated with appropriate secondary antibodies (at dilutions of 1:2000) for 2 hours at room temperature: goat anti-rabbit IgG (IRDye 800CW, 926-

TABLE 1. Differential Expression of Genes From Microarray Analysis

Genes	FD vs. FSK		FSK vs. AM	
	FC	P Value	FC	P Value
<i>Rxrg</i>	2.00	<0.05	-1.71	<0.05
<i>Rarb</i>	-2.06	<0.05	-1.61	<0.05
<i>Fzd5</i>	No significant change		-2.05	<0.01
<i>Ctnnd2</i>	No significant change		-1.89	<0.05
<i>Rspo1</i>	-1.60	<0.05	No significant change	
<i>Wnt9a</i>	1.91	<0.001	No significant change	
<i>Dkk2</i>	No significant change		-1.57	<0.05
<i>Dkk3</i>	2.40	<0.05	-1.89	<0.001

32211; LI-COR Biosciences, Lincoln, NE, USA) and goat anti-mouse IgG (IRDye 680RD, 926-68070; LI-COR Biosciences). The protein bands were visualized with an imaging system (Odyssey Infrared Imaging System; LI-COR Biosciences). ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used to carry out densitometric analysis of the captured images. The protein bands were quantified relative to the loading control, α -tubulin.

RESULTS

Effects of FSK Treatment on Major Scleral ECM Genes

Numerous collagen subtypes (*Col1a1*, *Col2a1*, *Col3a1*, *Col5a1*, *Col6a5*, *Col6a6*, *Col8a2*, *Col10a1*, *Col11a1*, *Col13a1*, *Col14a1*, and *Col15a1*) were differentially regulated between the following conditions: (1) FSK versus AM eyes and (2) FD versus FSK eyes (Fig. 1). The results of microarray analysis carried out between each of the treatment groups are presented in Supplementary Tables S1, S2, S3, S4, and S5, which list the probe IDs, *P* values, FC (at least 1.5), and direction of regulation.

Compared to the AM eyes, FSK downregulated the expression of the indicated collagen subtypes; the myofibroblast marker, *Acta2*; and its transdifferentiation activator, *Tgf- β 2* (Fig. 1). Relative to AM controls, collagen subtypes (*Col1a1* and *Col1a2*) and the growth factor, *Tgf- β 2*, were downregulated in the FD group by -1.5-fold, -3.06-fold, and -1.8-fold, respectively, whereas, the expression of *Acta2* remained unchanged and *Acta1* was upregulated (+1.5). While Figure 1 is suggestive of collagen upregulation during FD compared to FSK treatment, a greater downregulation of collagen subtypes in the latter group accounts for such a finding. For example, compared to AM controls *Col1a1* was downregulated by -1.5-fold and 2.78-fold in response to FD and FSK treatments, respectively. These declines were -3.06 (FD versus AM eyes) and -6.38 (FSK versus AM eyes) for *Col2a1*. Only the above two collagen subtypes showed significant differences in expression during FD compared to AM controls and thus are presented here for comparison. Even though downregulation of other collagen subtypes in response to FD was not statistically significant, a larger and significant downregulation of these genes during FSK treatment could account for the differential gene expression patterns between these two treatments as seen in Figure 1.

Effects of FSK Treatment on Genes Implicated in Ocular Growth

FSK downregulated the expression of the genes linked to axial elongation, such as retinoic acid receptor- γ (*Rxrg*) and - β (*Rarb*), compared to untreated AM controls. Wnt signaling pathway genes such as *Fzd5*, *Ctnnd2*, *Dkk2*, and *Dkk3* (Table 1) were also downregulated by FSK treatment. Only *Rarb* (-1.61) and *Wnt9a* (+2.21) were differentially regulated during FD compared to AM eyes. Similar to the ECM genes, compared to the AM eyes FSK treatment resulted in a larger and significant downregulation of *Rxrg* and *Dkk3* (-1.71 and -1.89, respectively, *P* < 0.05). On the other hand, FD failed to significantly downregulate these two genes (-1.17 and -1.05, respectively, *P* > 0.05). Such differences in gene expression pattern between FSK and FD treatments are presented as upregulation of these genes during the latter condition (Table 1), while in fact both these treatments led to varying magnitudes of downregulation. On the other hand, the expression of *Fzd5*, *Ctnnd2*, and *Dkk2* was not different in this comparison, with two additional genes from the Wnt

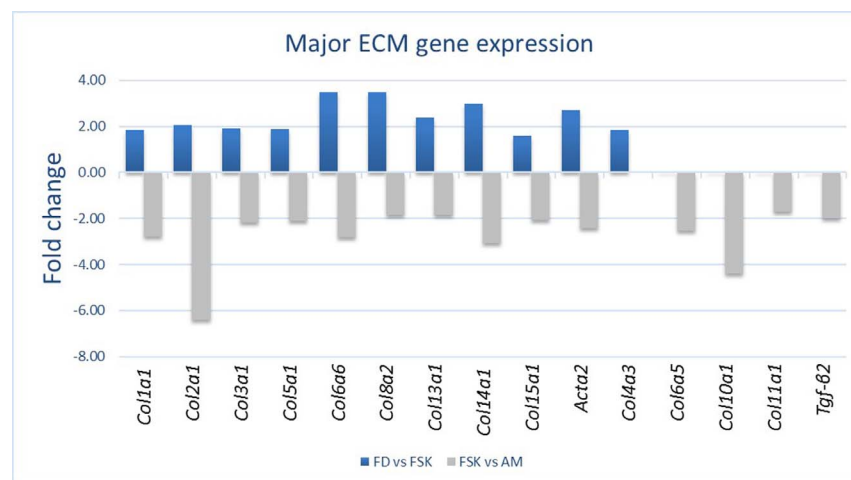


FIGURE 1. Effects of FSK on major scleral matrix genes. Significant downregulation of collagen, *Tgf- β 2*, and *Acta2* was observed following FSK injection compared to AM eyes (blue-colored bars), whereas the opposite effect was observed on comparing FD and FSK treatments (except for *Tgf- β 2*; gray-colored bars).

TABLE 2. Most Significant Biological Processes Underlying the Differentially Expressed Genes Between FSK-Treated and AM Eyes

GO Term	Description	P Value
GO:0006613	Cotranslational protein targeting to membrane	<0.001
GO:0006612	Protein targeting to membrane	<0.001
GO:0030198	ECM	<0.001
GO:0043062	Extracellular structure organization	<0.001
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	<0.001
GO:0019083	Viral transcription	<0.001
GO:0090150	Establishment of protein localization to membrane	<0.001
GO:0006413	Translational initiation	<0.001
GO:0000184	Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	<0.001
GO:0006605	Protein targeting	<0.001

P value was estimated based on hypergeometric (HG) model.²⁴ SRP, signal recognition particle.

pathway, *Wnt9a* and *Rspo1*, being up- and downregulated, respectively, on comparing the FD and FSK-treated groups. However, these genes were not differentially expressed between FSK-treated and AM eyes.

Enrichment Analysis

GO enrichment analysis classifies genes in large datasets based on their location and involvement in various biological processes. Figure 2A shows the results of such an analysis comparing FSK-treated and AM eyes. Most of the differentially expressed genes seem to be associated with the ECM (yellow-colored bars) and are involved in biological processes that maintain the structure and organization of ECM components (red-colored bars) along with possibly being involved in cell morphogenesis and cell adhesion. On the other hand, genes showing differential expression between the FD and AM

control groups do not appear to be associated with biological processes related to ECM remodeling (Fig. 2B). Almost all cellular components and biological processes seem to be equally enriched on comparing FD and FSK treatment groups. The functions modulated by these genes are not delimited to a specific cellular location, as evidenced by the consistently higher P values across all the yellow- and red-colored bars (Fig. 3). This concordance may be because both FD and FSK treatments increase cAMP levels.

Additional analysis of the differentially expressed genes induced by FSK treatment was performed by comparing (1) FSK versus AM and (2) FD versus FSK eyes using the GO enrichment analysis and visualzation (GORILLA) tool. In the first case, the biological processes associated with these genes play possible roles in mediating ECM structure and organization through modulating the gene expression of collagen isoforms, growth factors, and matrix metalloproteinases (Table 2). In the second case, the differentially expressed genes seem to be associated with growth and oxygen balance (Table 3).

Microarray Analysis Validation

qPCR was used to compare the scleral gene expression levels of *Tgf-β2*, *Acta1*, *Col2a1*, *Col12a1*, *Wnt9a*, and *Rarb* during FD with those in AM controls. On the other hand, differences in *Col6a5*, *Gjd2*, *Igf1*, and *Tbbs1* expression were evaluated in eyes exposed to FD compared to their fellow controls (see Table 4 for primer sequences). Microarray analysis revealed significant changes in the expression of these genes, which have been implicated in mediating scleral ECM remodeling and/or control of ocular growth. Except for *Col12a1* and *Wnt9a*, the direction of gene regulation in 8 out of 10 other genes was similar between the results of microarray and qPCR (Figs. 4A, 4B), which is exemplified by a high correlation (Pearson's coefficient: 0.84, $P < 0.01$). From the above pool of 10 genes, microarray analysis showed that the collagen subtypes, *Col2a1*, *Col6a5*, and the major ECM growth factor *Tgf-β2* were differentially regulated between the FSK-treated and AM eyes. However, PCR results show no change in the expression of these three genes in the vehicle-injected eyes compared to AM controls (Fig. 4C).

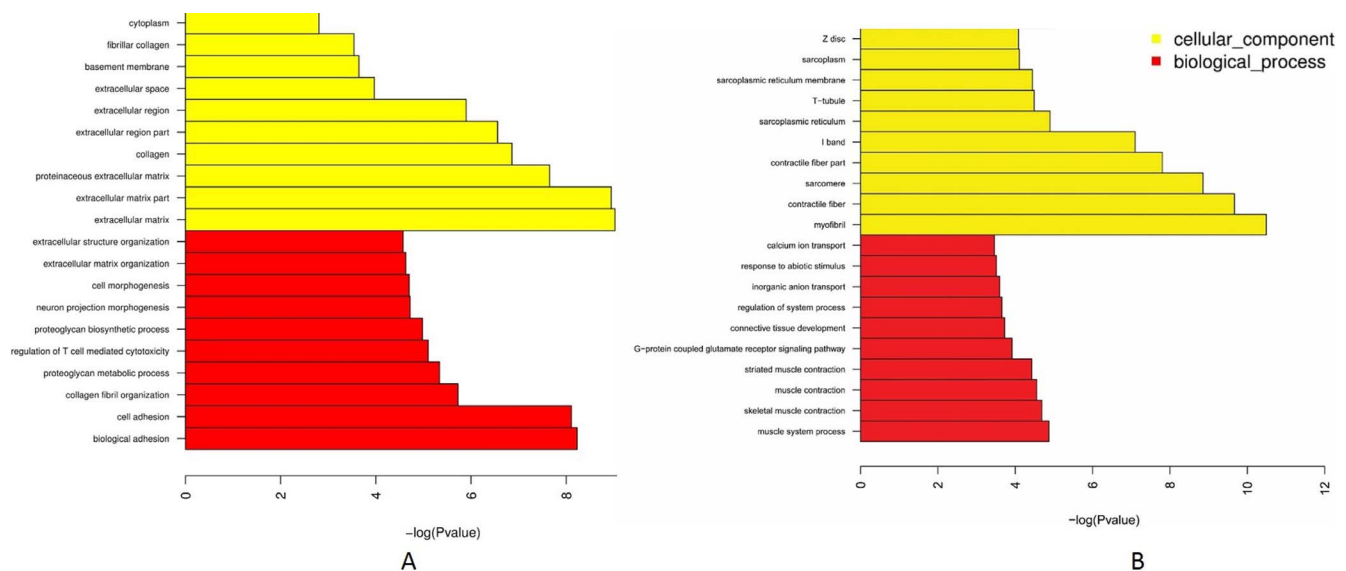


FIGURE 2. Enrichment analysis using the GO tool. (A) Differentially expressed genes in response to FSK treatment were predominantly grouped into cellular components and biological processes (y -axis) involved in the synthesis and organization of ECM components such as collagen and proteoglycans as indicated by their levels of statistical significance (x -axis). (B) Cellular components and biological processes related to the turnover of major ECM components could not be identified in the enrichment analysis of differentially expressed genes during FD compared to AM controls.

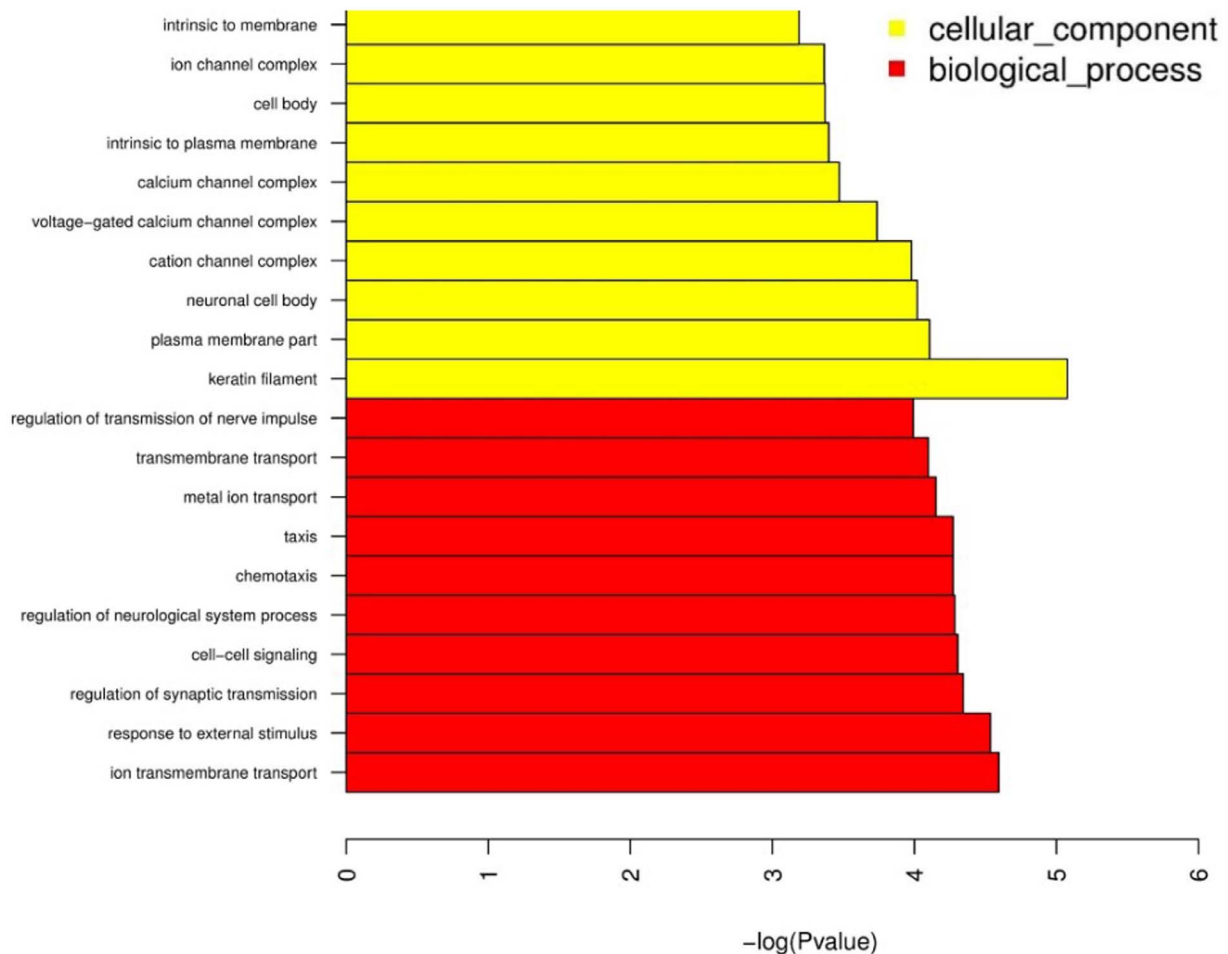


FIGURE 3. Enrichment analysis using GO tool (FD versus FSK treatments). Enrichment analysis of the differentially expressed genes between FD and FSK treatments showed that the top cellular components and biological processes (y -axis) had similar high levels of statistical significance (x -axis).

DISCUSSION

Because scleral collagen content declined following 2 weeks of FSK injections in guinea pigs,¹² the present study used microarray analysis to examine the gene expression changes that occur after 4 days of FSK injection. This treatment regimen produced a downregulation of collagen, *Acta2*, and *Tgf- β 2* in the FSK-treated sclerae compared to the AM controls. Even though FD and FSK treatments resulted in myopic refraction and ocular elongation, the FSK-induced downregulation of collagen as well as *Acta2* was larger than that resulting from FD. Both these treatments also produced differential expression of genes that have a potential role in controlling ocular growth: *Rxrg*, *Rarb*, *Fzd5*, *Ctnnd2*, *Rspo1*, *Wnt9a*, *Dkk2*, and *Dkk3*. On the other hand, only a few genes were differentially regulated in eyes exposed to either FD or FSK treatments compared to their fellow untreated eyes. This invariance may be due to a possible yoking effect in the fellow eye due to injections or visual manipulations in the treated eye of the same animal.

Our correlation of 80% between the gene expression profiles identified in the microarray and qPCR data is similar to those reported in other studies.^{29,30} Additionally, the scleral α -SMA protein expression increased during FD compared to AM controls (Supplementary Fig. S1), which is consistent with the gene expression patterns identified in the qPCR and microarray data. Nevertheless, our inability to validate all of the gene expression changes with qPCR may be attributable to

different normalization methods used in analyzing the microarray and qPCR results. The microarray data were normalized using the raw probe signals generated for each gene to those in the 75th percentile in the microarray assay, whereas qPCR employed a housekeeping gene for this purpose.

Microarrays have been used to characterize changes in gene expression levels in various animal model studies of myopia, such as in marmosets, mice, and chicks. Microarray analysis reliably resolves numerous gene expression changes in a single experiment employing a small sample size. The obtained large datasets are usually authenticated by comparing changes of a few candidate genes with those obtained using qPCR. Shelton et al.³¹ reported over 200 differentially expressed genes in the

TABLE 3. Most Significant Biological Processes Underlying the Differentially Expressed Genes During FD Compared to FSK-Treated Eyes

GO Term	Description	P Value
GO:0007204	Positive regulation of cytosolic calcium ion concentration	<0.001
GO:0051480	Regulation of cytosolic calcium ion concentration	<0.001
GO:0040008	Regulation of growth	<0.001
GO:0032364	Oxygen homeostasis	<0.001
GO:0009605	Response to external stimulus	<0.001

P value was estimated based on HG model.²⁴

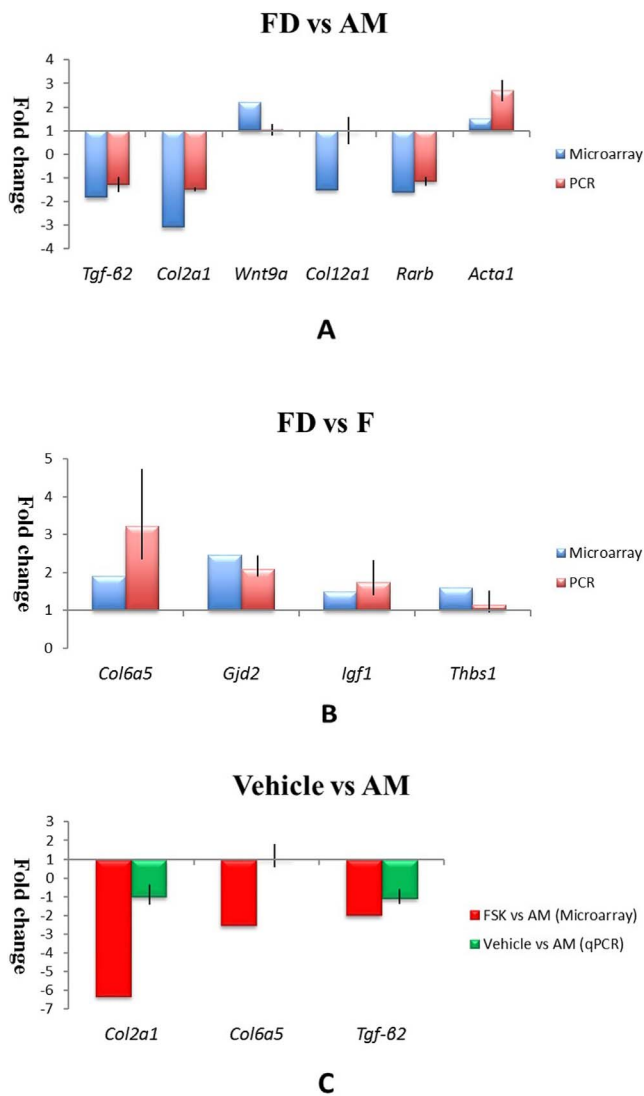


FIGURE 4. Gene expression data using qPCR. FC estimates of genes in FD group ($n = 4$) compared to that in AM controls (A) ($n = 4$) and their fellow controls (B) expressed as mean \pm SD. Eighty percent of the genes probed with microarray and qPCR techniques showed similar directions of gene regulation. No significant gene expression changes were observed when comparing the vehicle-injected group (0.1% DMSO) with those of AM controls ($n = 4$ each) (C). Gene expression differences between FSK and AM eyes from microarray analyses are presented for comparative purposes (C).

combined choroid and RPE of myopic marmosets using this technique. Additionally, a microarray was used to characterize the retinal gene expression changes in macaque monkeys³² and chicks.³⁰ We employed a microarray to determine the scleral gene expression changes associated with cAMP activation by FSK in guinea pigs and compared them to those induced by FD. A small sample size of four animals per group was used in our study, similar to those that employed the microarray technique to identify gene expression changes in mice and marmosets earlier.^{29,31} Moreover, the direction of regulation of several candidate genes from our microarray analysis agrees with that obtained from qPCR, thus confirming the validity of the large dataset.

Both FSK treatment and FD increased scleral collagen degradation and turnover, which are major changes occurring during myopia development.^{12,33} However, the current study

TABLE 4. Primer Sequences for qPCR Analysis

Genes	Primer Sequence (5'–3')	T _m (°C)	Product Size
<i>Acta1</i> _F	GAAGGAGTAGCCACGCTCAG	60.18	319 bp
<i>Acta1</i> _R	TGCTGTCCCTCTATGCCTCT	60.03	
<i>Rarb</i> _F	GCTACGAGATGACTGCTGAGT	59.6	111 bp
<i>Rarb</i> _R	ATGCACCTGGTGGCCAGTTC	61.18	
<i>Tgf-β2</i> _F	ATCCCGCTTGAAATCAATGT	55.69	206 bp
<i>Tgf-β2</i> _R	AGACCCACATCTCCTGCTA	59.66	
<i>Col12a1</i> _F	AGGCGAAAGGAAATCAGCCAC	61.22	355 bp
<i>Col12a1</i> _R	AGCAGGGCATTTTGCTTCATC	59.79	
<i>Col2a1</i> _F	CTGTGACGAAGGATGTGCTC	59.72	287 bp
<i>Col2a1</i> _R	GGAGGTCCTTGGGTCTTACA	60.83	
<i>Wnt9a</i> _F	TTTCTGGGCCGACGATCAAG	60.39	234 bp
<i>Wnt9a</i> _R	TTGCAGTCTGCTCCACTC	59.97	
<i>Gjd2</i> _F	GCAGCAGCACTCCACTATGA	60.11	256 bp
<i>Gjd2</i> _R	AGCGTGTGCACACAAACAT	59.54	
<i>Col6a5</i> _F	TGGCCACGTCTCAGTTTCAT	59.6	424 bp
<i>Col6a5</i> _R	ACACGAGGTCAGCAAGTGAAT	59.93	
<i>Thbs1</i> _F	AGGGATACTCGGGCCTTTCT	60.03	228 bp
<i>Thbs1</i> _R	CGCCATCTGTAGGCAAGTAA	60.11	
<i>Igf1</i> _F	TGTCTGGGTGACCAAGTGAAC	60.16	164 bp
<i>Igf1</i> _R	GAAGAGGTGGGAAGAGGACAC	59.72	

T_m, temperature.

showed that the collagen downregulation was greater in response to FSK treatment than that observed during FD. One such example of this difference is that the magnitude of *Col2a1* downregulation in the FSK-treated group compared to that in the AM eyes was almost twofold greater than that between FD and AM groups (FC: -6.38 , $P < 0.05$ vs. -3.06 , $P < 0.05$). Still other collagen subtypes showed significant downregulation in FSK-treated eyes but not during FD (gene expression data with $P > 0.05$ comparing FD and AM groups are not presented). Nevertheless, FSK injections, as with FD, resulted in a myopic shift in refraction accompanied by increased eye size.¹² This agreement shows that either model is relevant for characterizing myopic scleral changes. Incidentally, this FSK-induced scleral collagen downregulation is consistent with its antifibrotic effects in cardiac fibroblasts.^{14,17}

A recent study demonstrated that stimulation of cAMP-dependent protein kinase induced by 8-Br-cAMP intravitreal injections reduced myopia progression in a negative lens-induced model to such an extent that it resulted in a hyperopic refraction in chicks.³⁴ This study also showed that positive lens defocus inhibited ocular growth and increased both retinal cAMP and *ApoA1* levels. These results differ from those previously reported by our group, in which the putative rise in scleral cAMP levels induced by FSK had no additional effect on the refraction induced by FD, and the retinal cAMP levels were unaltered in these guinea pigs.¹² One possible reason for this difference may be attributable to variations in drug delivery routes. An intravitreal route was used in the aforementioned chick study, whereas in our case FSK was injected subconjunctively. While intravitreal injection is an effective method to deliver drugs to posterior ocular tissues such as the retina, drugs injected subconjunctively are dispersed locally throughout the sclera.³⁵ The sclera is a critical determinant of eye size and undergoes extensive ECM remodeling during myopia development. This current finding that cAMP inhibits collagen expression, a major scleral ECM constituent, agrees with our previous finding that the subconjunctival FSK injections promote myopia development and collagen degradation,¹² which is also consistent with our results showing that numerous collagen subtypes undergo downregulation.

FSK treatment may have inhibited fibroblast transdifferentiation into myofibroblasts as evidenced by downregulation of both *Acta2*, a myofibroblast marker, and *Tgf-β2*, one of the mediators of this phenotypic change. While *Tgf-β1* mediates myofibroblast transdifferentiation in various connective tissues,^{36,37} Jobling et al.⁹ found *Tgf-β2* to be the major isoform in the mammalian sclera that was significantly downregulated during myopia. Reduced myofibroblast transdifferentiation is also consistent with earlier studies showing that FSK treatment increased cAMP levels,¹⁶ leading to inhibition of myofibroblast transdifferentiation in pulmonary fibroblasts.¹⁵ In addition to scleral ECM genes, FSK also induced differential expression of other genes implicated in ocular growth. *Rarb* and *Rxrg* were both downregulated when comparing the FSK-treated and AM eyes. *Rxrg* underwent larger downregulation during FSK treatment as opposed to when exposed to FD. The Wnt signaling inhibitors, *Dkk2* and *Dkk3*, were downregulated in the FSK-treated eyes compared to the AM controls. This difference suggests that Wnt signaling may also be activated by stimulation of AC that in turn increases cAMP accumulation. Expression of *Dkk2* in the sclera of human infants was upregulated in the anterior segment compared to the posterior region, suggesting a role of this Wnt inhibitor in controlling anterior scleral development.³⁸ After 8 days of FD, myopic changes were characterized by rapid scleral expansion centrally (around the optic nerve), with relatively less scleral expansion in the periphery (+5.3 μm/deg versus +1.5 μm/deg) in guinea pigs. Prolonging the FD periods up to 2 and 3 weeks resulted in inhibition of scleral growth in the periphery (Zeng G, et al. *IOVS* 2013;54:ARVO E-Abstract 5180). Our findings regarding the effects of 4 days of either FD or daily FSK injections, suggest a possible role of Wnt signaling pathway inhibitors, *Dkk2* and *Dkk3*, during the initiation phase of such peripheral inhibition. However, further experiments with various myopia induction periods are necessary to test this hypothesis. Other evidence of Wnt signaling involvement in myopia development is the reported upregulation of *Fzd5* and β-catenin in the retina of myopic mice³⁹ and downregulation of scleral *Fzd5* and *Ctnnd2* (Delta-catenin) expression in our current study. Furthermore, *CTNND2* has been shown to be associated with high myopia in replication studies among Asian populations.⁴⁰⁻⁴² A comparison of FD and FSK treatments also identified differential regulation of *Rspo1*. This Wnt signaling pathway gene was implicated in ocular growth in a human genome-wide association study.⁴³ These findings suggest a possible role for cAMP in mediating the expression of genes in the Wnt signaling pathway that were previously implicated in regulating ocular growth in humans and animal models of myopia.

An unusually small number of genes were differentially expressed in comparisons of the treated and fellow eyes: (FSK versus fellow eyes: 200 genes; FD versus fellow eyes: 273 genes; FSK versus AM: 2092 genes; and FD versus AM: 1771 genes). This could be due to possible yoking effects in these animals. Howlett and McFadden⁴⁴ demonstrated an increase in vitreous chamber depth (VCD) in the fellow eyes in response to monocular FD, suggestive of interocular yoking in guinea pigs. Induction of myopia in one eye has been shown to affect the VCD, axial length, and refractive error in the fellow eye of animal models such as the chicks⁴⁵ and macaque monkeys.⁴⁶ However, the mechanism behind this yoking effect is still unknown. Such an effect could explain the lack of significant change in the expression of ECM and axial length-related genes in the treated eyes compared to the fellow controls in our microarray data. The omission of a vehicle control for the FSK injection group is a potential limitation. However, this may not be a major issue as the qPCR results failed to identify any significant differences in the expression of a few selected genes

(*Col2a1*, *Col6a5*, and *Tgf-β2*) between the vehicle-injected and AM eyes.

In the current study, gene microarray technology was applied to investigate differences in gene expression patterns induced by FSK treatment and FD in the guinea pig sclera. Both of these protocols induce myopia and increase ocular elongation, with FSK treatment inducing larger gene expression changes than FD. Nevertheless, despite such varying magnitudes, the effects of FSK are consistent with previous reports that showed an increase in cAMP accumulation suppressed collagen expression and promoted myopia development. Given the possible involvement of cAMP and Wnt signaling pathways in mediating these gene expression changes, targeting genes in these pathways may lead to the development of novel treatment modalities to inhibit excessive ocular elongation and myopia progression.

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