

Form-deprivation myopia induces decreased expression of bone morphogenetic protein-2, 5 in guinea pig sclera

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Received: 2014-08-26 Accepted: 2014-11-13

Abstract

- **AIM:** To identify the presence of various bone morphogenetic proteins (BMPs) and their receptors in normal sclera of human, rat and guinea pigs, and to determine whether their expression changed with form-deprivation myopia (FDM) in guinea pig sclera.
- **METHODS:** The expression of BMPs and BMP receptors were detected using reverse transcription polymerase chain reaction (RT-PCR) and immunofluorescence. Two-week-old guinea pigs were monocularly form-deprived with a translucent lens. After fourteen days induction of FDM, total RNA was isolated and subjected to RT-PCR to examine the changes of BMPs and BMP receptors in tissues from the posterior sclera. Western blotting analysis was used to investigate their changes in protein levels.
- **RESULTS:** Human sclera expressed mRNAs for BMP-2, -4, -5, -7, -RIA, -RIB and BMP-RII. Conversely, rat sclera only expressed mRNA for BMP-7 and BMP-RIB, while the expression of BMPs and BMP receptors in guinea pigs were similar to that of humans. Human sclera also expresses BMP-2, -4, -5, -7 in protein level. Fourteen days after the induction of myopia, significant decreased expressions for BMP-2 and BMP-5 in the posterior sclera of FDM-affected eyes ($P < 0.05$ vs internal control eyes).
- **CONCLUSION:** Various BMPs were expressed in human and guinea pig sclera. In the posterior sclera, expressions of BMP-2 and BMP-5 significantly

decreased in FDM eyes. This finding indicates that various BMPs as components of the scleral cytokines regulating tissue homeostasis and provide evidence that alterations in the expression of BMP-2 and BMP-5 are associated with sclera remodeling during myopia induction.

• **KEYWORDS:** form-deprivation myopia; bone morphogenetic protein; human sclera; guinea pig sclera

DOI:10.3980/j.issn.2222-3959.2015.01.07

Wang Q, Xue ML, Zhao GQ, Liu MG, Ma YN, Ma Y. Form-deprivation myopia induces decreased expression of bone morphogenetic protein-2, 5 in guinea pig sclera. *Int J Ophthalmol* 2015;8(1):39-45

INTRODUCTION

Myopia has emerged as a major health issue in east Asia because of its high prevalence and high sight-threatening pathologies associated with high myopia^[1]. In South east Asia high myopia is closer to 20% of the general population^[2]. Strong evidence from clinical and experimental studies indicates that the sclera plays an important role in influencing the size of the eye and the refractive state of the eye^[2,3].

Postnatal scleral growth, like other connective tissues, is under the control of growth hormone or its downstream effectors^[4]. Transforming growth factor beta (TGF- β) had been shown to play a major role in sclera remodeling as well as biomechanical changes^[2,5,6]. Bone morphogenetic proteins (BMPs), which belong to the TGF- β super family had been found in a number of ocular tissues and play an important role in eye development and differentiation of ocular tissues^[7]. Despite the involvement of BMPs in eye development and adult eye tissues, to our knowledge, the homeostasis of BMPs in the sclera has not been fully documented, the relationship between BMPs and axial elongation in myopia remains obscure.

It is well known that form-deprivation myopia (FDM) guinea pigs are considered as a suitable animal model to be used for the study of human myopia^[8]. In this paper we examined

human, rat and guinea pigs sclera for BMP-2 to BMP-7 and reported their changes during myopia induction in guinea pigs sclera.

SUBJECTS AND METHODS

Ethics Statement All experimental procedures were conducted in conformity with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No.85-23, revised 1985). The study protocol was approved by the Review Committee for the Use of Human or Animal Subjects of Medical College of Qingdao University.

Animals Twenty pigmented guinea pigs (approximately 15d old) were reared on a cycle of 12h illumination and 12h darkness daily during the experimental period. Water supplemented with vitamin C and food (guinea pig pellets and fresh vegetables) were freely available. The room temperature was 22°C.

Experimental Design and Myopia Induction The animals were randomly assigned to either of 2 groups: diffusers ($n=10$) and normal controls ($n=10$). The diffusers (white translucent hemispheric occluder with a diameter of 12 mm) were mounted on a matching plastic ring and glued to the periorbital fur of the eye for the whole two week period of this experiment. In diffusers group, the fellow eye was untreated as a matched control.

Biometric Measurements Biometric measurements included streak retinoscopy and ultrasonography. Retinoscopy was performed in a dark room using a streak retinoscope and trial lenses approximately 15min after the eyes had been fully cyclopleged with 2 drops of tropicamide. The refraction was recorded as the mean value of the horizontal and vertical meridians. Axial length of the eye was measured by an A-scan ultrasonograph with 11 MHz. The measurements were performed under topical anaesthesia by 0.4% Oxybuprocaine Hydrochloride (Alcon, Belgium). Ocular refraction and axial length were collected at the start and end of the experiment.

Tissue Preparation Human sclera tissues were excised from healthy adult human donor eyes ($n=3$; age of 18 and 19 years old) obtained from the Eye Bank of the Affiliated Hospital of Qingdao Medical College, Qingdao University, with the approval of Ethics Committee of Qingdao University (Qingdao, China) and complied with the tenets of the Declaration of Helsinki for biomedical research involving human subjects. Human sclera was embedded with optimum cutting temperature compound (Sigma, St. Louis, MO, USA), and cut into 8 μm sections at -20°C. Sections were subsequently tiled onto slides (Corning Ltd, Tokyo, Japan), fixed with cool acetone for 15min, air-dried, and kept frozen at -20°C until use. Rat and guinea pigs were killed by overdoses of Chloral Hydrate. The eyes were removed and cut into halves about 1 mm posterior to the ora serrata on the ice plate. The anterior segment of the eye was discarded. The

posterior sclera was excised by using a 6 mm-diameter trephine around the head of the optic nerve. The head of the optic nerve was discarded. The sclera were stored at -80°C.

Immunofluorescence The slides were washed three times with phosphate buffered saline (PBS), nonspecific binding was blocked by incubation in 10% (v/v) goat serum in PBS for 30min at room temperature. The slides were incubated at 4°C overnight with primary antibodies BMP-2 to BMP-7 (Polyclonal rabbit antibodies, Boster Biological Technology) diluted in PBS. The antibody-treated and negative control slides were washed with PBS and exposed to fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit IgG antibodies (Boster Biological Technology) diluted in PBS and incubated at room temperature for 1h. Fluorescent signals were detected using a fluorescent microscope (TS-100, Nikon).

Nuclear Acid Extraction, Reverse Transcription and Polymerase Chain Reaction Sclera was grinded in liquid nitrogen. Total RNA were extracted from sclera [normal human sclera $n=3$; normal rat sclera $n=3$; normal guinea pigs sclera $n=3$; form deprived guinea pig sclera $n=5$; contralateral control sclera $n=5$ with a kit (NcneoSpin RNA II; Macherey-nagel; Germany)] according to the manufacturer's instructions. The total RNA was quantified by measuring OD₂₆₀ and OD₂₈₀ (optical density 260/280 higher than 1.9). cDNA was synthesized with 5 μg total RNA, 2 μL random 6 mers, 0.5 μL Oligo dT Primer, 2 μL PrimeScript™ Buffer, and 0.5 μL PrimeScript™ RT Enzyme Mix I (TaKaRa, Dalian, China) at 37°C for 15min. The reaction was suspended at 85°C for 5s. Conditions for PCR were 95°C for 30s, 35 cycles of 95°C for 5s, 55°C (56°C for BMP-3, BMPR-1B respectively) for 30s and 72°C for 30s. The final extension step was at 72°C for 7min. The PCR products were then electrophoresed on a 2% agarose gel containing 10 \times Gelred reagents in parallel with 50 bp DNA markers. The nucleotide sequences of the primers used in the experiments are summarized in Table 1. β -actin was used as an internal control. Images were captured and relative band intensities were quantitated and compared using the AlphaImager-3400 Computerized Densitometry System (Biozym, Oldendorf, Germany). Because guinea pigs nucleotide sequences haven't been reported, we designed probes to cover regions where the human and rat BMP sequences show high homology. It is therefore reasonable to expect that our primers specifically recognize the examined BMPs. To prove the identity, all the experiments were performed at least 3 times and the products were sequenced in TaKaRa, Dalian, China.

Western Blotting Analysis Tissues were homogenized in ice-cold extraction buffer (0.01 mol Tris-HCl at pH 7.4, 0.15 mol NaCl, 1% w/v Triton X-100, 0.1% SDS, 1% deoxycholic acid, 1 mmol EDTA) as well as protease inhibitors (1 μmol pepstatin, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 0.2 mmol

Table 1 Sequences, annealing temperatures (Tan.) and predicted product sizes of the primers used

Gene NCBI accession number (rat and human)	Upstream primer	Downstream primer	Tm (°C)	Size (bp)
BMP-3 NM_017105.1 NM_001201.2	TTGGCTGGAGCGAATGGATTA	GCTCAGGAATCCCAGAGACGAC	56	159
BMP-4 NM_017105.1 NM_001201.2	TTTGTTC AAGATTGGCTCCCAAG	AAACGACCATCAGCATTGGTGA	55	101
BMP-5 NM_012827.2 BC020546.2	TCCACAGAACAATTGGGGCTTACA	ACCATGAACGGGCTGCTTTGAC	55	120
BMP-6 NM_013107.1 NM_001718.4	CGCCTTCCTCAACGACCGCGG	GGAATCTGGGATAAGTTGAA	55	120
BMP-7 NM_001191856.1 NM_001719.2	TCCGGTTTGATCTTCCAAGA	CCCGGATGTAGTCCTTATAGATCCT	55	81
BMPR-IA S75359.1 NM_004329.2	GCCGTTTTGAAGCTGATGTCA	TCTTTGCGAGCGTCTTCTTGA	55	88
BMPR-IB NM_001024259.1 D89675.1	TGGCTGACATGTACAGCTTTGG	GGCACTCGTCACTGCTCCAT	56	198
BMPR-II NM_080407.1 NM_001204.6	ACTGCAGATGGACGCATGG	AATCTCGATGGGAAATTGCAG	55	199
β -actin NM_031144.2 NM_001101.3	GGCACCACACTTTCTACAATG	GGGGTGTGAAGGTCTCAAAC	55	133

PMSF). After homogenization, samples were placed on ice for 30 min and centrifuged at $12\,000 \times g$ for 15min. The supernatant was decanted and the precipitate was discarded. Protein concentrations were determined according to the BCA method by using an Enhanced BCA Protein Assay Kit (Boster, Wuhan, China). Each protein sample (30 μ g) was mixed with $5 \times$ sample buffer for SDS polyacrylamide gel electrophoresis. The mixture was boiled for 3min, electrophoresed on 10% SDS polyacrylamide gel, and transferred to nitrocellulose membranes (Pall Corporation, East Hills, NY, USA). Protein loading and transfer efficiency were monitored by staining the membranes with 1% Ponceau S. The membranes were washed three times with Tris Buffered Saline Tween (TBST pH 7.6) and soaked in a blocking solution (100 mL, Beyotime Institute of Biotechnology, Haimen, china. 5% w/v skim milk powder in 2.5 mmol Tris-HCl and 14 mmol NaCl plus 0.05% Tween-20) for 1h at room temperature. Then overnight incubation with primary antibodies to the BMP-2, BMP-4 and BMP-5 (Polyclonal goat antibodies) at a 1:100 dilution (0.2 mL, Boster Biological Technology, Wuhan, China) at 4°C in blocking solution was performed. The following day, the membranes were washed three times with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody at a 1:1000 dilution (0.4 μ g/mL; Boster, Wuhan, China) for another 1h at room temperature. After three times of washing with TBST, the reaction products were visualized with BeyoECL Plus western blotting

detection reagents (eyotime Institute of Biotechnology, Haimen, China). Images were captured with Fuji Film LAS3000 imaging system and analyzed with MultiGauge software (Fuji Film, Tokyo, Japan). β -actin (Kang Chen, China) was used as a housekeeping protein to normalize the protein load.

Statistical Analysis Ocular refraction and biometric measures data were expressed as means \pm SD for each group. Differences between both eyes of each animal and between the groups were statistically compared using paired sample t -test and one-way ANOVA with Bonferroni Correction. Statistical analysis was performed using the SPSS 15.0 statistical software. The results were considered statistically significant at $P < 0.05$.

RESULTS

Expression mRNA of BMPs and BMPs' Receptors in Human, Guinea Pig and Rat Sclera Distinct bands specific for BMP-2, BMP-4, BMP-5, BMP-7, BMPR-IA, BMPR-IB and BMPR-II were observed after reverse transcription PCR using total RNA extracted from normal human sclera (Figure 1). mRNA coding for BMP-5 was found to have the strongest expression, followed by BMP-2, BMP-4 and BMP-7, whereas expression of BMP-3 and BMP-6 was not observed after 35 PCR cycles ($n=3$). Expression levels of mRNA coding for BMPR-IA and BMPR-IB were higher than those of BMPR-II. However, only a distinct band specific for BMP-7 was found in normal rat sclera (Figure 1). The expressions of BMPs and BMPRs

Table 2 Refractive error and axial length between the eyes of the two groups

Parameters	Refraction		<i>P</i>	Axial length		<i>P</i>
	R	L		R	L	
NOR						
0wk	3.25±0.63	3.25±0.63	0.654	7.91±0.04	7.89±0.05	0.404
2wk	2.27±0.70	3.02±0.54	0.386	8.06±0.06	8.05±0.06	0.595
FDM						
0wk	3.22±0.74	3.42±0.60	0.516	7.92±0.04	7.91±0.05	0.531
2wk	-0.48±0.51	2.72±0.61	0.000	8.30±0.05	8.11±0.06	0.000

in guinea pig sclera were similar to those of human. Immunofluorescence further confirmed the expression of BMPs in guinea pig sclera (Figure 2).

Refractive Error and Axial Length Prior to the diffuser wear, there was no significant difference between the two eyes of the animals in refraction and axial length within each group ($P>0.05$, paired sample *t*-test). The differences between the eyes of each animal in axial length were also not significant between the 2 groups at the beginning of the experiment ($P>0.05$, one-way ANOVA with Bonferroni correction).

At 14d, monocularly deprived eyes had myopia of -0.48 ± 0.51 D and an axial length of 8.30 ± 0.05 mm ($P<0.001$ versus normal and internal control eyes, ANOVA and Tukey post-hoc test; Figure 3). Refractive error and axial length between the eyes of the two groups were presented in Table 2.

Changes of the BMPs and BMPRs mRNA Expression in Guinea Pig Sclera Normal guinea pig sclera express mRNA for BMP-2, BMP-4 and BMP-5. The BMPs and BMPRs mRNA expression normalized to the expression of β -actin showed that mRNA expression of FDM eyes was significantly lower than that of internal control eyes for BMP-2 ($P=0.017$ or $P<0.05$), and BMP-5 ($P=0.028$ or $P<0.05$). However, mRNA expression for the BMP-4 was not significantly altered in FDM eyes ($P=0.46$; Figure 4).

Changes of BMP-2 and BMP-5 Protein Expression in Each Group Immunoblotting for BMP-2, BMP-4 and BMP-5 showed a band at about 30 kDa. Figure 5 showed the effect of myopia induction on the protein expression for BMP-2, BMP-4 and BMP-5. After 14d of visual deprivation, significant decrease were found for BMP-2 (-21.25%, $P=0.030$) and BMP-5 (-17.11%, $P=0.031$) compared with the internal control eyes of the same animals, while the expression for BMP-4 was not significantly changed ($P=0.365$; Figure 5).

There was no significant difference between the two eyes of the animals in refraction and axial length within each group ($P>0.05$, paired sample *t*-test). The differences between the eyes of each animal in axial length were also not significant between the 2 groups at the beginning of the experiment ($P>0.05$, one-way ANOVA with Bonferroni correction). At

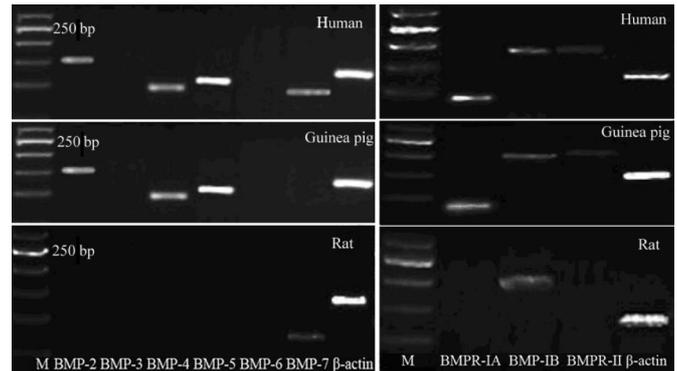


Figure 1 Semi-quantitative RT-PCR analysis of total RNA from 3 normal human sclera, 3 normal guinea pig sclera and 3 normal rat sclera using specific primers for BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMPR I A, BMPR I B, BMPR II normalized to β -actin at 35 cycles (M: Molecular size marker).

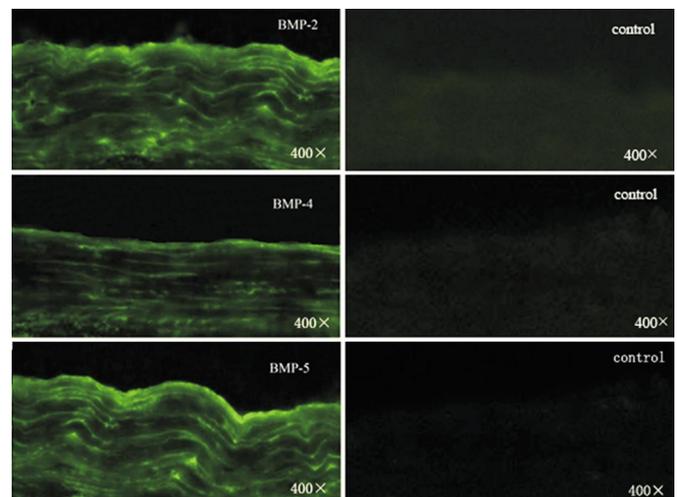


Figure 2 Distribution of BMPs in guinea pig sclera by indirect immunofluorescence FITC marked the secondary antibody.

14d, monocularly deprived eyes had myopia of -0.48 ± 0.51 D and an axial length of 8.30 ± 0.05 mm ($P<0.001$ versus normal and internal control eyes).

DISCUSSION

BMPs are a large group of multifunctional growth factors which play a crucial role during embryonic development and morphogenesis, and equally essential in a variety of biological processes during the postnatal life, such as cell

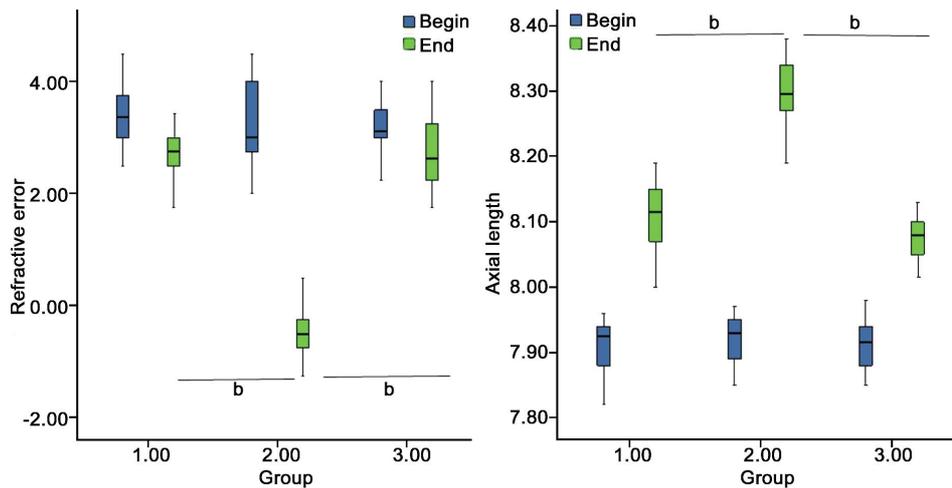


Figure 3 Differences in refractive error and axial length after induction of myopia Differences in refractive error (A) and axial length (B) among FDM (group2), internal control (group1) and normal (group 3) eyes of guinea pigs is illustrated. At 14d, monocularly deprivation produced relative myopia, and axial lengths of FDM eyes were significantly greater than the others. ^b $P < 0.001$. Data are the mean \pm SD.

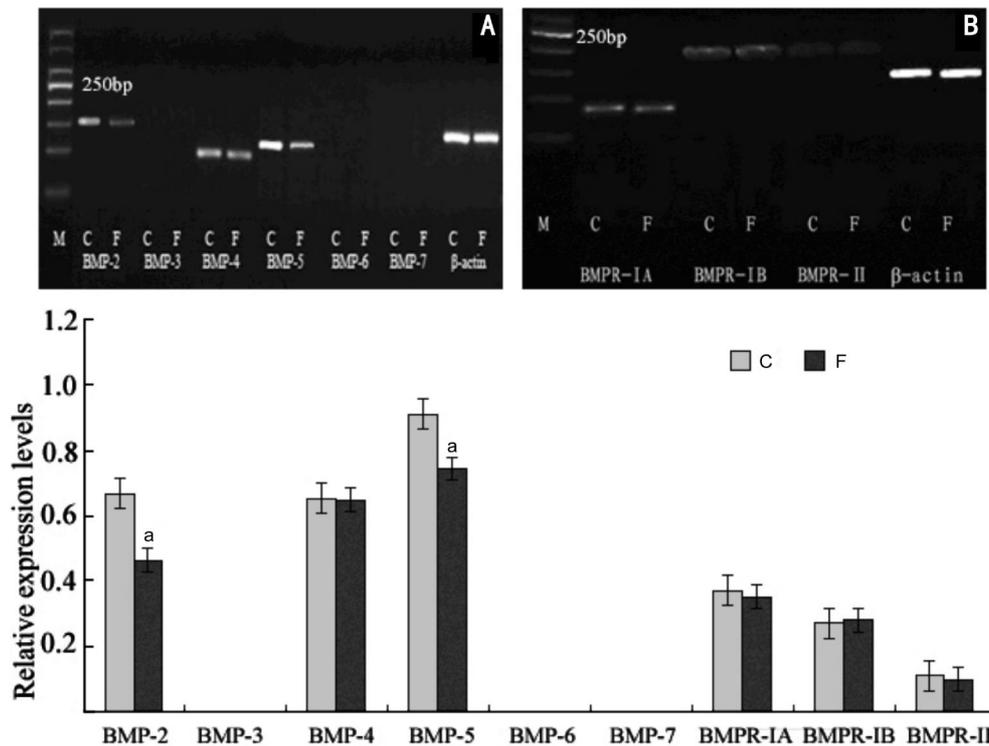


Figure 4 Effect of myopia induction on the mRNA expression for BMP-2 to BMP-7 and BMPRs in guinea pigs sclera 2wk after visual deprivation, semi-quantitative RT-PCR showed a significant decrease in mRNA expression in BMP-2 (-26.48%, $P = 0.017$) and BMP-5 (-18.67%, $P = 0.028$) but not for BMP-4 compared with the internal control eyes (C) in the same animals. Bar graph shows changes in mRNA expression of BMP subtypes 2 to 7 and BMPRs in the posterior sclera during FDM (F) in guinea pigs. Values (mean \pm SD) were normalized for β -actin and expressed as ratios of optical density. ^a $P < 0.05$.

proliferation, differentiation, apoptosis, angiogenesis, and secretion of extracellular matrix components^[10,11]. BMPs exert their biologic effects through binding to the cell-surface serine-threonine kinase receptors BMP-RI and BMP-RII, activation of which leads to phosphorylation of intracellular signaling molecules including Smad1, Smad5, and Smad8^[11,12]. The present study showed that human sclera expressed mRNA for BMP-2, BMP-4, BMP-5, and BMP-7, whereas BMP-3 and BMP-6 were not found. We only found BMP-7

in the rat sclera, which similar to Thomadakis *et al*'s^[13] results. The expression of BMPs mRNA in guinea pig sclera was similar to that of human, which was BMP-2, BMP-4 and BMP-5, but without BMP-7. The additional expression of BMP receptors (BMPR-IA, BMPR-IB, BMPR-II) in human sclera indicates that BMPs in fact play a role in normal sclera homeostasis.

The activation of sclera fibroblasts, predominantly mediated by the transgenic growth factor TGF- β , has been generally

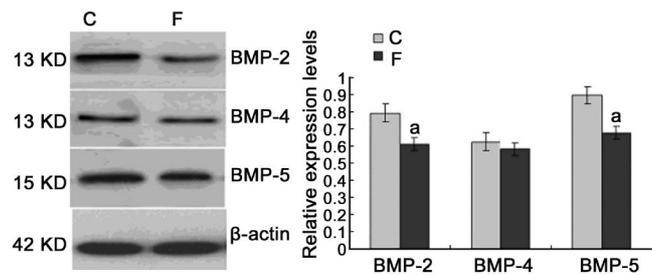


Figure 5 Effect of myopia induction on the protein expression of BMP -2, -4, -5. Western blotting showed a significant decrease in protein expression for BMP-2 and BMP-5 but not BMP-4, compared with the internal control (C) eyes. Values (mean±SD) were normalized for β-actin and expressed as ratios of optical density. ^a $P < 0.05$.

regarded to be responsible for sclera remodel during myopia induction [6,14,15]. In this study, we provided evidence for a significant down regulation of BMP-2 and BMP-5, suggesting an additional role for these members of the TGF-β superfamily in the scleral remodeling.

BMP-2 was found to promote sclera fibroblast proliferation *in vitro* [16,17]. It is also expressed in human cornea and is considered a heparin-binding cytokine that can modulate corneal fibroblast apoptosis [18]. A gene microarray analysis was used to identify gene expression changes in human sclera fibroblasts in response to mechanical loads and possible mechanisms of sclera remodeling in the development of myopia and showed that mechanical stresses induced BMP-2 mRNA expression after 30min and 24h [19]. McGlenn *et al* [20] reported that chicks following 6h and 3d of diffuser-wear, BMP-2 mRNA levels were significantly down-regulated in the retina and RPE. Another research showed that variations in the BMP2K gene were strongly correlated with high myopia in the Taiwanese population [21]. Most recently, BMP-2 in chick RPE showed bidirectional defocus sign-dependent changes which further suggested an important role for BMP-2 in eye growth regulation [22]. In our study, we detected BMP-2 in guinea pigs sclera, and our data showed that BMP-2 was decreased in posterior sclera of myopia eyes, which further illustrated this kind of growth factor was involved in the growth of human sclera. Further research on BMP-2 and its effects on the alteration of scleral proteoglycan and collagen synthesis will contribute to the understanding of its role on the remodeling of the sclera.

BMP-2, BMP-4 and BMP-5 were also found in human trabecular meshwork (TM), optic nerve head and may be associated with glaucoma pathogenesis [23]. Wordinger *et al* [24] found that BMP-4 could blocked the TGF-β2 induction of fibronectin in TM cells, the result suggests that BMPs can modulate the effects of TGF-β2 in TM cells. Further study found that in primary open angle glaucoma, elevated BMP antagonist expression by TM cells inhibits BMP-4

antagonism of TGF-β2 and this inhibition leads to increased ECM deposition and elevated IOP [25]. BMP-4 also enhanced the secretion of vascular endothelial growth factor (VEGF) by human RPE cells and may be involved in the regulation of ocular angiogenesis associated with diabetic retinopathy [26]. Mutations in BMP-4 cause ocular malformations including anophthalmia-microphthalmia (AM) and may be a candidate gene for myopia [27]. However, in our research, we did not find significant changes in BMP-4 expression in sclera during myopia induction. Further research may focus on the relationships of BMP-4 and its antagonist on the effect of TGF-β in sclera ECM remodeling.

In the present study, we found a very high expression of BMP-5 in normal guinea pigs sclera and there was a significantly decreased expression of BMP-5 in FDM. Other than TM and optic nerve head, BMP-5 is also expressed in adult cornea [28]. BMP-5 and its function in ocular tissues were not clarified yet. The function of BMP-5 in other tissues has been wildly investigated which indicate BMP-5 is an important growth factor in maintain normal tissue homeostasis. BMP-5 expression increases during chondrocyte differentiation *in vivo* and *in vitro* and could promotes proliferation and cartilage matrix synthesis through Smad1/5/8 and p38 MAP kinase pathway [29]. In synovial tissue of patients with osteoarthritis and rheumatoid arthritis, the expression of BMP-5 decreased [30]. BMP-5 is also expressed by rat granulosa cells (GCs) and affects on the cell's proliferation and steroidogenesis in an autocrine manner [31]. Most recently, it was reported that BMP-5 modulate adrenal cell proliferation and steroidogenesis and was down-regulated in adrenocortical carcinoma [32]. Our results of decreased expression of BMP-5 in FDM guinea pigs sclera indicate that BMP-5 was involved in sclera remodeling during myopia induction.

In conclusion, human sclera express various BMPs and BMPRs. BMP-2, BMP-4 and BMP-5 are also expressed in normal guinea pigs sclera and were found to be difference expressed in posterior sclera of guinea pig during myopia induction. These results suggest that BMPs may be an important role in sclera homeostasis and are therefore potential candidates for myopia control.

ACKNOWLEDGEMENTS

The authors thank Xiang-Tian Zhou for his technical assistance in animal model and De-Quan Li for his helpful suggestions pertaining to this manuscript.

Foundations: Supported by The National Youth Science Fund Project (No. 81300790), The Development Project of Shandong Province Medical Science and Technology (No. 2013WS0262) and The Youth Innovation Team Project of Medical College Qingdao University (No. 600201304).

Conflicts of Interest: Wang Q, None; Xue ML, None; Zhao GQ, None; Liu MG, None; Ma YN, None; Ma Y, None.

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