Bidirectional, Optical Sign-Dependent Regulation of BMP2 Gene Expression in Chick Retinal Pigment Epithelium

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Purpose. We explored the role of bone morphogenic protein 2 (BMP2) in defocus-induced ocular growth using gene expression changes in RPE as a surrogate.

Methods. Young White-Leghorn chickens were used in this study. Normal gene expression of BMP2 and its receptors was examined in retina, RPE, and choroid, and BMP2 protein expression assessed in the same tissues using Western blots and immunohistochemistry. Quantitative PCR (qPCR) was used to assess the effects of short-term exposure (2 or 48 hours) to monocular +10 and −10 diopter (D) lenses, on RPE gene expression of BMP2 and its receptors. Ocular growth was assessed using A-scan ultrasonography.

Results. In the eyes of untreated chickens, BMP2 mRNA was expressed more highly in RPE compared to retina and choroid and all three tissues expressed BMP2 protein. The gene expression for all three receptors also was detected in these tissues, with BMPR2 showing highest and BMPR1B lowest expression. BMP2 was up-regulated in the RPE from eyes wearing +10 D lenses, which exhibited shorter than normal vitreous chambers (VCDs) and thickened choroids, while BMP2 was down-regulated in the RPE from eyes wearing −10 D lenses, which developed enlarged VCDs. These treatments did not induce differential expression of BMP receptors in RPE.

Conclusions. That mRNA expression of BMP2 in chick RPE shows bidirectional, defocus sign-dependent changes is suggestive of a role for BMP2 in eye growth regulation, although the diffuse ocular expression of BMP2 and its receptors suggests complex growth-modulatory signal pathways. (*Invest Ophthalmol Vis Sci.* 2012;53:6072–6080) DOI:10.1167/iovs.12-9917

Uncorrected refractive errors are one of the world's leading causes of blindness and significant contributors to the global burden of eye disease.[1–4] Ocular refractive errors reflect the balance between the refracting power of the eye, to which the cornea and crystalline lens contribute, and its axial length, which defines the position of the retina relative to the latter optical elements. Mismatches between these parameters can result in either myopia, where the eye is too long in relative terms, or hyperopia, where the eye is too short. Babies typically are born with refractive errors, which are corrected during early development through a process of coordinated ocular growth known as emmetropization.[5–9] However, myopia also may occur in childhood as a failure of emmetropization, when the eye continues to elongate after emmetropia is achieved.[4,10]

Studies using animal models have provided convincing evidence for the role of visual input in the emmetropization process and its abnormalities.[11–13] For example, spatial form deprivation and negative defocusing lenses accelerate the rate of eye elongation, while positive defocusing lenses slow eye elongation. The net results in refractive terms are induced myopia and hyperopia, respectively. A variety of studies, including neural lesioning ones, support a model of local regulation of eye growth, with the retina being the presumed origin of growth modulatory signals, linked via one or more local signal cascades directed at the two outer layers of the eye wall—the choroid and sclera, which ultimately determine eye size.[14–17] Although the nature of these regulating pathways remains poorly understood, one investigational approach has been to look for genes showing differential regulation in one or more of these key tissues during altered eye growth.[18–21] Because emmetropization is bidirectional, at least in chicks, bidirectional, optical defocus sign-dependent regulation of genes has been interpreted as evidence of their roles in emmetropization.[11] To date, only expression of the *ZENK* gene in a subset of retinal amacrine cells exhibits this profile (i.e., optical defocus sign-dependence).[11,22,23]

The RPE is a unique tissue, lying between the retina and choroid, and comprising a single layer of polarized cells interconnected by tight junctions. It serves not only to absorb stray light within the eye, but to regulate tightly the exchange of molecules, including ions and water, between the retina and choroid. Thus, the RPE hosts a variety of receptors and transporters.[24,25] Our interest in the RPE is as a likely conduit for growth regulatory signals originating in the retina. By examining gene expression patterns in the RPE from eyes undergoing altered growth, we hoped to obtain insight into how such retinal signals are relayed to the choroid/sclera complex, with the possibility of identifying key growth regulatory molecules underlying myopic eye growth.[19,25]

BMPs represent a large family of multifunctional growth factors that belong to the transforming growth factor-β superfamily, with important roles in embryogenesis and osteogenesis.[26–30] Of this family, bone morphogenic protein 2 (BMP2) already has been linked to ocular development and growth regulation.[31–33] Importantly, BMP2 gene expression in chick retina/RPE is down-regulated in form-deprivation myopia.[33] BMP2 also has been reported to inhibit serum-induced human RPE cell proliferation, consistent with the profile of a negative growth regulator,[34] although BMP2 is reported to stimulate the proliferation and differentiation of human scleral fibroblasts in vitro – the opposite action.[35]

Our interest in BMP2 and its receptors stems in part from a related chick gene microarray study, in which we observed

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Table 1. Primer Gene Symbols, NCBI Access Numbers, Sequences, Efficiencies, and Sizes of Amplicons

Gene	NCBI Access Number	Sequences (5′–3′)	Efficiency	Amplicon
BMP2	NM_204358.1	Forward: 5′-AGCTTCCACCACGAAGAAGTTT-3′ Reverse: 5′-CTCATTAGGGATGGAAGTTAAATTAAAGA-3′	93.6%	96 bp
BMPR1A	NM_205357.1	Forward: 5′-TGTCACAGGAGGTATTGTTGAAGAG-3′ Reverse: 5′-AAGATGGATCATTTGGCACCAT-3′	93.8%	68 bp
BMPR1B	NM_205132.1	Forward: 5′-GGGAGATAGCCAGGAGATGTGT-3′ Reverse: 5′-GGTCGTGATATGGGAGCTGGTA-3′	105%	66 bp
BMPR2	NM_001001465.1	Forward: 5′-GCTACCTCGAGGAGACCATTACA-3′ Reverse: 5′-CATTGCGGCTGTTCAAGTCA-3′	100%	62 bp
GAPDH	NM_204305.1	Forward: 5′-AGATGCAGGTGCTGAGTATGTTG-3′ Reverse: 5′-GATGAGCCCCAGCCTTCTC-3′	95.6%	71 bp

bp, base pairs.

changes in the expression of BMP2 in the RPE of very enlarged, myopic eyes, the result of prolonged exposure to optical defocus (38 days; Zhang Y, et al. *IOVS* 2010;51:ARVO E-Abstract 3680). Two possible explanations for the observed changes in BMP2 expression were considered: (1) that they are a byproduct of stretching and, thus, altered function of the RPE during this enlargement process, or (2) that they reflect activation of a signal pathway linked to eye growth regulation. To distinguish between these possibilities in the follow-up study reported here we used very short exposures to positive and negative lenses, to limit the magnitude of induced ocular dimensional changes. We observed defocus sign-dependent, bidirectional regulation of BMP2 gene expression, but not its receptors in RPE, although expression of BMP2 and three BMP receptors was confirmed in RPE as well as retina and choroid.

Materials and Methods

Animals and Lens Treatments

White-Leghorn chickens were obtained as hatchlings from a commercial hatchery (Privett, Portales, NM) and raised under a 12-hour light/12-hour dark cycle. To induce myopic and hyperopic growth patterns, 19-day-old (adolescent) chickens wore monocular −10 and +10 (diopter) D lenses, respectively, for either 2 or 48 hours. To characterize the effects of the lens treatments on eye growth, the axial ocular dimensions of both eyes of individual birds were measured under isoflurane anesthesia (1.5% in oxygen), at the beginning and end of the lens treatment periods, using high-frequency A-scan ultrasonography ($n = 53$). Only data for the parameters showing significant change are reported, that is vitreous chamber depth (VCD), choroidal thickness (CT), and axial length (AL, the distance between of the anterior corneal and posterior scleral surfaces). The same treatments were applied to a separate set of chickens for use in gene expression studies, to avoid the potentially confounding influence of anesthesia on gene expression. In this case, each of the 4 treatment groups comprised a total of 4 to 6 birds, made up from 3 independent repetitions of the experiment ($n = 16$ for 2 hours of −10 D lens treatment group; $n = 14$ for all three other treatment groups); age-matched untreated birds, that is no lens treatment, also were included ($n = 24$).

Experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Animal Care and Use Committee (ACUC) at University of California, Berkeley, CA.

Gene and Protein Expression Studies

BMP2 and its receptors (BMPR-1A, -1B, -2) were targeted in this study. The normal expression profiles of these genes as well as of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), used as the housekeeping gene, were first established using retina, choroid, and RPE samples isolated from the eyes of untreated birds. BMP2 protein expression also was examined in the same three tissues. Additional posterior eyecups were collected for immunohistochemistry. Only RPE was collected in the lens study in the interest of obtaining samples in minimal time; the expression of BMP2 and its receptors was examined in samples from lens-treated and untreated fellow eyes of experimental subjects, after lens-wearing periods of 2 and 48 hours, as well as in samples from untreated birds. The validity of our selection of chick *GAPDH* as the housekeeping gene in the latter experiments also was assessed.

Tissue Isolation and RNA Extraction. In all cases, birds were sacrificed, eyes enucleated quickly, and tissues collected separately over ice. First, the anterior segment of the eye was cut away. The remaining posterior eye cup was immersed in cold Ringer's buffer, the retina peeled off from the RPE with forceps, and then the RPE collected by rinsing cells gently off the choroid with buffer. In experiments requiring retina, pieces visibly contaminated with RPE were discarded. Choroids were collected last, by peeling them away from the adjacent sclera. Lysed retina, RPE, and choroid samples were stored in RLT buffer from RNeasy Mini kits (Qiagen, Valencia, CA), at −80°C for later use. Total RNA from retina and RPE samples was purified using RNeasy Mini kits, while total RNA from choroid was purified using RNeasy Fibrous Tissue Mini Kits (Qiagen), with on-column DNase digestion, according to the manufacturer's protocol. RNA concentration and A260/A280 optical density ratio were measured for quantification and quality control with a spectrophotometer (NanoDrop 2000; NanoDrop Technologies, Inc., Wilmington, DE). RNA quality also was examined by gel electrophoresis, using a 1.2 % agarose gel, with ethidium bromide staining.

Real-Time PCR. Primers for these studies were designed using Primer Express 3.0 (Table 1; Applied Biosystems, Foster City, CA). QuantiTect SYBR Green PCR Kits (Qiagen) and a StepOnePlus Real-Time PCR System (Applied Biosystems) were used for gene expression quantification. Total RNA was first reverse transcribed to cDNA (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA). Genomic DNA contamination was examined using RNA samples without RT enzymes. Ten-fold serial dilutions of cDNA were used for generating standard curves for each pair of primers. Amplification of each gene was performed in triplicate. Melt curves were performed for all genes examined; all PCR tests yielded single peak products. The efficiency (E) of each primer was calculated using the following equation, $E = 10^{(-1/slope)}$. Mean normalized expression (*MNE*) values were used to compare gene expression levels in RPE from lens-treated eyes, their fellow (control) eyes, and normal eyes from untreated birds. MNE values and fold changes were derived as follows[36];

$$\text{MNE} = \frac{(\text{E}_{\text{reference}})^{\text{CT}_{\text{reference,mean}}}}{(\text{E}_{\text{target}})^{\text{CT}_{\text{target,mean}}}} \quad \text{and} \quad \text{Fold} = \sum_{i=1}^{N} \frac{\text{Treatment}_i}{\text{Fellow}_i}.$$

PCR products also were sequenced (DNA Sequencing Facility, University of California, Berkeley, CA).

Western Blot. Normal BMP2 protein expression profiles were established for retina, RPE, and choroid using Western blots and samples from untreated birds. The ocular tissues were collected and lysed at 4°C with RIPA buffer (Sigma-Aldrich, St. Louis, MO), containing a protease inhibitor cocktail (Sigma-Aldrich). Total protein concentration was measured using a BCA assay (Pierce Biotechnology, Rockford, IL). For Western blots, protein samples were prepared in NuPAGE LDS sample buffer (Invitrogen), with or without DTT as a reducing agent, and heated at 95°C for 5 minutes. Protein samples (20 μg) then were electrophoresed under non-reducing and reducing conditions on 4%–12% gradient gels (NuPAGE 4-12% Bis-Tris Gel; Invitrogen), before being transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks; Invitrogen). Membranes were blocked (StartingBlock T20 [TBS]; Pierce Biotechnology), then incubated with mouse anti-human monoclonal antibody against BMP2 (#ab6285, 1:500–1:8000 dilution; Abcam, San Francisco, CA), and finally labeled with HRP-conjugated goat anti-mouse IgG (#31430; Pierce, Rockford, IL). The choice of antibody was based on results of database searches; mature human BMP2 and chicken BMP2 have 96.5% identity, and human and mouse BMP2 have 100% identity. The specificity of the BMP2 primary antibody also was verified using commercial BMP2 protein (#ab87065; Abcam). As a negative control the same protein also was used as a preabsorbed blocking peptide for the BMP2 antibody. Mouse brain lysates were used as positive controls. Immunoreactive bands were detected with chemiluminescence (Supersignal Pico ECL; Pierce Biotechnology), and images developed using a bioimaging system (FluorChem Q, Alpha Innotech; San Leandro, CA). Assays involved three independent biologic samples and triplicate repeats.

Immunohistochemistry. Posterior eyecups were prepared from enucleated eyes, immersed in optimal cutting temperature (OCT) compound (Ted Pella, Inc., Redding, CA) and stored at −80°C immediately for later use. Then, 7 μm cryostat sections were dried at room temperature, fixed with acetone, washed with PBS, and then blocked with 10% normal goat serum in PBS containing 2% BSA. Immunostaining used as the primary antibody, a mouse anti-human monoclonal antibody against BMP2 (ab6285, 1:50-1:300 dilution; Abcam), and as a secondary antibody, an Alexa Fluor 546 goat anti-mouse IgG conjugated antibody (Invitrogen); an isotype control (Invitrogen) also was included. Sections were labeled and then mounted on glass slides with medium containing the nuclear stain, 4′6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA), and photodocumented with a Zeiss Axioplan 2 microscope (Carl Zeiss, Inc., Oberkochen, Germany).

Statistical Analysis

Data were expressed as mean ± SEM. Paired Student's *t*-tests were used to compare lens-treated eyes with their fellow (contralateral) control eyes; one-way ANOVAs combined with post-hoc analysis (Fisher's least significant difference) were used for comparisons involving more than 2 groups. In analyzing gene expression data, comparisons were made between the two eyes of treated birds, as a measure of the primary treatment effects, and also between the fellow eyes of treated birds and eyes of untreated birds, to look for effects on the fellow untreated eyes that would imply interocular yoking influences.

Results

Expression of BMP2 and BMP Receptors in Normal Retina, RPE, and Choroid

RNA Yield and Quality. Mean RNA concentrations and A260/A280 optical density ratios for retina, RPE, and choroid are shown in Table 2. Retinal samples had highest RNA yield (16.9 ± 0.8 μg/eye), followed by choroidal samples (8.7 ± 1.1 μg/eye), with RPE samples giving the lowest yield (1.9 ± 0.1 μg/eye). A260/A280 ratios for all three tissues were approximately 2.0. Gel electrophoresis confirmed the integrity of RNA in the samples (Fig. 1).

Table 2. RNA Concentration, A260/A280 Ratio, and Yield/Eye for Retina, RPE, and Choroid Samples

	RNA Concentration (ng/μL)	A260/A280	Total yield (ng)/eye
Retina	338.8 ± 17.0	2.0 ± 0.003	16,942 ± 848
RPE	38.7 ± 1.79	2.0 ± 0.009	1,935 ± 90
Choroid	173.1 ± 22.7	2.0 ± 0.008	8,655 ± 1,135

mRNA Expression of BMP2 and BMP Receptors. BMP2 and all three BMP receptor subtypes examined, BMPR1A, BMPR1B, and BMPR2, were expressed in all three tissue types examined, retina, RPE, and choroid (Fig. 2). That the primary PCR product of interest was BMP2 was confirmed by DNA sequencing. In relative terms, BMP2 appeared more highly expressed in RPE compared to retina and choroid, as reflected in the following MNE values: 0.21 ± 0.02 for RPE, 0.0007 ± 0.00008 for retina, and 0.017 ± 0.003 for choroid. For BMP receptors, BMPR2 and BMPR1A showed much higher expression than BMPR1B across all 3 tissues. Note that differences in baseline expression of GAPDH between these ocular tissues also were evident when Ct values were normalized against total RNA amount. The ratio of GAPDH expression (retina: RPE:choroid) was 15:3:1.

Protein Expression of BMP2. Western blots indicated the presence of BMP2 protein in chick retina, RPE, and choroid (Fig. 3). To understand the complex banding patterns observed under non-reducing and reducing conditions, it is important to note that mature and proprotein of BMP2 have been reported for the chick (in the public domain at http://www.uniprot.org/uniprot/Q90751), as well as other animals.[27,37] The mature protein has 114 amino acids (aa; 13 kDa) while the propeptide is much larger (353 aa, 40.3 kDa), with some glycosylation sites at which further protein modification may occur.[38,39] The presence of the amino acid, cysteine, also allows dimers to form from monomers via disulfide bonds.[37,40] In describing our results, we have made tentative assignments to observed bands, based on this background knowledge. Under non-reducing conditions (Fig. 3A), the retinal sample (lane 2) showed 4 strong bands corresponding to the dimer of the proprotein (~80 kDa), a modified (glycosylated) monomer of the proprotein (~50 kDa), a monomer of the proprotein (~40 kDa), and a dimer of the mature BMP2 (~28 kDa). The dimer of the mature BMP2 (~28 kDa) was not detected in either RPE (lane 3) or choroid (lane 4). Interestingly, in lane 4 (choroid), there was an additional weak band at ~39 kDa, which may represent either a trimer of the mature or other forms of BMP2.[41] In lane 5, to which BMP2 protein (0.02 μg) was added as a control, a band at ~13 kDa was detected. Compared to the non-reducing conditions, the reducing conditions (Fig. 3B), generated stronger bands and in some cases, additional bands

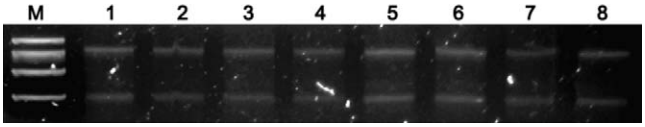

Figure 1. Results of electrophoresis using a 1.2% agarose gel and ethidium bromide staining, for 8 RPE RNA samples checked for RNA integrity. Lane M, marker; lanes 1 to 8, RNA samples.

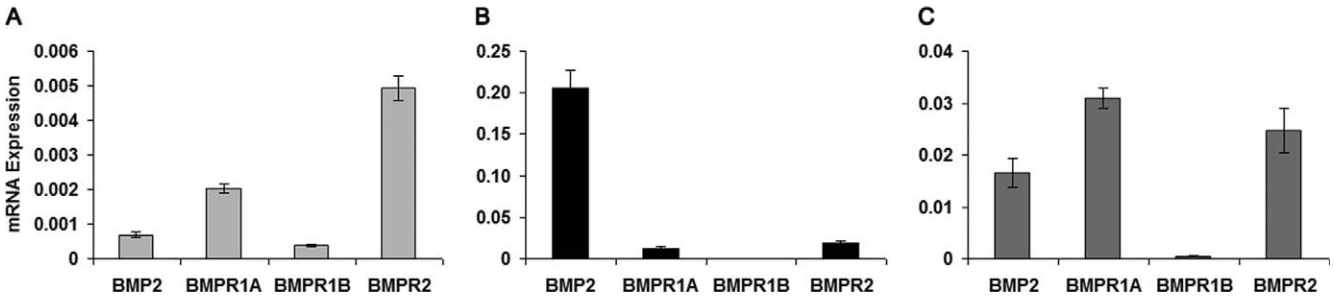

Figure 2. mRNA expression of BMP2, and BMP types I and II receptors (BMPR1A, BMPR1B, BMPR2) in normal untreated chick retina (**A**), RPE (**B**), and choroid (**C**); *GAPDH* used as the housekeeping gene. Data are expressed as mean MNE ± SEM.

(e.g., lane 4, choroid), presumably reflecting improved binding of the antibody, although the results for the two conditions generally were similar. In both cases, no mature BMP2 was detected in RPE. No obvious bands were visible in the negative control test, for which the BMP2 primary antibody first was neutralized, implying very low nonspecific binding.

Immunohistochemistry. BMP2 labeling was observed in all of the layers making up the wall of the posterior eyecup (Figs. 4A, 4B). In the retina, there was intense BMP2 labeling in regions corresponding to the photoreceptor outer segments, outer plexiform layer (OPL), inner part of inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL), as well as weak labeling in the outer nuclear layer (ONL). In the RPE, labeling appeared limited to the basal (choroid) side of RPE, although this may represent an artifact of the heavy pigmentation elsewhere in this layer. The choroid showed diffuse labeling while in the sclera, there was intense labeling throughout the outer fibrous component and at the choroid-sclera boundary, as well as localized labeling confined to the chondrocytes in the inner cartilaginous layer. The negative control, which was prepared by incubating sections in mouse isotype IgG and secondary antibody, showed only very low background labeling (Fig. 4E). These immunostaining data were consistent with the above Western blot results, which detected the BMP2 protein in retina, RPE, and choroid.

Effects of Lens Treatments on Eye Growth and Gene Expression in RPE

Ocular Dimensional Changes. With the +10 D lenses (Fig. 5A), VCD was decreased significantly in treated eyes relative to their fellows after only 2 hours of wear ($P < 0.001$, $n = 6$). The longer exposure period of 48 hours yielded a similar response pattern although the change in VCD was significantly larger than that recorded with the shorter, 2-hour period of lens wear and CT was now increased significantly ($P < 0.001$ in both cases, $n = 6$). Although this lens treatment was expected to slow axial elongation, no significant change was seen over the short treatment durations used in this study, and thus interocular differences in AL did not reach statistical significance for either treatment duration.

The −10 D lens treatment also induced changes in ocular dimensions (Fig. 5B), although they reached statistical significance only after the longer, 48-hour period of lens wear. At this time, lens-treated eyes had longer VCDs and ALs, and thinner choroids compared to their fellows, with interocular differences reaching statistical significance in all 3 cases ($P < 0.001$, $n = 18$). The interocular VCD and AL difference data for 2 ($n = 11$) and 48 hours also were significantly different from each other ($P < 0.001$).

The eyes of normal, untreated birds typically had similar dimensions and, thus, as expected, no significant interocular differences in VCD, CT, and AL were observed (data not

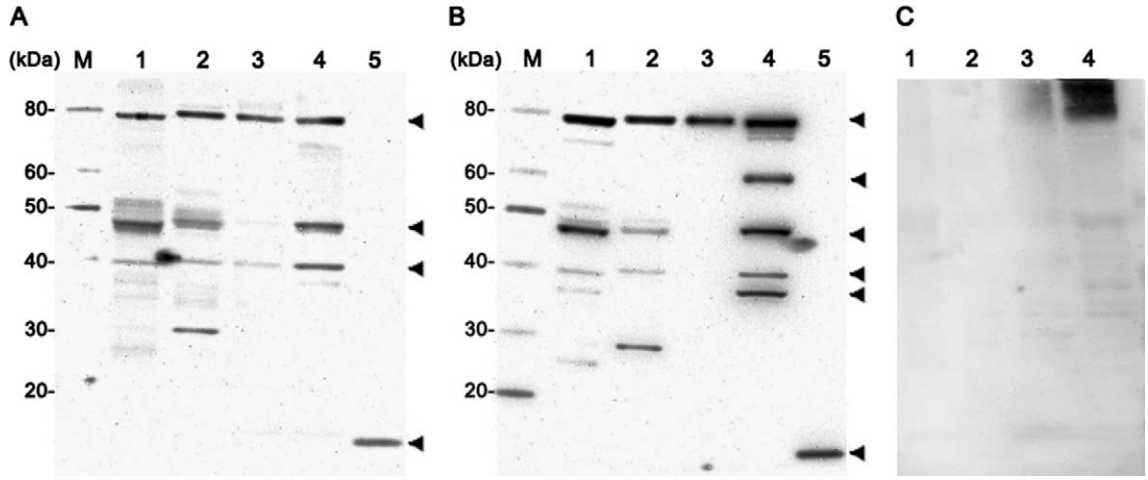

Figure 3. Western blots showing protein expression of BMP2 for non-reducing (**A**) and reducing (**B**) conditions. In cases, lane M was loaded with marker, lane 1 with mouse brain lysates (positive control), and lanes 2 to 4, with chick retina, RPE, and choroid, respectively, while lane 5 was loaded with commercial BMP2 protein. Differences in BMP2 expression, between tissues and between conditions, were evident, with the choroid showing the highest expression and multiple forms. Molecular weights of main mature and proprotein forms of BMP2 are 13.0 and 40.3 kDa, respectively. Negative control using BMP2 peptide preabsorbed primary antibody was included (**C**). Primary antibody concentration 1:500.

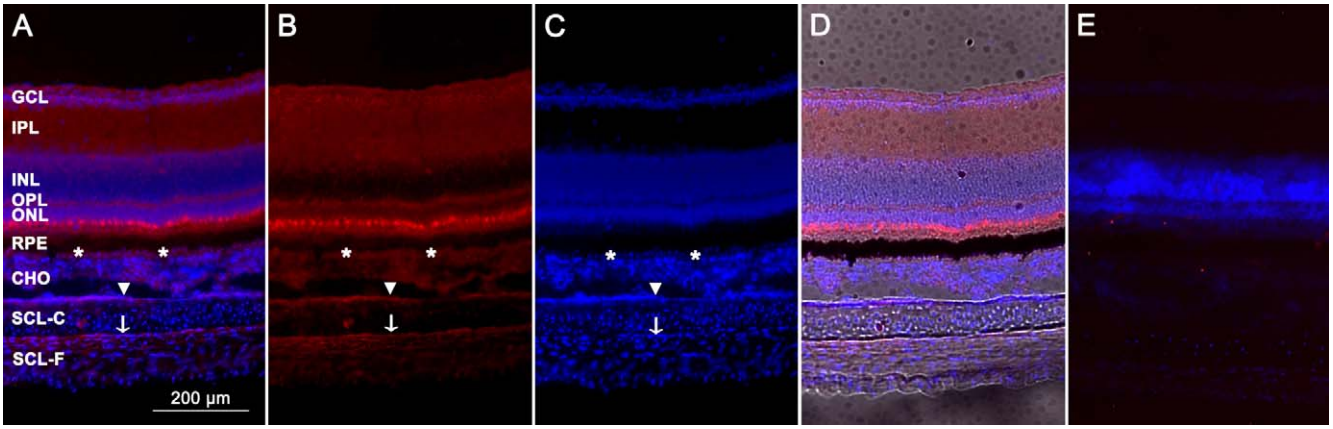

FIGURE 4. Labeled section from the wall of the posterior eyecup, either double-labeled for BMP2 (*red*) and DAPI (*blue*, **A**), with BMP2 alone (**B**), with DAPI alone (**C**), with light microscopy image overlaid (**D**), or isotype control (**E**). CHO, choroid; SCL-C, sclera cartilaginous layer; SCL-F, sclera fibrous layer. *Basal side of RPE. ▼Inner boundary between choroid and sclera. ↓Border between cartilaginous and fibrous layers of sclera. *Scale bar* = 200 μm.

shown, $n = 12$). Chicks used for the collection of ocular biometry data were not used in gene expression experiments, to avoid potentially confounding effects from the measurement procedure, which included brief exposure to isoflurane and unobstructed vision.

Defocus-Induced Gene Expression Changes of BMP2 in RPE. The +10 D lens treatment induced an up-regulation of BMP2 in the RPE, with the largest increase being recorded after only 2 hours (Fig. 6A). Expression of BMP2 was increased by 7.2- and 4.1-fold in treated eyes compared to their fellow (control) eyes, with 2 and 48 hours treatments, respectively ($P < 0.001$, $n = 14$ for both cases). The opposite trend was observed with the −10 D lens treatment, which induced a down-regulation of BMP2, and here also, the change recorded with the 2-hour treatment was larger (Fig. 6B). Expression of BMP2 was decreased by 13.3- and 3.7-fold in treated eyes compared to their fellow eyes, with 2 and 48 hours of treatments, respectively ($P < 0.001$, $n = 16$; $P < 0.01$, $n = 14$). No significant interocular difference in BMP2 gene expression was observed in RPE from eyes of age-matched untreated birds (data not shown).

Yoking Effects of Lens Treatments on BMP2 Gene Expression in RPE. BMP2 expression in RPE from the contralateral fellow eyes of lens-treated birds and the eyes of untreated birds was compared to look for indirect evidence of interocular yoking effects, which cannot be detected by within-bird interocular comparisons as described above. Figure 7 shows expression data (MNEs) for all eyes and treatments. With the 48 hours, −10 D lens treatment (Fig. 7B), BMP2 expression levels in treated eyes and their fellows appeared reduced relative to levels in the eyes of untreated birds, hinting at yoking; however, the difference between fellow and untreated eyes did not reach statistical significance ($P = 0.077$). No equivalent trends were apparent in the +10 D lens treatment BMP2 expression data (Fig. 7A).

Gene Expression Changes of BMP Receptors in RPE after Lens Treatments. None of the three genes, *BMPR1A*, *BMPR1B*, *BMPR2*, showed differences in expression between treated eyes and their fellows, for either of the lens treatments, irrespective of their duration (Fig. 8A). However, when gene expression (MNEs) in the eyes of lens-treated birds was compared to equivalent data for untreated birds, BMPR2 was significantly down-regulated in treated and fellow eyes with −10 D lens treatment, for the 2- and 48-hour treatment durations, implying a yoked down-regulation of this receptor ($P < 0.01$, Fig. 8B).

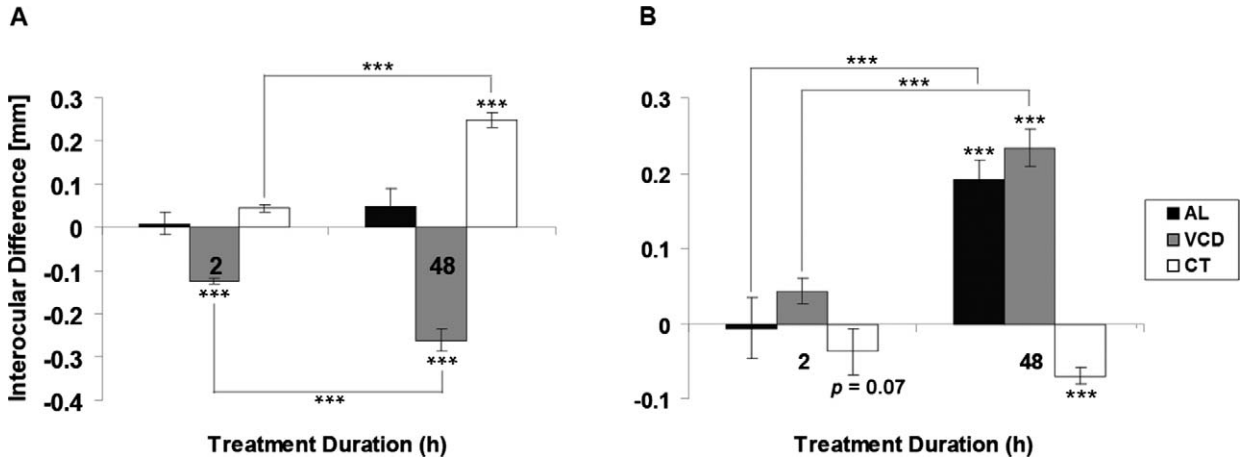

FIGURE 5. Effects of +10 D (**A**) and −10 D (**B**) lens treatments on axial length (AL), vitreous chamber depth (VCD), and choroidal thickness (CT) following 2 hours ($n = 6$, 11 respectively) and 48 hours ($n = 6$, 18 respectively) of lens wear, shown as interocular differences (treated-control eyes, mean ± SEM). Asterisks are placed on top of data when ocular dimensions after treatment were compared with before treatment. ***$P < 0.001$.

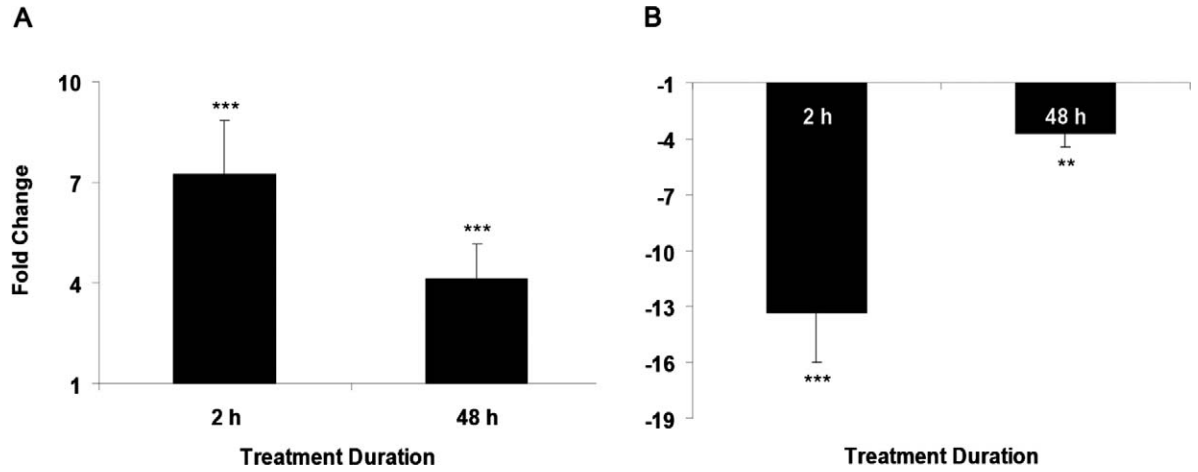

Figure 6. Differential expression of BMP2 mRNA in RPE after 2 and 48 hours of imposed defocus (+10 D, **A**; −10 D, **B**). Ratios of values for treated and fellow eyes expressed as mean ± SEM. $^{**}P < 0.01$. $^{***}P < 0.001$.

Validation of Using GAPDH as a Housekeeping Gene. The stability of *GAPDH* expression across different treatment conditions was assessed by comparing the expression of GAPDH/total RNA (μg) in RPE from untreated, treated, and fellow eyes. Its expression was not affected significantly by the lens treatment conditions (Fig. 9).

Discussion

In our study, we demonstrated in normal chickens the gene and protein expression of BMP2, as well as the gene expression of three different subtypes of BMP receptors in three posterior ocular tissues (retina, RPE, and choroid) and for the first time to our knowledge, the optical defocus-sign-dependent, bidirectional regulation of BMP2 gene expression in chick RPE. The latter results are consistent with, although not definitive evidence for, a role of BMP2 in defocus-induced modulation of eye growth.

BMP2 is one of the most widely studied growth factors in the BMP family, which has important roles in embryogenesis and osteogenesis, as noted in the introduction.[26–30] While several studies have focused on the roles of BMPs in embryonic eye development, investigations into their roles in postnatal ocular development and function in adult eyes are very limited (Zhang Y, et al. *IOVS* 2010;51:ARVO E-Abstract 3680).[28,33,34] To our knowledge, our study represents the most comprehensive study to date of BMP2 and BMP receptor gene expression in the posterior ocular tissues of adolescent chickens, complementing and expanding on an earlier investigation of retina/RPE BMP2 expression in 7 day-old chicken.[33] While BMP2 expression was found to be only low in the retina, BMP2 was highly expressed in the RPE, consistent with RPE being a major ocular source of this growth factor. Furthermore, the gene expression profiles for the receptors of BMP2 suggest that it acts at multiple sites with potentially multiple functions within the posterior layers of the eye. Specifically, we were able to confirm the presence in all three ocular tissues of the receptors involved in downstream signaling of BMP2 - the heterodimerized type I (either BMPR1A or BMPR1B) and type II (BMPR2).[26] Although there were receptor-related differences in gene

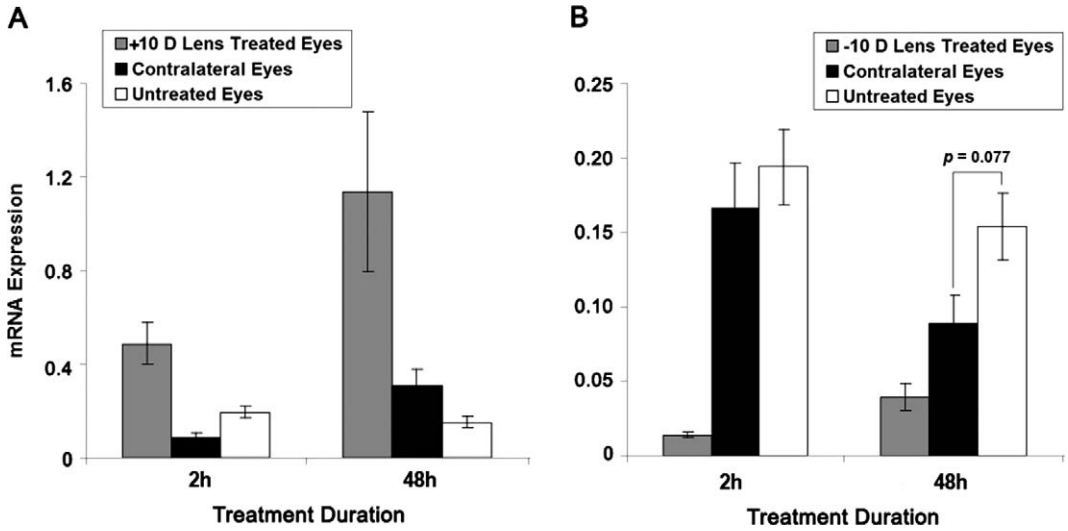

Figure 7. BMP2 mRNA expression in RPE after +10 D (**A**) and −10 D (**B**) lens treatments. Expression relative to *GAPDH* plotted as mean MNE ± SEM. Differences in gene expression between lens-treated and fellow eyes reached statistical significance for all four groups. Expression in −10 D fellow eyes of −10 D lens-treated eyes decreased relative to eyes from untreated birds but did not reach statistical significance; no such yoking is evident in the +10 D lens data.

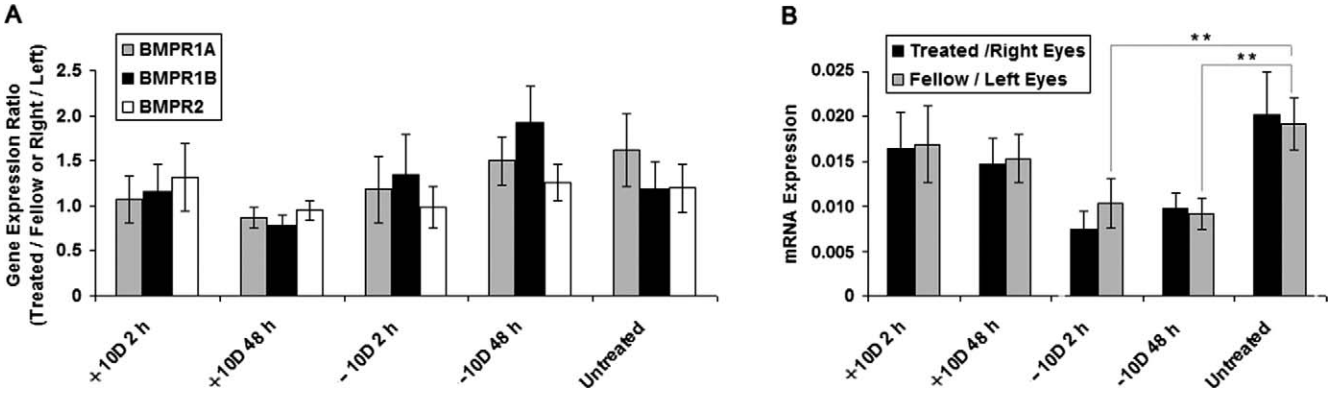

Figure 8. BMP receptor mRNA expression in RPE after +10 and −10 D lens treatments and in eyes of untreated birds. No differences in gene expression between lens-treated and fellow eyes or between right and left eyes of untreated birds were observed (**A**, $P > 0.05$). mRNA expression of *BMPR2* was significantly down-regulated in treated and fellow eyes compared to untreated eyes after −10 D lens treatment for 2 and 48 hours ($P < 0.01$). $^{**}P < 0.01$.

expression, nonetheless the implied broad ocular distribution of these receptors is compatible with paracrine and autocrine signaling.

Because BMP2 exists in multiple forms, all but one of which are inactive, and further, because gene expression levels do not predict reliably translation into protein, we also examined BMP2 protein expression and its localization in posterior ocular tissues. The Western blots detected various forms of BMP2 in all three tissues, retina, RPE, and choroid, and also showed tissue-related differences in protein expression profiles. Of note was the detection of the biologically active mature BMP2 in retina and possibly also in choroid, but not in RPE. Thus, it seems likely that the RPE contributes to the BMP2 stores of these neighboring tissues. Indeed, the high level of BMP2 gene expression in the RPE compared to the two adjacent tissues is consistent with it being a major source of BMP2 for these tissues, which nonetheless also synthesize and secrete BMP2 locally. The immunohistochemistry data in our study lend further support for this interpretation; BMP2 labeling was found throughout the retina, choroid, and adjacent sclera, and there was intense labeling at the RPE/choroid boundary. This profile also is consistent with secretion of BMP2 by RPE as part of a paracrine signaling pathway, modulating as yet unknown ocular functions.

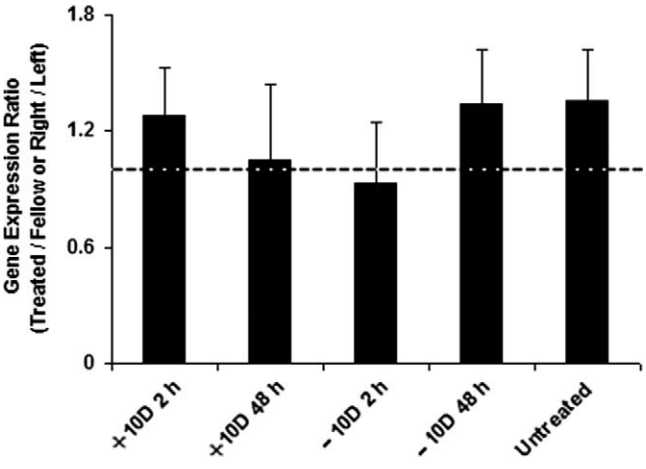

Figure 9. Expression of *GAPDH* in RPE normalized to total RNA (μg); data plotted as the ratio of expression levels in treated and fellow eyes for treated birds, and for untreated birds, the ratio of levels in right and left eyes. Dotted line indicates a ratio of 1.0.

The most significant finding from our study is the apparent optical defocus, sign-dependent regulation of BMP2 gene expression in chick RPE. BMP2 gene expression was significantly up-regulated by the +10 D lens treatment and significantly down-regulated with the −10 D lens treatment, with the greatest effects seen after only 2 hours of lens wear in both cases. These temporal patterns, and importantly, the rapid onset of the gene expression changes, are compatible with a role for BMP2 in initiating defocus-driven eye growth changes. That the same pattern of differential gene expression still was evident after 48 hours of negative lens wear, when eyes were growing faster than normal, as evident from ultrasonography data, suggests a further role for BMP2 in maintaining this altered growth pattern. Note that the apparent reduction in the magnitude of the change after 48 compared to 2 hours of treatment is at least partly a product of yoked changes in the fellow eye at the latter time point (Fig. 7). A role for BMP2 as a growth inhibitor, as suggested by this gene expression profile, is in line with the results of another study describing BMP2 as a negative growth regulator.[34] However, the mechanisms underlying the regulation of BMP2 expression in RPE remain largely unknown and its role in postnatal eye growth regulation is yet to be demonstrated directly. While high basal level of BMP2 expression, as observed, is not a necessary pre-requisite for bidirectional changes in expression, it could plausibly extend the range of response, although this point has not been emphasized in relevant previous gene expression studies.

Of the few other molecules known to be regulated bidirectionally in the eye by optical defocus,[23,42–45] *ZENK*, an immediate early gene, has been shown to undergo optically-modulated expression changes in retina. For example, the number of *ZENK*-expressing glucagon amacrine cells was found to be increased with positive lenses, after as little as 30 minutes of wear, and decreased after 2 hours of negative lens wear in chicks. It remains to be determined whether or not these cells are part of a signal pathway mediating the observed changes in BMP2 expression in the RPE. It is possible that BMP2 expression is regulated by an independent, yet-to-be identified retinal cell population.

It also is noteworthy that retinoic acid (RA), which has been put forward as a potential eye growth regulator, also has been linked to the regulation of BMP2 expression in other studies.[46–49] The data tying RA with eye growth regulation in chicks also exhibit bidirectionality; retinal RA levels are increased in eyes wearing negative lenses and diffusers and levels are decreased in eyes wearing positive lenses, with the opposite trends being true for choroidal RA levels (Mertz JR, et

al. *IOVS* 1999;40:ARVO Abstract 4473).[43–45] While RA could be acting upstream from BMP2 on the retinal and/or choroidal side of RPE (these possibilities are not distinguishable based on currently available data), a signal pathway linked to eye growth regulation would argue for an upstream retina-RA, RPE-BMP2 association. Nonetheless, while retina, choroid, and sclera all are plausible sites of action of BMP2 based on our immunohistochemistry results, Western blots detected the mature BMP2 (as a dimer) only in the retina. Local tissue-specific manipulations of BMP2 levels may be required to dissect this apparently very complex signaling cascade.[41]

In our study, we found no evidence of defocus-dependent differential regulation of BMP receptor expression, although we did observe yoked down-regulation of *BMPR2* in response to the negative lens treatment. We have observed similar interocular yoking of gene expression changes in the RPE from birds subjected to monocular lens treatments, for the somatostatin receptor 2, while not for the somatostatin ligand (Hammond D, et al. *IOVS* 2012;53:E-Abstract 3429). At this time, we do not have an explanation for these observations, but suffice to say that these data also point to a very complicated signaling cascade downstream from BMP2.

Interestingly, BMP2 gene expression was reported to be down-regulated in chick retina/RPE with form-deprivation myopia,[33] in the same direction as that induced by our negative lens treatment, which also induces myopia. While there is accumulating evidence that the mechanisms underlying these two types of myopia are different,[50,51] our results add to other data suggesting that some components of the regulatory pathways are shared.[23,52] As the effects on retinal image quality of these treatments generally are quite different and, thus, likely to elicit different retinal responses, we speculate that the RPE was the site of BMP2 gene expression changes in the previous form deprivation study, with the RPE serving as a conduit or point of convergence of different retinal signal pathways.

In summary, we demonstrated the expression of genes for BMP2 and its receptors, as well as of the BMP2 protein in the posterior tissues of adolescent chick eye, and found BMP2 gene expression to be bidirectionally regulated by optical defocus, according to its sign. Our findings open up a new avenue for investigation into the regulation of eye growth during emmetropization. The possibility that BMP2 could be used in therapeutic intervention for myopia also may be worthy of consideration.

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