Crosstalk between EP2 and PPARα Modulates Hypoxic Signaling and Myopia Development in Guinea Pigs

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PURPOSE. Cyclic adenosine monophosphate (cAMP) and peroxisome proliferator-activated receptor alpha (PPARα) levels mediate extracellular matrix (ECM) changes by altering the levels of hypoxia-inducible factor 1-alpha (HIF-1α) in various tissues. We aimed to determine, in the sclera of guinea pigs, whether a prostanoid receptor (EP2)-linked cAMP modulation affects PPARα and HIF-1α signaling during myopia.

METHODS. Three-week-old guinea pigs (n = 20 in each group), were monocularly injected with either an EP2 agonist (butaprost 1 μmol/L/10 μmol/L), an antagonist (AH6809 10 μmol/L/30 μmol/L) or a vehicle solution for two weeks during normal ocular growth. Separate sets of animals received these injections and underwent form deprivation (FD) simultaneously. Refraction and axial length (AL) were measured at two weeks, followed by scleral tissue isolation for quantitative PCR (qPCR) analysis (n = 10) and cAMP detection (n = 10) using a radioimmunoassay.

RESULTS. Butaprost induced myopia development during normal ocular growth, with proportional increases in AL and cAMP levels. FD did not augment the magnitude of myopia or cAMP elevations in these agonist-injected eyes. AH6809 suppressed cAMP increases and myopia progression during FD, but had no effect in a normal visual environment. Of the diverse set of 27 genes related to cAMP, PPARα and HIF-1α signaling and ECM remodeling, butaprost differentially regulated 15 of them during myopia development. AH6809 injections during FD negated such differential gene expressions.

CONCLUSIONS. EP2 agonism increased cAMP and HIF-1α signaling subsequent to declines in PPARα and RXR mRNA levels, which in turn decreased scleral fibrosis and promoted myopia. EP2 antagonism instead inhibited each of these responses. Our data suggest that EP2 suppression may sustain scleral ECM structure and inhibit myopia development.

Keywords: EP2, HIF-1α, PPARα, myopia and sclera

Myopia presents with excessive ocular elongation resulting in a mismatch between the eye size and the refractive power of the cornea and lens, thus obscuring normal vision. Global myopia prevalence has risen rather rapidly in the past 50 years. In East Asian countries such as Taiwan, Hong Kong, China, Singapore and Japan, its incidence now ranges between 85% and 95%.[1,2] The severity of this rise is exemplified by the fact that the prevalence of high myopia (over −6.00 diopters [D]) ranges approximately between 5% and 20% in some Asian populations, although it is also on the rise in western countries.[3] In the year 2000, the global prevalence of myopia and high myopia was estimated to be 22.9% and 2.9%, respectively, which is predicted to increase dramatically to 49.8% and 9.8%, respectively, by 2050.[4] The cause of myopia remains unclear, even though its incidence is lower among individuals who spend more time outdoors compared with those exposed to less daylight. Also, a genetic component cannot be ruled out, as high-

lighted by studies on parental myopia, genetic linkage, and candidate gene analyses and genome-wide association studies.[5,6]

Animal models exposed to visual distortions to induce myopia are often interrogated for molecular cues underlying this condition.[7] Irrespective of its cause, increased eye size accompanying extensive extracellular matrix (ECM) remodeling of the sclera, resulting from changes in the scleral architecture and composition, are common features in humans and animal models of myopia. Such effects ultimately lead to excessive ocular elongation.[8] Among other changes to the scleral ECM, degradation of collagen and proteoglycans, reduced levels of growth factors, such as transforming growth factor–β (TGF-β) isoforms and increased expression and activity of matrix metalloproteinases (MMPs) are observed during myopia.[7,9]

Adenylyl cyclase (AC) stimulation increases cyclic adenosine monophosphate (cAMP) content, which in turn

degrades scleral collagen content during myopia in guinea pigs.[10,11] We also reported that AC activation and presumed increases in scleral cAMP levels promote myopia development through inhibition of genes that promote collagen synthesis and ECM fibrosis in guinea pigs.[12] Although numerous studies focus on studying the roles of various signaling pathways involved in myopia development, we investigated the role of prostaglandins (PGs) in this process. These lipid mediators are synthesized from arachidonic acid by cyclooxygenases (COX).[13,14] Prostaglandin E_2 (PGE$_2$) is a naturally occurring major PG subtype that is most abundant in the human body.[15] PGs interact with prostanoid E receptor subtypes (EP1-4) to perform specific functions.[16,17] EP1 activation increases intracellular Ca^{2+} levels, while EP2 and EP4 elevate cAMP levels and EP3 reduces cAMP.[18] PGE$_2$ agonism promotes proliferation of NIH/3T3 and 3T6 cells, but it inhibits the proliferation of lung fibroblasts and the synthesis of collagen,[19,20] thus highlighting a cell type-dependent response of its EP receptors. Such differences also exist between EP2 and EP4 receptors, both of which elevate cAMP levels[21] that in turn affect collagen levels and fibroblast proliferation. In the eye, EP2 activation reduced intraocular pressure (IOP) in glaucomatous monkeys by increasing the uveoscleral outflow.[22] A significant body of research has shown a strong relation between myopia and glaucoma with myopic individuals having a higher risk of developing glaucoma.[23] The role of EP2 in mediating cAMP, and thus collagen levels combined with their role in reducing the IOP intrigued us to study the role of this particular receptor in myopia development.

In HEK-293 cells, cAMP-induced protein kinase A (PKA) activation enhanced the activity of peroxisome proliferator-activated receptor alpha (PPARα), which is an antifibrotic transcription factor.[24] PPARα forms a heterodimer with retinoid X receptor (RXR) and regulates gene transcription and inhibits ECM accumulation in various tissues.[25–27] However, in the guinea pig sclera, PPARα agonism had a profibrotic effect that suppressed myopia progression, whereas PPARα antagonism inhibited fibrosis and promoted myopia.[28,29] We also earlier reported that a hypoxic environment in the sclera promotes myopia in mice and guinea pigs, while an inhibitor of hypoxia-inducible factor 1-alpha (HIF-1α), a known hypoxia marker, suppressed myopia development. Such myopia inhibitions were accompanied by COL1α1 downregulations in the latter model.[30] Hypoxia has also been shown to decrease RXR levels in cardiac myocytes, which inhibits the formation of a PPARα:RXR complex,[31] thus suppressing the transcriptional ability of PPARα.[32] In HeLa and C33a cervical carcinoma cells, hypoxia increased cAMP levels by increasing AC activity.[33] Thus we predicted that the scleral EP2 modulates cAMP levels and myopia development in guinea pigs through a crosstalk with PPARα and HIF-1α signaling pathways.

In this study, EP2 activity modulation had corresponding effects on cAMP accumulation and myopia progression. The mechanisms underlying such EP2-involvement were studied by monitoring the expression profiles of a few major scleral ECM genes and downstream genes in the PPARα and HIF-1α signaling pathways. The results allowed us to conclude that EP2 activation in a normal visual environment increased cAMP levels, which activated HIF-1α. These effects in turn inhibited fibrosis and PPARα/RXR signaling events that led to myopia development. Alternatively, EP2 antagonism during induced myopia suppressed the myopia-associated cAMP increases and negated the inhi-

bition and activation of PPARα/RXR and HIF-1α signaling, respectively. These reciprocal effects offset declines in scleral ECM content during form deprivation (FD), ultimately preventing myopia progression. Such findings highlight the essential role of EP2 in altering scleral cAMP content, which affects both PPARα and HIF-1α levels that are key modulators of scleral ECM changes during myopia development.

METHODS

Three-week-old pigmented guinea pigs from Bi Kai experimental animal farm (Danyang, Jiangsu) were reared in standard cages under a 12-hour light/dark cycle with food and water provided *ad libitum*. Right eye of each animal was used for drug injections and/or FD, while the fellow eye was untreated. The treatment and care of animals were 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, P.R. China).

Drug Injections and Myopia Induction

Daily peribulbar injections of either an EP2 agonist (butaprost), an EP2 antagonist (AH6809) or a vehicle (0.1% dimethyl sulfoxide [DMSO]), were administered monocularly in mildly restrained guinea pigs. The drugs were dissolved in DMSO, diluted in Milli-Q water to a final working concentration of 0.1%, and 100 µL of the drug or vehicle was injected for two weeks. Animals in groups 1 and 2 received the agonist, "N + Butaprost (1 µmol/L)" and "N + Butaprost (10 µmol/L)," respectively, whereas groups 3 and 4 received the antagonist, "N + AH6809 (10 µmol/L)" and "N + AH6809 (30 µmol/L)," respectively. Group 5 was injected with a vehicle solution of 100 µL Milli-Q water in 0.1% DMSO ("N + DMSO"). Groups 1 through 5 were exposed to normal visual environment. Group 6 was subjected to monocular FD using latex facemasks, whereas groups 7 through 11 received butaprost, AH6809 or vehicle injections along with FD for two weeks (Table 1). Although butaprost has been studied extensively as a therapeutic agent in the management of glaucoma,[34] AH6809 is a potent EP2 antagonist[35,36] that acts via suppressing cAMP elevations in other tissues/cells such as in lung fibroblasts[37] and in the hippocampus of rats during brain injury.[38] Hence, this drug was used in our study to identify the antagonistic effects of EP2 receptor in the sclera during myopia development. These aforementioned drug concentrations were chosen on the basis of studies showing they were effective in mediating ECM remodeling and cell proliferation without causing cellular toxicity in various tissue systems.[37,39] A separate set of untreated animals (Group 12) served as age-matched (AM) controls (Table 1).

Ocular Measurements and Sclera Isolation

At the end of two weeks, the refraction was measured in minimal lighting using a custom-built eccentric infrared photoretinoscope.[40] Each eye was measured thrice, and the mean value was considered the final refraction. Axial length (AL) and vitreous chamber depth (VCD) were estimated using an A-scan ultrasonograph (AVISO Echograph Class I-Type Bat, frequency:11 MHz; Quantel Medical, Clermont-Ferrand, France) as previously described.[29] The refraction

TABLE 1. Groups and Treatment Paradigms for Agonist, Antagonist, and Vehicle Injections in Guinea Pigs

Group Number	Group Name	Treatment	Sample Size
1	N+Butaprost (1 μmol/L)	EP2 agonist injections in a normal visual environment	17
2	N+Butaprost (10 μmol/L)	EP2 agonist injections in a normal visual environment	18
3	N+AH6809 (10 μmol/L)	EP2 antagonist injections in a normal visual environment	18
4	N+AH6809 (30 μmol/L)	EP2 antagonist injections in a normal visual environment	18
5	N+DMSO	Vehicle injection in a normal visual environment	18/19*(in butaprost/ AH6809 groups, respectively)
6	FD	Form deprivation	19
7	FD+Butaprost (1 μmol/L)	EP2 agonist injections with form deprivation	15
8	FD+Butaprost (10 μmol/L)	EP2 agonist injections with form deprivation	12
9	FD+AH6809 (10 μmol/L)	EP2 antagonist injections with form deprivation	18
10	FD+AH6809 (30 μmol/L)	EP2 antagonist injections with form deprivation	17
11	FD+DMSO	Vehicle injection with form deprivation	15/18* (in Butaprost/ AH6809 groups, respectively)
12	AM	Untreated age-matched controls	20

*Butaprost and AH6809 injection groups had separate sets "N+DMSO" and "FD+DMSO" controls for parallel comparison across drug and vehicle injection treatment paradigms.

and axial length measurements were also obtained at the start of the experiment. After completing the two-week ocular measurements, the guinea pigs were terminally anesthetized with an overdose of chloral hydrate and euthanized via cervical dislocation. Their eyes were immediately enucleated on ice with cornea, iris, lens, vitreous body, retina, and choroid discarded to isolate the sclera. Some scleral tissues were stored in RNA*later* for gene expression analysis (n = 10), and some were snap-frozen in liquid nitrogen for cAMP assay (n = 10) and stored at −80°C.

Scleral cAMP Assay

The scleral samples were weighed and homogenized in a ball mill (MM400; Retsch, Haan, Germany) with 200 μL acetate buffer (50 mmol/L Na$^+$ acetate, 50 mmol/L H$^+$ acetate, and 4 mmol/L ethylenediaminetetraacetic acid, pH 4.75) for 10 minutes at a frequency of 30 Hz. The homogenized samples were spun in a centrifuge at 13,000*g* for two minutes at 4°C onto which a 900-μL aliquot of acetate buffer was added and further lysed at 200 watts for 15 seconds in an ultrasonic crusher (JY92-2D; Ningbo Scientz Biotechnology, China) on ice. The homogenate was spun in a centrifuge at 13,000*g* for five minutes at 4°C to collect the supernatant. Acetate buffer (1 mL) and 100% ethanol (2 mL) were added to the supernatant and spun in a centrifuge at 13,000*g* for 15 minutes at 4°C. The supernatants were collected and dried at 60°C overnight. The dried and powdered residue was dissolved in 1 mL acetate buffer with 0.1 mL being assayed using the ^{125}I-cAMP radioimmunoassay kit (Institute of Isotopes Ltd, Budapest, Hungary) according to the manufacturer's protocol. The cAMP concentrations were estimated using a gamma scintillation counter (xh6080; Xi'an Nuclear Instrument Factory, Xi'an, Shaanxi, China) and presented as fmol/mg.

RNA Extraction, cDNA Synthesis and Gene Expression Analysis

Total RNA was isolated using the RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Samples with an A260/A280 ratio of at least 1.8 as measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) were used for gene expression analysis. Total RNA (0.5 μg) was converted to cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) as described earlier.[12] Guinea pig–specific primers (Table 2) were designed using primer3 software[41] and quantitative polymerase chain reaction (qPCR) was performed in duplicates using a SYBR Green master mix (Applied Biosystems, Carlsbad, CA, USA) on an Applied Biosystems ViiATM 7 PCR System. The mRNA level of each target gene was estimated separately in treated and control samples and normalized to the reference gene, 18S rRNA (that was amplified in a separate well to that of the target genes). Relative gene expression values were converted to fold change estimates using the $2^{-\Delta\Delta Ct}$ formula.[42,43] Also the results of gene expression data was compared with the already existing data from Guo et al.[44] that used a similar targeted gene expression analyses from diverse pathways using the PCR technique that we used here. The differentially expressed gene list, along with their fold changes, from four days of FD in tree shrews was used to perform a network analysis using STRING (v10) database[45] that shows interactions between molecules (Supplementary Fig. S2).

Statistical Analysis

Mean differences in refraction, VCD, and AL between the treated and fellow eyes (i.e., interocular difference) at the end of two weeks were determined for each group and

TABLE 2.   Primer Sequences for qPCR Analysis

Genes	Sequence (5′- 3′)	Tm (°C)	Product Size
Creb1_F	GATCTTAGTGCCCAGCAACC	58.62	175 bp
Creb1_R	TAGGCGAACTTCTCGCTTTC	58.01	
Creb2_F	GGGGCTGAAGAAAGCTTAGG	58.24	329 bp
Creb2_R	GGGGGCTCCGTATTAGTCTC	59.03	
Creb3_F	CTCCAGGCCATGGTAATTGA	57.27	97 bp
Creb3_R	CAGGTACAAAGAGGAGGCAGA	59.10	
Pparα_F	GGTGAGGACTTCGGCTTTTA	57.53	110 bp
Pparα_R	AGTACTGCCGCTTCCACAAG	60.32	
Cpt1a_F	ATGTGGAAGTGGATGAAGTGC	58.56	176 bp
Cpt1a_R	TTATCAGAGCCTTGGCGATT	56.99	
Rara_F	GTGAGGAACGACCGCAATAA	58.28	247 bp
Rara_R	CGGTCTTGATGATGCACTTG	57.18	
Rarb_F	GCTACGAGATGACTGCTGAGT	59.60	111 bp
Rarb_R	ATGCACTTGGTGGCCAGTTC	61.18	
Rarg_F	CGAGCTCAGAGGAGATGGTT	58.89	215 bp
Rarg_R	CCTGGTCACCTTGTTGATGA	57.43	
Rxra_F	CTCTCCCATGAACCCTGTGA	58.72	170 bp
Rxra_R	AGCTGTACACGCCATAGTGC	60.46	
Rxrb_F	GCTCCTTATCGCCTCCTTTT	57.67	178 bp
Rxrb_R	CATCCTCATGTCACGCATTT	56.50	
Rxrg_F	GAATGACCTGGTCCTCCAAG	57.59	205 bp
Rxrg_R	TGAAGACATGCCTGTGGAGA	58.65	
Hif1α_F	AGCTGCTGGAGACACAATCAT	59.72	212 bp
Hif1α_R	CTTGATTGAGTGCAGGGTCA	57.81	
Bmp6 _F	AAGAAGGCTGGCTGGAATTT	57.68	205 bp
Bmp6 _R	ACGTGTACCTCGCTCACCTT	61.18	
Gata4_F	AGCTTCATGTAGAGGCCACAG	59.79	180 bp
Gata4_R	TGGCCTCTACCACAAGATGA	58.05	
Abca1_F	ACCCGCGTATTTTTCTCCAT	57.59	121 bp
Abca1_R	GAGGGAGCATGTGGAGTTCT	59.09	
Pml_F	GTGCCCATCTATGCCTTCTC	58.11	110 bp
Pml_R	ATGATCTTTCGGGAGCACTG	57.68	
Ptprn2_F	AAGCTTCGCACCAGAAAGTC	58.77	80 bp
Ptprn2_R	GGCTCCAACCTCTACCACAT	59.09	
Chd4_F	AGGCATGTCCTACTGGCACT	60.91	144 bp
Chd4_R	CGGCTCTTCTCTTCATCACC	58.07	
Lrpap1_F	AGGCTGACGACCTATACGACA	60.41	188 bp
Lrpap1_R	CTCCAGCTCCTTCTTGGTGA	59.02	
Col1a1_F	GGTCCTGATGGCAAAACTG	56.50	121 bp
Col1a1_R	CACCTTTAGGTCCAGGGAAT	56.50	
Col2a1_F	CTGTGACGAAGGGATTGTCCT	59.72	287 bp
Col2a1_R	GGAGGTCCTTTGGGTCCTACA	60.83	
Col6a5_F	TGGCCACGTCTCAGTTTCAT	59.60	424 bp
Col6a5_R	ACACGAGGTCAGCAAGTGAAT	59.93	
Col12a1_F	AGGCGAAAGGAAATCAGCCAC	61.22	355 bp
Col12a1_R	AGCAGGGCATTTTGCTTCATC	59.79	
Acta1_F	GAAGGAGTAGCCACGCTCAG	60.18	319 bp
Acta1_R	TGCTGTCCCTCTATGCCTCT	60.03	
TGF-β1_F	ATGAATAGCAGCCAGGTCAC	57.66	95 bp
TGF-β1_R	GTGCTCACTGCTCCTGTGAT	60.04	
TGF-β2_F	ATCCCGCTTGAAATCAATGT	55.69	206 bp
TGF-β2_R	AGACCCCACATCTCCTGCTA	59.66	
TGF-β3_F	GAGAGTTGCTCCACCTTTGG	58.47	209 bp
TGF-β3_R	GTCCACGAACCTAAGGGCTA	58.81	

presented as mean ± standard deviation (SD). Statistical significance of the change in ocular refraction and biometrics between groups was estimated using one-way analysis of variance (ANOVA) and corrected for multiple testing using Bonferroni correction, unless otherwise stated. Significant changes in cAMP levels and gene expression levels were assessed using paired sample t-test for animals in the same group and independent sample t-test for intergroup comparisons.

RESULTS

There were no significant differences in refraction and axial length between the right and left eyes of guinea pigs in each group before the start of the experiment, which is consistent with our earlier published work.[46,47] Guinea pigs subjected to FD, with or without vehicle (0.1% DMSO) injections, developed significant amounts of myopia characterized by increased VCD and AL compared to their fellow eyes ($P < 0.0001$, paired sample t test) and AM controls ($P < 0.0001$, independent sample t test). Interocular differences in ocular refraction and biometrics of animals in group 6 were not significantly different from those in group 11 (FD vs. FD + DMSO: $P > 0.05$, one-way ANOVA), suggesting a lack of vehicle effect. Data on group 6 are not shown, whereas those on group 11 are presented in comparisons involving the effects of drug injections on myopia.

EP2 Agonist Promotes Myopia Development and Axial Elongation During Normal Ocular Growth

Guinea pigs injected with butaprost (1 μmol/L and 10 μmol/L) and exposed to an unobstructed visual environment progressed towards myopic refractions compared to the vehicle-injected eyes (N + Butaprost (1 μmol/L) and N + Butaprost (10 μmol/L) versus N + DMSO: −4.15 ± 1.82 D and −4.3 ± 1.36 D versus 0.06 ± 0.85 D, respectively; $P < 0.0001$, Fig. 1A). Butaprost or vehicle injections during FD similarly resulted in a significant myopic shift compared to group 5 (N + DMSO) (Fig. 1A). Consistent with refractive error changes, VCD and AL were significantly elongated after 1 μmol/L and 10 μmol/L butaprost injections during normal ocular growth (Figs. 1B and 1C) compared with group 5. During FD, either dose of the drug did not significantly alter the magnitude of myopia induced or ocular elongations compared those injected with the vehicle (Groups 7 vs. 8 vs. 11: −5.09 ± 1.80 D vs. −5.74 ± 1.07D vs. −5.44 ± 1.53 D, $P > 0.05$, Figs. 1A through 1C).

EP2 Antagonist Inhibits Myopic Shift and Axial Elongation During FD

Refraction, VCD and AL were not significantly different between the AH6809-injected and their respective fellow eyes during normal ocular growth (data not shown). In contrast, AH6809 injections during FD (Groups 9 and 10) significantly inhibited the progression of myopia compared with the vehicle-injected group (FD + DMSO vs. FD + AH6809 [10 μmol/L] vs. FD + AH6809 [30 μmol/L]: −5.07 ± 1.66D vs. −3.16 ± 1.48 D vs. −2.79 ± 1.94 D, at least $P < 0.0001$, Fig. 2A). Although both dosages suppressed the myopic shift in refraction, only the highest dosage significantly inhibited AL elongations (FD + DMSO vs. FD + AH6809 [30 μmol/L]: 0.19 ± 0.06 mm vs. 0.14 ± 0.05 mm, $P < 0.05$, Fig. 2C). Refraction and ocular length in the fellow eyes of guinea pigs in treatment groups that developed myopia or in those that presented with myopia inhibition were not significantly different from each other (Supplementary Fig. S1).
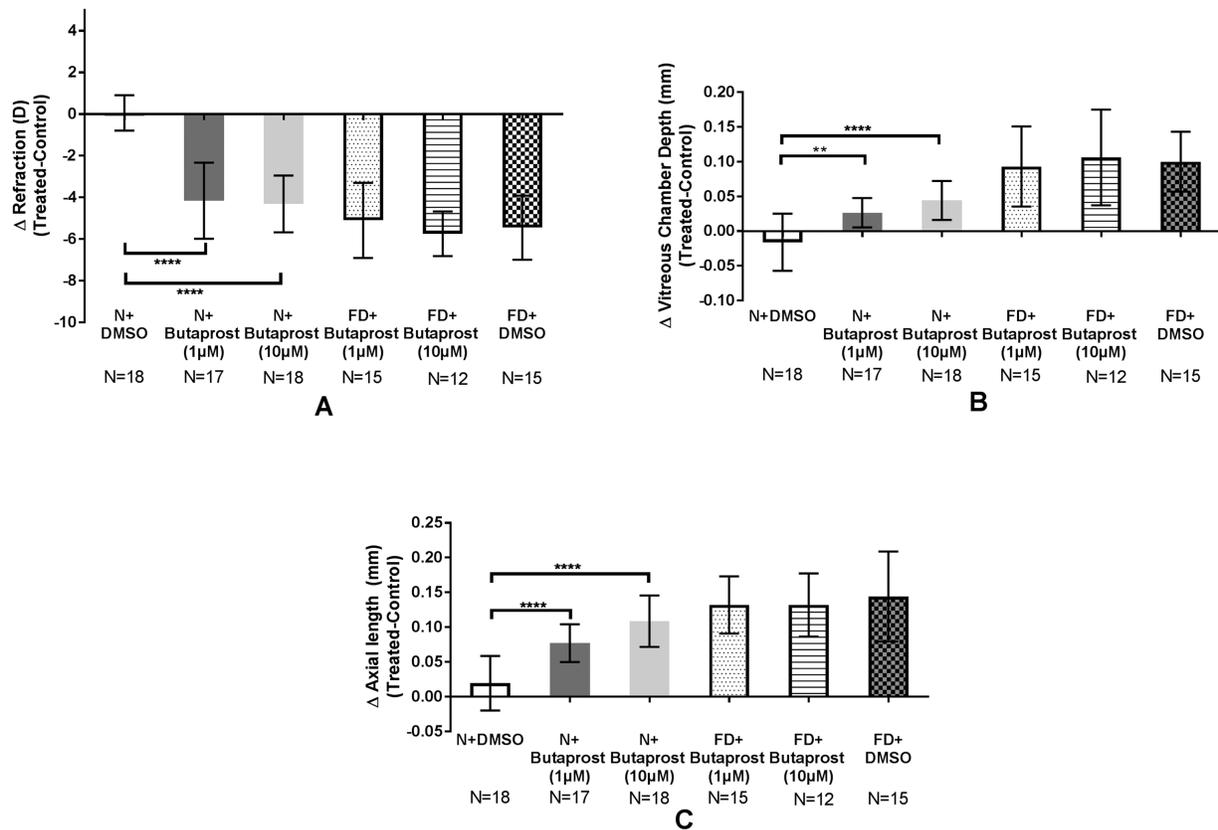
FIGURE 1. Butaprost induces myopia development. Significant myopic shift in refraction (**A**) combined with increases in VCD (**B**) and AL (**C**) were associated with butaprost injections and FD. Data presented as mean interocular difference between treated and fellow eyes ± SD. **$P < 0.01$ and ****$P < 0.0001$; one-way ANOVA.

EP2 Agonism Increases Scleral cAMP Levels

Compared to the vehicle injected eyes, scleral cAMP levels were significantly higher in eyes injected with butaprost (N+DMSO vs. N + Butaprost (1 μmol/L) and N + Butaprost (10 μmol/L): 60.41 ± 13.35 vs. 94.75 ± 28.9 and 102.6 ± 34.89 fmol/mg, respectively, at least $P < 0.05$, Fig. 3A, one-way ANOVA). Intragroup comparison showed significant cAMP increases ($P < 0.05$, paired sample t-test) in eyes injected with 1 μmol/L and 10 μmol/L butaprost ("T") compared with their respective fellow ("F") eyes during normal ocular growth (Fig. 3A) and FD (Fig. 3B). Similarly, FD increased cAMP levels relative to their fellow controls (FD + DMSO-T vs. F: 83.47 ± 17.24 vs. 49.01 ± 12.83 fmol/mg, $P < 0.05$, Fig. 3B). Intergroup comparison among the fellow eyes of the drug and DMSO-injected groups showed increased cAMP levels on 1 μmol/L of butaprost injection alone (FD + Butaprost-F [1 μmol/L] vs. FD + DMSO-F: 99.2 ± 15.84 vs. 49.01 ± 12.83 fmol/mg, $P < 0.05$, one-way ANOVA with Bonferroni correction, Fig. 3B). A possible yoking effect between the treated and fellow eyes of each treatment group, as reported earlier in guinea pigs,[12,48] could account for such a finding. Consistent with the lack of significant refractive and biometric changes among groups 7, 8, and 11, butaprost injections during FD did not significantly alter the cAMP levels relative to vehicle injected eyes ($P > 0.05$ one-way ANOVA with Bonferroni's correction).

EP2 Antagonism Suppresses FD-Induced Scleral cAMP Elevations

A separate set of experiments was carried out to determine the effect of AH6809 injections on scleral cAMP levels compared to that of vehicle, during FD. As reported above, scleral cAMP levels were significantly increased during myopia development relative to fellow controls (FD + DMSO-T vs. FD + DMSO-F: 187.8 ± 50.61 vs. 137.7 ± 35.37 fmol/mg, mean ± SD, $P < 0.05$, paired sample t-test, Fig. 4). In contrast, compared to the vehicle injected eyes (187.8 ± 50.61 fmol/mg), scleral cAMP levels were significantly decreased in response to 10 μmol/L (135.3 ± 34.01 fmol/mg, $P < 0.05$) and 30 μmol/L AH6809 (111.3 ± 26.06 fmol/mg, $P < 0.01$, one-way ANOVA with Bonferroni's correction, Fig. 4) during FD. On the other hand, intragroup comparison showed no significant changes in cAMP levels between the treated ("T") and fellow ("F") eyes of animals receiving either dose of AH6809 ($P > 0.05$, paired samples t-test, Fig. 4) during FD. A possible yoking effect in guinea pigs as reported earlier[12,48] could account for increases in cAMP levels in the fellow eyes of drug/vehicle injected groups during FD relative to AM controls. However, an intergroup comparison showed no significant changes in cAMP levels across the fellow eyes of vehicle and drug-injected groups ($P > 0.05$, one-way ANOVA with Bonferroni's correction). Consistent with the lack of refractive and AL changes, cAMP levels in AH6809-injected eyes ("T") did not signifi-
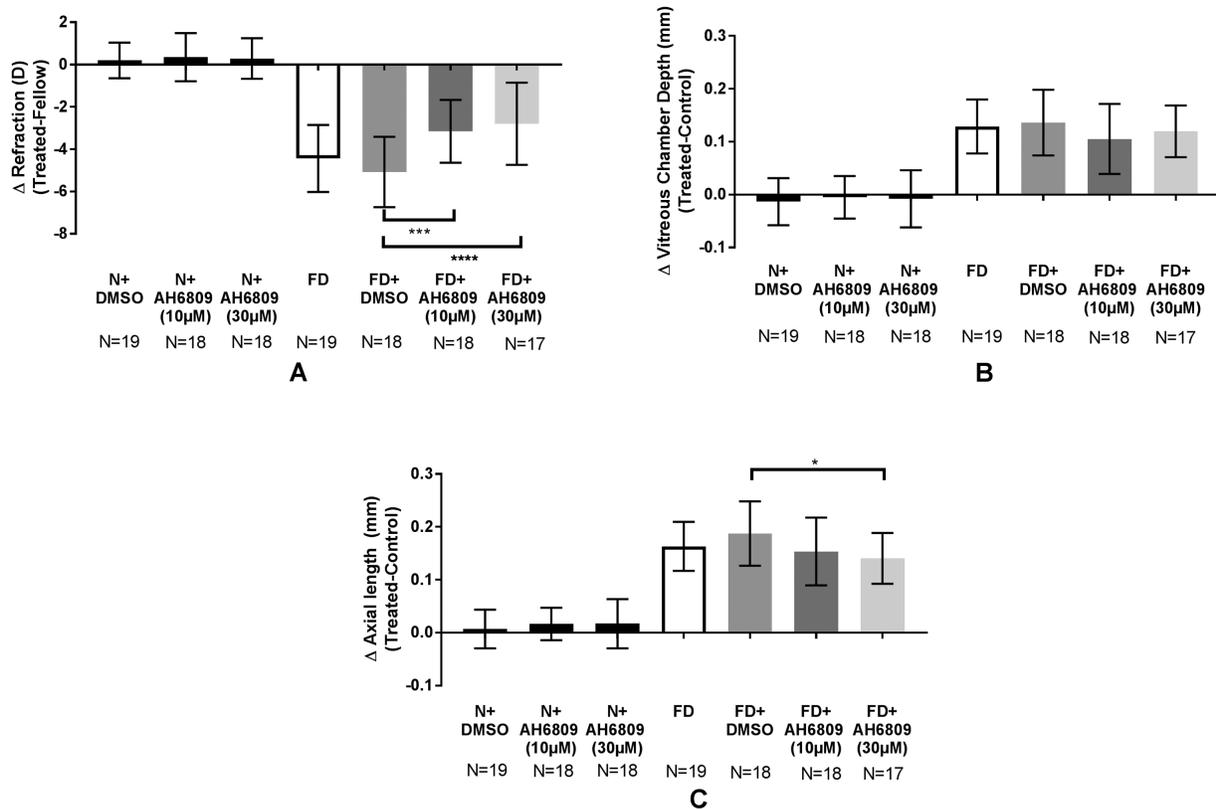
FIGURE 2. AH6809 suppresses FD-induced myopia progression. Myopia development was significantly inhibited after injections with an EP2 antagonist, AH6809 (**A**). VCD was unchanged (**B**), but 30 μmol/L AH6809 during FD inhibited AL elongation (**C**). The antagonist did not alter these parameters in an unobstructed visual environment. Data presented as mean interocular difference between treated and fellow eyes ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; one-way ANOVA).

cantly differ from those of the vehicle-injected ones ("T") during normal ocular growth (Fig. 4). Similarly, no significant changes in cAMP levels were observed among their fellow eyes (N + DMSO-F vs. N+AH6809-F (10 μmol/L) and N + AH6809-F (30 μmol/L): $P > 0.05$, one-way ANOVA).

Differential Gene Expression Patterns Underlie Butaprost-Mediated Myopia Development

Scleral gene expression changes associated with butaprost-induced myopia development were evaluated. Given that no significant changes were observed in refraction, AL and cAMP levels between the butaprost and vehicle-injected eyes during FD, gene expression analysis was not carried out in groups 7 and 8.

Butaprost increased the mRNA levels of cAMP Responsive Element Binding Protein, *Creb1* and *Creb2*, in parallel with scleral cAMP elevations. On the other hand, *Pparα* expression was downregulated with 1 and 10μM of butaprost. Two subtypes of RXR receptors, *Rxra* and *Rxrb*, were also downregulated (Fig. 5). Among the retinoic acid receptors, butaprost injections only downregulated *Rarb*. Overall, such declines may hinder formation of a PPARα:RXR complex that is critical to gene transcription. Also, butaprost increased the *Hif1α* expression levels. Out of the hypoxic signaling pathway genes that earlier showed significant interactions with human myopia genes,[30] butaprost (1 μmol/L) only upregulated *Gata4* (Fig. 5). Among the scleral ECM-related genes,

only *Acta1*, which encodes the smooth muscle protein, α-smooth muscle actin (α-SMA), was upregulated. On the other hand, butaprost (1 μmol/L) downregulated collagen subtypes (*Col1a1* and *Col2a1*) and TGF-β isoforms (*TGF-β1*, *-β2*, *-β3*). Although collagens were similarly downregulated, only *TGF-β2* mRNA was reduced significantly with 10 μmol/L butaprost (Fig. 5). In a normal visual environment, the expression levels of all target genes were unaltered between the DMSO-injected and fellow control eyes.

AH6809-Induced Myopia Inhibition Accompanies Suppressed Gene Expression Changes

FD and FD + DMSO groups did not show any significant differences in refraction, AL and cAMP levels between them suggesting a lack of vehicle effect. Hence, the effect of AH6809 injections on gene expression was compared with animals that received vehicle injections in the same form-deprived environment (i.e., Group 11).

FD-induced (FD + DMSO) *Pparα* downregulations were suppressed with AH6809 injections (Fig. 6). Myopic downregulations of *Rarb* and RXR receptor subtypes (*Rxra*, *Rxrb* and *Rxrg*) also returned to baseline on myopia inhibition with the antagonist (Fig. 6). AH6809 (30 μmol/L) also suppressed the increases in *Hif1α* mRNA levels. Although *Acta1* upregulation and TGF-β isoform downregulations in group 11 were inhibited by both doses of AH6809, only 30 μmol/L of the drug reduced *Col1a1*, *Col2a1* and *Col12a1*
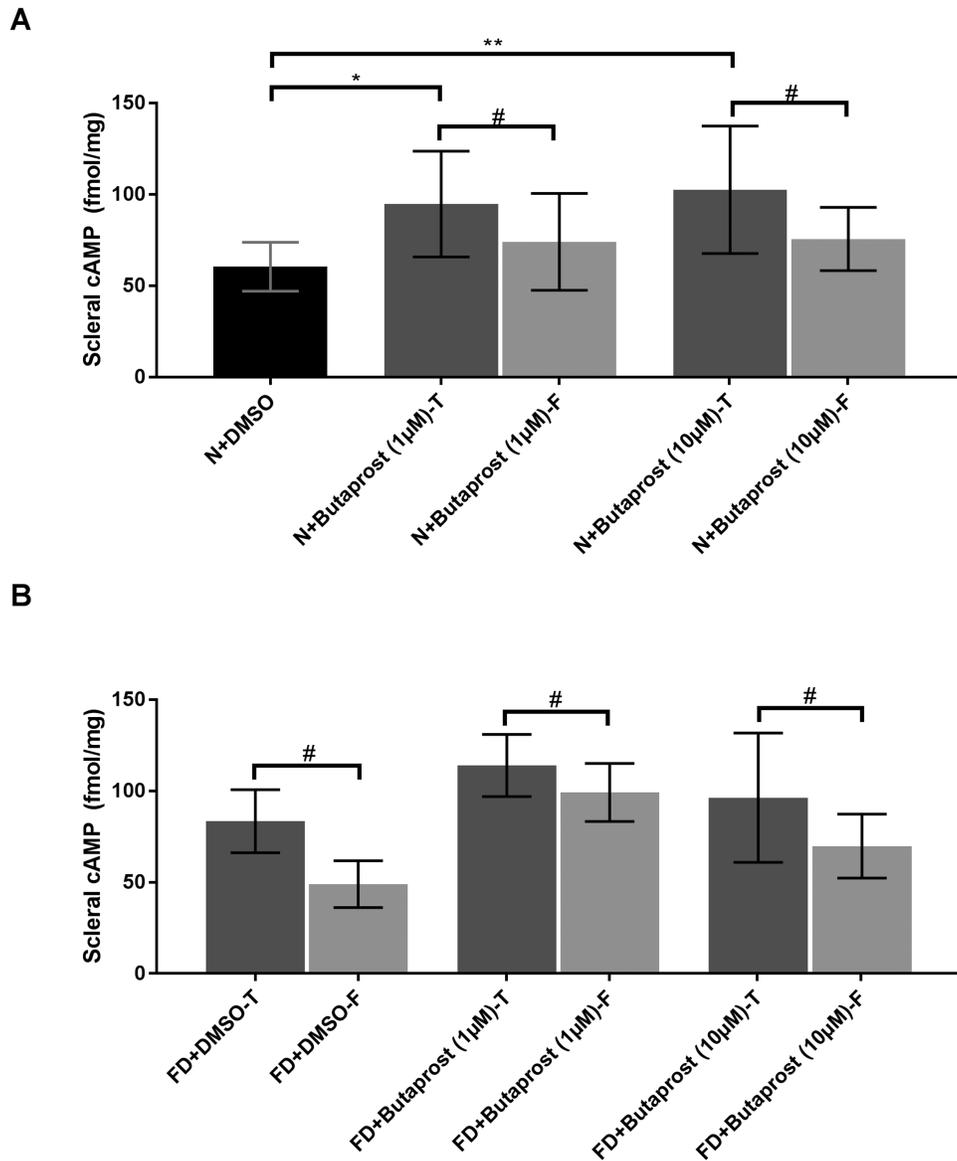
Figure 3. Butaprost increases cAMP levels. Butaprost increased scleral cAMP relative to the fellow and vehicle-injected eyes during normal ocular growth (**A**). FD increased scleral cAMP relative to their fellow eyes with no significant differences amongst the treated ("T") eyes (**B**). Mean cAMP data presented as femtomole/milligram of the tissue (± SD). #$P < 0.05$, paired sample t-test, *$P < 0.05$; **$P < 0.01$; one-way ANOVA with Bonferroni correction.

downregulations (Fig. 6). Overall, during FD, AH6809 negated significant myopic-gene expression changes. Such reversals are consistent with the inhibitory effects of AH6809 on both myopia development and cAMP increases. Given that this EP2 antagonist did not induce any significant changes in a normal visual environment, gene expression analyses were not carried out in groups 3 and 4.

DISCUSSION

A balance between ECM synthesis and degradation is critical in maintaining the structure and integrity of the sclera. During myopia development, this balance is disrupted by increased degradation and reduced ECM synthesis in mammalian models. We here showed that the activation of prostaglandin receptor subtype, EP2 induces myopic scleral ECM remodeling through increases in scleral cAMP levels

and suppression of PPARα/RXR signaling. Such an EP2-mediated crosstalk mechanism stimulates the HIF-1α signaling pathway that promotes myopia development in guinea pigs.

Activation of EP2 led to scleral cAMP increases and myopia development in an unobstructed visual environment. Although FD-mediated myopia development also led to increased cAMP levels, such myopic shifts and cAMP increases were suppressed with an EP2 antagonist in a form-deprived environment. These results are consistent with our earlier study that showed increased cAMP levels to be associated with a myopic refraction, whereas declines in cAMP levels suppressed the progression of induced myopia in guinea pigs.[10] Our findings suggest that the EP2 agonist can only increase baseline cAMP levels provided they are not pre-elevated by FD. On the other hand, the EP2 antagonist only reduced the FD-induced cAMP elevations to levels
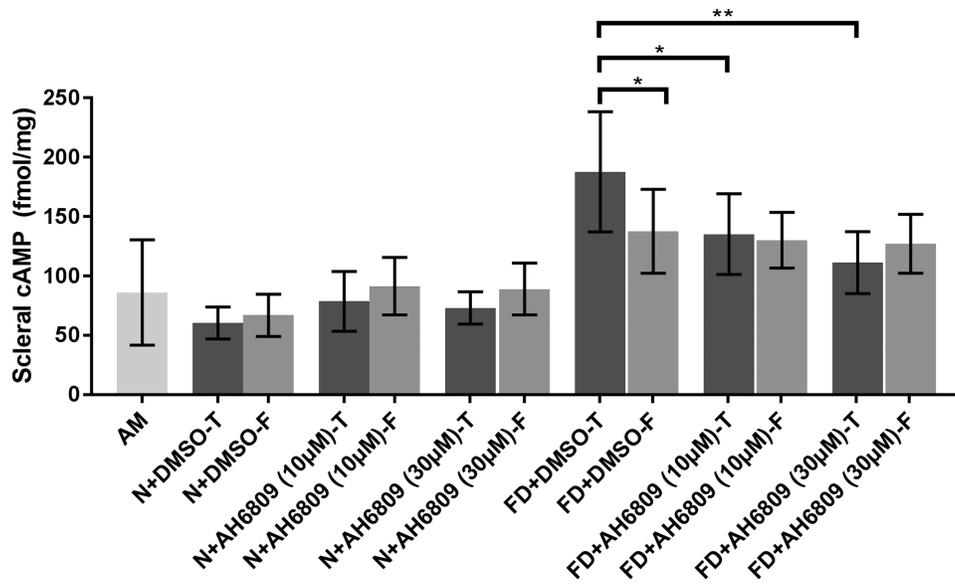
Figure 4. AH6809 suppresses scleral cAMP increases during FD. Myopic scleral cAMP increases were inhibited by AH6809 injections during FD. AH6809 did not significantly affect cAMP levels during normal ocular growth. Mean cAMP data presented as femtomole/milligram of the tissue (± SD). *$P < 0.05$; **$P < 0.01$; one-way ANOVA with Bonferroni correction.
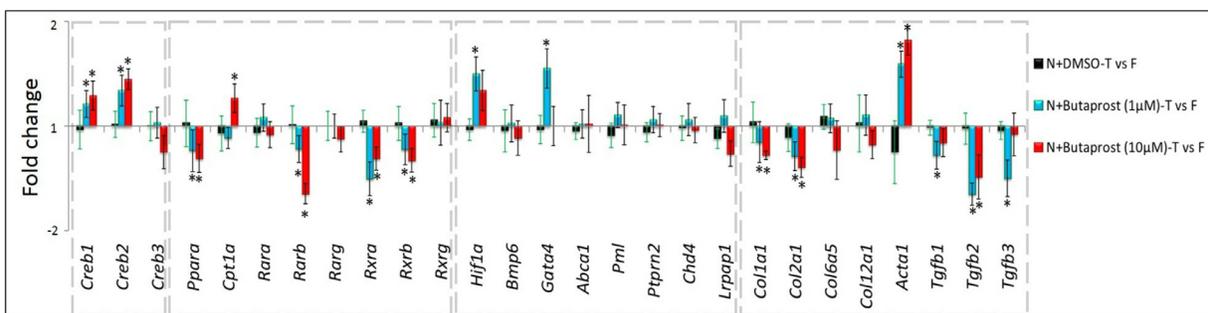

Figure 5. Differential gene expression patterns induced by butaprost during normal ocular growth. Significant gene expression changes, presented as fold changes, were observed in cAMP-related (**A**), PPAR/RXR-related (**B**), hypoxia-related (**C**), and ECM-related (**D**) genes in response to butaprost injections. Gene expression data of "N+DMSO" group are provided for visual comparison of the effect of vehicle and drug injections, with the other experimental conditions being the same (*$P < 0.05$, paired sample *t*-test).

that were not lower than those seen in AM controls. Such a phenomenon is in agreement with "ceiling" and "floor' effects of cyclic guanosine monophosphate (cGMP) levels observed in the sclera of guinea pigs during myopia.[49] In the myopic model, we earlier showed that retinal prostaglandin

F2α receptor (FP) expression levels declined during FD in guinea pigs. Treatment with a FP antagonist resulted in a myopic refraction, whereas one of its agonist inhibited FD-induced myopia development.[47] However, we here found that butaprost induced a myopic shift whereas AH6809
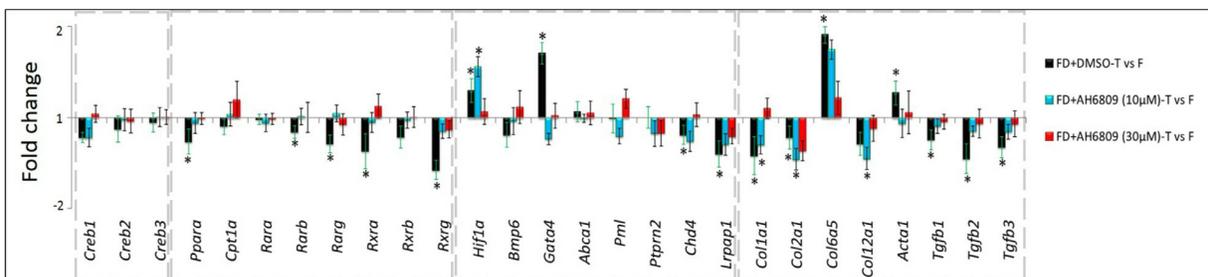

Figure 6. Differential gene expression patterns during AH6809-mediated myopia inhibition. Gene expression changes, presented as fold changes, in cAMP-PKA-related (**A**), PPAR/t RXR-related (**B**), hypoxia-related genes (**C**), and ECM-related (**D**) genes during myopia (FD+DMSO) were inhibited AH6809 injections during FD. *$P < 0.05$, paired sample *t*-test.

inhibited the progression of FD-induced myopia. This could be because of the diverse roles of prostaglandins that are mediated by their receptors (e.g., EP and FP) that perform specific functions. Given that the sclera is mainly composed of collagen as opposed to retina that is multilayered with different cell types, the opposing effects of EP and FP receptors towards myopia development is not surprising. This is in agreement with tissue/cell-specific response of prostaglandins receptors reported earlier.[19,20]

To gain additional insights into EP2 mechanisms, some of the scleral gene expression changes associated with EP2 receptor agonism and antagonism were characterized. Butaprost injections upregulated *Creb1* and *Creb2*, which is consistent with increased cAMP levels; however, FD did not alter their gene expression levels. This could be due to the mechanism of the action of butaprost that impacts directly on cAMP levels, whereas FD depends on visual cues, such as blur to induce myopia. This highlights possible differences between the type of myopic stimulus and the mechanisms underlying myopia development, similar to the diversities reported earlier between negative lens and diffuser-induced myopia.[50,51] Butaprost upregulated *Hif1α* during FD, which is consistent with our earlier data that showed hypoxia to be a key regulator of myopia development in mice and guinea pig models.[30] Also, hypoxia has been shown to increase cAMP levels in carcinoma cell lines[33] and activate CREB in mice lungs[52] and HeLa cell lines.[53] Our current data showing upregulation of scleral *Hif1α* and increased cAMP levels suggest that hypoxia promotes myopia development through increased cAMP levels. Upregulation of the transcription factor, *Pparα* inhibits fibrosis in various tissues.[27] However, we here found that downregulation of *Pparα* by butaprost instead promoted antifibrotic ECM changes. Although it is well established that the sclerae of humans and animal models are characterized by antifibrotic events during myopia, downregulation of scleral *Pparα* is consistent with an earlier study wherein PPARα antagonism induced myopia development in guinea pigs.[29] Formation of the PPARα:RXR complex is critical in mediating the expression of downstream genes. However, we showed here that butaprost injections reduced *Rxra* and *Rxrb* mRNA levels as well. Studies in cardiac myocytes have shown activation of *Hif1α* also downregulated *Rxra* that led to inhibited binding of PPARα/RXR complex and reduced *Pparα* expression.[31,54] Furthermore, activation of *Pparα* inhibited *Hif1α* expression in cardiac myocytes.[32]

These data provide a possible mechanism by which PPARα activity could be inhibited during myopia development. Namely, declines in RXR mRNA levels suppresses PPARα:RXR binding in TGF hypoxic scleral tissue. Furthermore, downregulation of collagen (*Col1a1* and *Col2a1*) and TGF-β subtypes (*β1*, *β2* and *β3*) and upregulation of the myofibroblast marker, *Acta1*, during EP2 activation and FD-mediated myopia development reiterates the degenerative changes to the scleral ECM during myopia. The changes in cAMP levels and the differential regulation of *Pparα, Rxra, Rxrb,* and ECM-related genes were not significantly different from that of the fellow eyes on myopia inhibition with AH6809 injections during FD. Surprisingly, lower dose (10 µmol/L) of this EP2 antagonist during myopia induction increased *Hif1α* expression, whereas the highest dose suppressed it completely along with inhibited axial elongation. Further research into linked pathways, such as the Wnt signaling channel that is implicated in ocular growth[55,56] and hypoxia,[57,58] may be required to understand the mechanisms

of *Hif1α* activation in the context of myopia development and axial elongation, given that only the highest dose of AH6809 suppressed *Hif1α* expression and ocular elongation.

The sclera is dynamic in nature, which maintains the ocular shape during ocular growth and myopia development. Scleral ECM undergoes rapid changes such as a significant decrease in its glycosaminoglycan content within a day after FD in tree shrews.[59] Also microarray analysis revealed that four days of FD or AC activation was adequate to alter the scleral gene expression patterns in guinea pigs.[12] Furthermore, 24 hours of FD increased the expression of α-SMA, a highly contractile protein in the sclera of tree shrews without affecting the ocular growth,[60] suggesting that scleral molecular changes could act as a predecessor for scleral structural changes. Such rapid scleral ECM changes ultimately lead to ocular elongation as hypothesized in the retinoscleral signaling cascade.[8] Our findings also implicate the presence of a dose-dependent response in the expression patterns of *Creb1, Creb2, Rxra, Hif1α, Col1a1, Col2a1, Acta1* and *TGF-β2* upon butaprost injection in normal eyes, without such a trend in ocular growth or refractive error development. Overall these findings reiterate that scleral ECM genes are capable of rapid remodeling that could precede excessive ocular elongation.

Although AH6809 interacts with the EP1 receptor,[16] this drug also inhibits PGE2-mediated cAMP increases in various tissues. Therefore such cAMP declines are attributable to AH6809 acting as an EP2 antagonist, because EP2 activation by butaprost increases cAMP levels.[38,61,62] Similar inhibition of cAMP increases in the sclera of guinea pigs reiterates the affinity of AH6809 towards EP2 receptor. It is tenable that the absence of data on refraction, gene expression and cAMP levels during recovery from myopia could be a limitation of this study. However, we did find that both doses of AH6809 injections during FD suppressed myopia progression, whereas only the highest dose (30 µmol/L) inhibited myopic axial length elongations that also negated FD-mediated gene expression changes. Longer periods of FD with AH6809 injections could potentially lead to significant VCD inhibition and AL suppression even with 10 µmol/L of AH6809. Yang et al.[47] showed that even though prostaglandin F receptor (FP) agonist inhibited myopia development during FD in guinea pigs, significant suppression of axial length was evident only at four weeks, but not at the two-week timepoint. Inhibited VCD growth was also more pronounced and significant after four weeks of treatment.[47] It must be noted that the guinea pigs used in our study were three weeks old compared with the two-week-old ones used by Yang et al.[47] above. The age of the guinea pigs at the start of treatment could also be detrimental in causing a bidirectional change in ocular growth pattern, with this EP2 antagonist, thus demanding longer treatment durations in future. Further investigations into the involvement of other PGE receptor subtypes, for example, EP3 that inhibits cAMP levels, will broaden our understanding of the overall contribution of prostaglandins and their cognate receptor subtypes in controlling myopia development.

We conclude that EP2-mediated scleral cAMP modulation affects myopia development through a crosstalk between PPARα and HIF-1α signaling in guinea pigs. Such EP2 activations are mediated by increases in cAMP levels that inhibit PPARα/RXR activity and increase HIF-1α levels, which ultimately lead to myopia. However, inhibition of EP2 negates these changes and retards myopic ocular

growth. Our study successfully identified the presence and significance of such an interaction in the sclera, which could be critical for myopia control.

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