·Basic Research·

Effects of 530 nm monochromatic light on basic fibroblast growth factor and transforming growth factor-β1 expression in Müller cells

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Abstract

• AIM: To expose rat retinal Müller cells to 530 nm monochromatic light and investigate the influence of varying light illumination times on basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1) expression.

• METHODS: Three groups of rat retinal Müller cells cultured *in vitro* under a 530 nm monochromatic light were divided into 6, 12 and 24h experimental groups, while cells incubated under dark conditions served as the control group. The bFGF and TGF-β1 mRNA expression, protein levels and fluorescence intensity of the Müller cells were analyzed.

• RESULTS: The bFGF mRNA expression and protein levels were significantly upregulated in Müller cells in all three experimental groups compared with the control group (P<0.05), while that of TGF-β1 was downregulated (P<0.05). Also, bFGF expression was positively correlated, but TGF-β1 expression was negatively correlated with illumination time. The largest changes for both cytokines were seen in the 24h group. The changes in bFGF and TGF-β1 fluorescence intensity were highest in the 24h group, and significant differences were observed among the experimental groups (P<0.05).

• CONCLUSION: The expressions of bFGF and TGF-β1 changed in a time-dependent manner in Müller cells exposed to 530 nm monochromatic light with 250 lx illumination intensity. Müller cells might play a role in the development of myopia by increasing bFGF expression or decreasing TGF-β1 expression. Changes in cytokine expression in retinal Müller cells may affect monochromatic light-induced myopia.

• KEYWORDS: monochromatic light; myopia; Müller cells; basic fibroblast growth factor; transforming growth factor-β1

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INTRODUCTION

Myopia has a high prevalence and its mechanism has been investigated [1]. The development of myopia is complicated and involves various factors. It is widely accepted that myopia development is promoted by environmental and genetic factors[2,3]. However, the details of these mechanisms remain to be elucidated. Form-deprivation myopia and myopia induced by optical defocusing are the two major myopia models, but myopia induced by monochromatic light exposure has recently attracted increasing attention. In recent years, studies found that exposure to different wavelengths of monochromatic lights could damage an animal's refractive development. Long wavelength monochromatic lights (*e.g.* 530 nm, 610 nm, 772 nm) can induce myopia, while short wavelength monochromatic lights (*e.g.* 430 nm, 455 nm) can induce hyperopia [3-6]. In fact, 530 nm monochromatic light could change an animal's refractive status and result in myopia[4,5]. Studies using different animal models demonstrated that abnormal visual signals could lead to scleral remodeling, vitreous cavity expansion, and a lengthened ocular axis *via* the scleral and choroidal pathways, resulting in changes to eyeball morphology and the development of myopia[5,7,8].

Müller cells, the major glial cells in the retina, are widely distributed among various cell bodies and fibers in the retina and essential for the formation of a neuronglia regulatory network among the three layers of retinal nerve cells. Müller cells are vital to normal retinal physiological function and involved in the pathological processes of related diseases[9,10]. Müller cells produce and secrete various neural active substances or cytokines [*e.g.* basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1)], and cytokine expression changes under monochromatic light illumination [11]. In an abnormal visual environment, changes in cytokine (*e.g.* TGF-β and bFGF) expression in the retina

can act on the sclera as primary signals for inducing scleral remodeling and myopia [12]. We speculated whether monochromatic light could induce cytokine synthesis and degradation in retinal Müller cell. The cytokine expression changes in these cell affect the formation of myopia *via* regulating the scleral rebuilding processes. To date, several studies have investigated how monochromatic light affects refractive development and eye growth [4,5], but whether Müller cells and the cytokines expression changes within them play a role in the formation of myopia remains to be elucidated. What effect does it have on the development of myopia? Thus, to provide a fundamental basis for developing myopia therapies, we established a rat retinal Müller cells model treated with monochromatic light *in vitro*.

In our previous study, exposure to a 530 nm monochromatic light with >250 lx illumination intensities inhibited rat retinal Müller cell proliferation *in vitro* and downregulated bFGF and TGF-β1 expression [11]. However, the impact of various illumination times using a 530 nm monochromatic light with identical illumination intensity on cytokine expression in Müller cells remains unknown. Thus, here we explored the impact of 250 lx illumination intensities and various illumination times on bFGF and TGF-β1 expression in Müller cells.

MATERIALS AND METHODS

Establishment of Cell Models Treated with Monochromatic Light

Establishment of cell culture incubator illuminated by a 530 nm monochromatic light The monochromatic light had an intensity of 250 lx (digital light meter, TES Electrical Electronic Corp) and peak wave of the light-emitting diode (LED) light (Gotek Optionics Technology Co., Ltd., Suzhou, Jiangsu Province, China) was 530 nm with 1W of power and a 20 nm wavelength width. The incubator box was composed of an opaque black Bakelite plate and was divided into two parts using a black Bakelite plate to create two independent culture spaces. A LED top light was added to each part to maintain the same illumination intensity for cells at the same horizontal level. The LED top light was controlled by a double circuit adjustable direct current (DC) stabilized power supply (Atten Technology Co., Ltd., Shenzhen, Guangdong Province, China) with high precision. The illumination intensity, time, and photoperiod could be modulated. Vents were located in the box front and back and a black-out cloth was used to cover the box to block the interference of other light (Figure 1). Before the start of the experiment, the monochromatic light cell incubator was placed into a normal cell incubator (temperature and CO_2 concentration were kept constant). We then compared the temperature and CO_2 concentration in the monochromatic light cell incubator with the normal cell incubator. But found no differences. The temperature and CO_2 concentration in the incubator remained constant. We have filed a patent application for the homemade cell culture incubator with the state intellectual property office of the People's Republic of China (Application No. ZL201420137671.8).

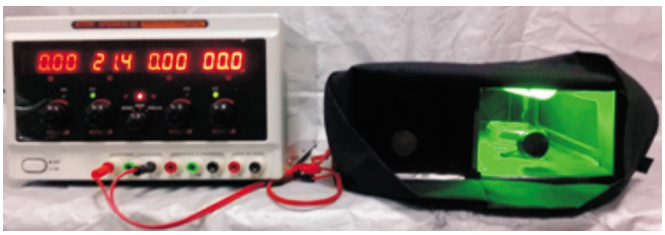

Figure 1 A homemade monochromatic light cell incubator The homemade cell culture incubator was used for culturing rat Müller cells *in vitro* and treating them with 530 nm monochromatic light. The cell incubator was placed into a normal cell incubator.

Culture of rat retinal Müller cells The SD rat retinal Müller cell line (rMc-1) was kindly donated by Dr Jian-Xin Ma from the University of Oklahoma Health Sciences Center. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. The Müller cells were cultured using Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) (Wisent Biotechnology Co., Ltd., Nanjing, China). Cells growing at the logarithmic phase were seeded into cell culture flasks at the final density of 1×10^6/mL. These cells were used for real-time polymerase chain reaction (PCR) and Western blotting or seeded into 12-well culture plates (Corning, USA) at the same density as for preparation of the slides that were used for confocal laser scanning microscope imaging. The cells were then cultured in a 50 mL/L CO_2 cell culture incubator at 37℃ overnight; after 70% cell adherence was achieved, the control group of cells was incubated in the homemade 530 nm monochromatic light cell culture incubator for 48h in the dark. Before being illuminated, the three experimental groups of cells were cultured 42, 36, 24h in the dark.

Experimental grouping Rat retinal Müller cells cultured *in vitro* under various illumination times using a 530 nm monochromatic light with identical illumination intensity were divided into the experimental 6, 12, and 24h groups and the 48h control group.

Real-time PCR to Analyze the Expression of bFGF and TGF-β1 mRNA in Müller Cells Primers were designed using Primer Express 3.0 software (Applied Biosystems, USA) according to the gene sequence from NCBI GeneBank (Table 1) and glyceraldehyde-3-phosphate dehydrogenase was chosen as an internal reference. Real-time PCR was performed using a 7500HT Fast Real-time PCR machine (Applied Biosystems) and the protocol used was as follows: incubation at 50℃ for 2min, and denaturation at 95℃ for 10min was followed by amplification for 40 cycles (denaturation at 95℃ for 15s, annealing and extension at

Table 1 Primer Sequence

Gene		Primer Sequence (5'-3')
bFGF	Sense	GCTTTGGTTGTGACCCAGTGAA
	Antisense	GGCTGAAATGGGAACAGATTGA
TGF-β1	Sense	CCAAGGAGACGGAATACAGGG
	Antisense	CCTCGACGTTTGGGACTGATC
GAPDH	Sense	CACGGCAAGTTCAACGGCACAGTC
	Antisense	CCATCACGCCACAGCTTTCCAGAG

bFGF: Basic fibroblast growth factor; TGF-β1: Transforming growth factor-β1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

53℃ for 1min). The amplification product specificity was determined using a dissociation program as follows: denaturation at 95℃ for 15s, annealing at 53℃ for 15s and then reaction at 95℃ for 15s. In this process, 1 μL of cDNA template was added, and then PCR amplification and fluorescence intensity were performed using the same program. A kinetic curve was created automatically and the Ct value was analyzed. All experiments were repeated three times. Data was analyzed using the $2^{-\Delta\Delta\alpha}$ method; ΔCt = Ct of target genes -Ct of internal reference and ΔΔCt = ΔCt of target genes -ΔCt of internal reference.

Protein Levels of bFGF and TGF-β1 in Müller Cells Analyzed by Western Blotting Lysis buffers (200 μL) were added to each group and incubated at 4℃ overnight. Ultrasonic treatment was then performed at 4℃ for 1min and supernatants were discarded. Samples were centrifuged at 12 000 r/min for 10min. The protein concentration of each group was determined using the Bradford method and adjusted to the same concentration for Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed and the gel was transformed to polyvinylidene fluoride membranes. After membrane blocking, the membrane was incubated with rabbit polyclonal anti-bFGF primary antibody (1:2000, Abcam, UK, ab72316), mouse monoclonal anti-TGF-β1 primary antibody (1 μg/mL, Abcam, UK, ab64715) and tubulin (an internal reference protein) primary antibody (0.5 μg/mL) at 4℃ overnight. The membranes were then washed three times for about 15min each with Tris buffered saline with Tween-20. The membrane was incubated with horseradish peroxidase labeled goat anti-rabbit/mouse secondary antibody (1:1000, Thermo, USA) at room temperature for 2h. After the three times wash, the signals were detected using the performing method. All experiments were repeated three times. Data were analyzed using Image J 1.42q software. The optical density A value = target protein A value-internal reference protein A value.

Signal Fluorescence Intensities of bFGF and TGF-β1 in Müller Cells Assessed Using a Confocal Laser Scanning Microscope The cell slides were fixed with 4% paraformaldehyde for 15min and then washed with phosphate buffered saline (PBS) for 2-3 times. The cell membranes were ruptured using Triton treatment for 15min and slides were washed with PBS for 2-3 times. The cells were then blocked with 1% bovine serum albumin (BSA) for 1.5h. Rabbit polyclonal anti-bFGF primary antibody (1:200, Abcam, UK, ab72316) and mouse monoclonal anti-TGF-β1 primary antibody (1:125, ab64715, Abcam, UK) were used and incubated at 4℃ overnight. The cell slides were incubated with mouse anti-rabbit and sheep anti-mouse secondary antibodies (1:1000, Thermo, USA) for 1h at 37℃ in the dark. After washing with PBS for 2-3 times, DAPI (1:100) was added and incubated for 15min. Slides were mounted using anti-quenching mounting medium (Beyotime Institute of Biotechnology, Jiangsu Province, China). In the negative control slides, the primary antibody was replaced with PBS. The slides were then observed using a confocal laser scanning microscope (TS-SP5, Leica, Germany).

The exciting light wavelength was 488 nm for the TGF-β1 imaging and 633 nm light wavelength was selected for the bFGF imaging. Thirty cells in each group were selected and imaged. Fluorescence intensity (mean value of intensity in cells-mean value of background intensity) of the bFGF and TGF-β1 signals were analyzed using Image J. Three independent experiments were performed.

Statistical Analysis Experimental data were statistically analyzed using SPSS19.0 software (International Business Machines Corporation，USA). The results were calculated as means±standard deviation (SD). Differences between control and experimental groups were determined by LSD t-test, while differences between subgroups were evaluated by single factor analysis of variance for the completely randomized experimental design. The statistical significance level was set at $P<0.05$.

RESULTS

Analysis of bFGF and TGF-β1 mRNA Expression in Müller Cells Using Real-time PCR Compared with the control group, bFGF mRNA expression in Müller cells in all three experimental groups was significantly upregulated, and extended illumination time further enhanced the upregulation ($^{a}P<0.05$). The upregulation was greatest in the 24h group and significant differences were observed among all three experimental groups ($^{c}P<0.05$). However, TGF-β1 expression was inhibited by illumination and the inhibition was enhanced with extended illumination time ($^{a}P<0.05$). The most significant changes were observed in the 24h group. Significant differences were observed among all the three experimental groups ($^{c}P<0.05$) (Figure 2).

Protein Levels of bFGF and TGF-β1 in Müller Cells Analyzed Using Western Blotting The bFGF protein level in Müller cells in all three experimental groups was significantly upregulated compared with the control group and positively correlated with illumination time ($^{a}P<0.05$). The most bFGF protein upregulationin was found in the 24h

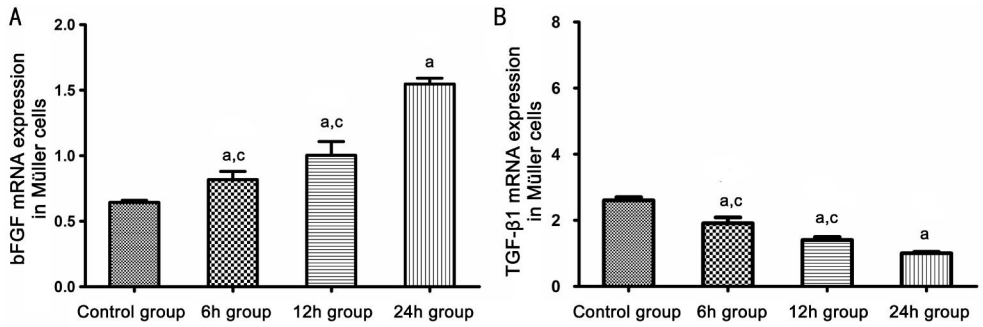

Figure 2 bFGF and TGF-β1 mRNA expression in Müller cells A: bFGF mRNA expression was significantly upregulated in all three experimental groups compared with the control groupand positively correlated with illumination time; B: The expression of TGF-β1mRNA was downregulated and negatively correlated with the control group. [a]P<0.05 *vs* control group; [c]P<0.05 *vs* 24h group.

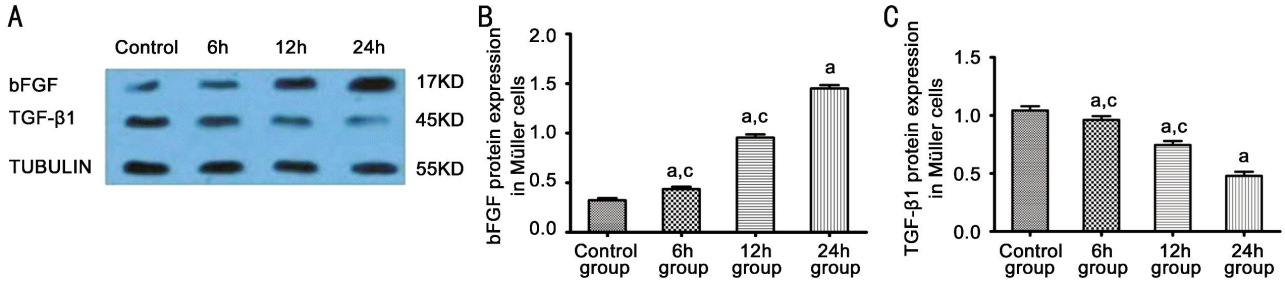

Figure 3 bFGF and TGF-β1 protein levels in Müller cells A: The Western blot band of bFGF TGF-β1 and TUBULIN; B: bFGF protein level was significantly upregulated in all three experimental groups compared with the control group and positively correlated with illumination time; C: The level of TGF-β1 protein was downregulated and negatively correlated with the control group. [a]P<0.05 *vs* control group; [c]P<0.05 *vs* 24h group.

group, while statistically significant differences were observed among all three experimental groups ([c]P<0.05). However, the TGF-β1 protein level in Müller cells in all three experimental groups was significantly reduced by light and this reduction was enhanced by extended illumination time ([a]P<0.05). Among all three experimental groups, TGF-β1 protein level downregulation was the highest in the 24h group and significant differences were observed among groups ([c]P<0.05) (Figure 3).

Fluorescence Intensity of bFGF and TGF-β1 in Müller Cells Analyzed Using a Confocal Laser Scanning Microscope Compared to the control group, bFGF fluorescence intensity in Müller cells was significantly higher in the 12 and 24h group ([a]P<0.001), while no significant difference was observed in the 6h group (P>0.05). Among the experimental groups, the fluorescence intensity was highest in the 24h group and the intensity was positively correlated with illumination time. Significant differences were observed among the three groups ([b]P<0.001). However, the TGF-β1 fluorescence intensity was reduced significantly in Müller cells in the three experimental groups compared to the control group ([a]P<0.001). Among experimental groups, the intensity was lower in the 24h group than in the 6h group ([b]P<0.001) while no significant reduction was observed between the 12 and 24h groups (P>0.05) (Figure 4).

DISCUSSION

Current theories show that the development of myopia is the result of scleral remodeling in the control of retina [13]. Many cytokines were involved in this process, including dopamine, nitric oxide, retinoic acid, TGF-β, and bFGF. TGF-β and bFGF are multi-functional factors that regulate cellular growth and differentiation. One of the most important physiological functions of bFGF and TGF-β is their involvement in the metabolism of the extracellular matrix (ECM), and their functions are contrasting in this process. *In vitro* experiments showed that the bFGF receptor and TGF-β receptor are expressed in scleral fibroblasts and that exogenous function occurs *via* interaction with these receptors [14]. TGF-β2 plays a major role in the biological activities involved in regulating the proliferation of sclera fibroblast cells and ECM production [15]. In ECM formation, bFGF is widely found throughout the body and can decrease the formation of collagens by inducing matrix metalloproteinase (MMP) expression [16]. Also, bFGF induces MMP production to enhance protein degradation, while TGF-β reduces MMP production and enhances tissue inhibitor of metalloproteinase (TIMP) production to reduce the MMP activity in scleral fibroblasts [17,18]. These results indicated that the balance disturbances between bFGF and TGF-β might lead to an abnormal MMP/TIMP ratio, which increased the degradation and reduced the production of extracellular collagen and proteoglycans. Ultimately, all of these phenomena induce scleral remodeling and myopia development.

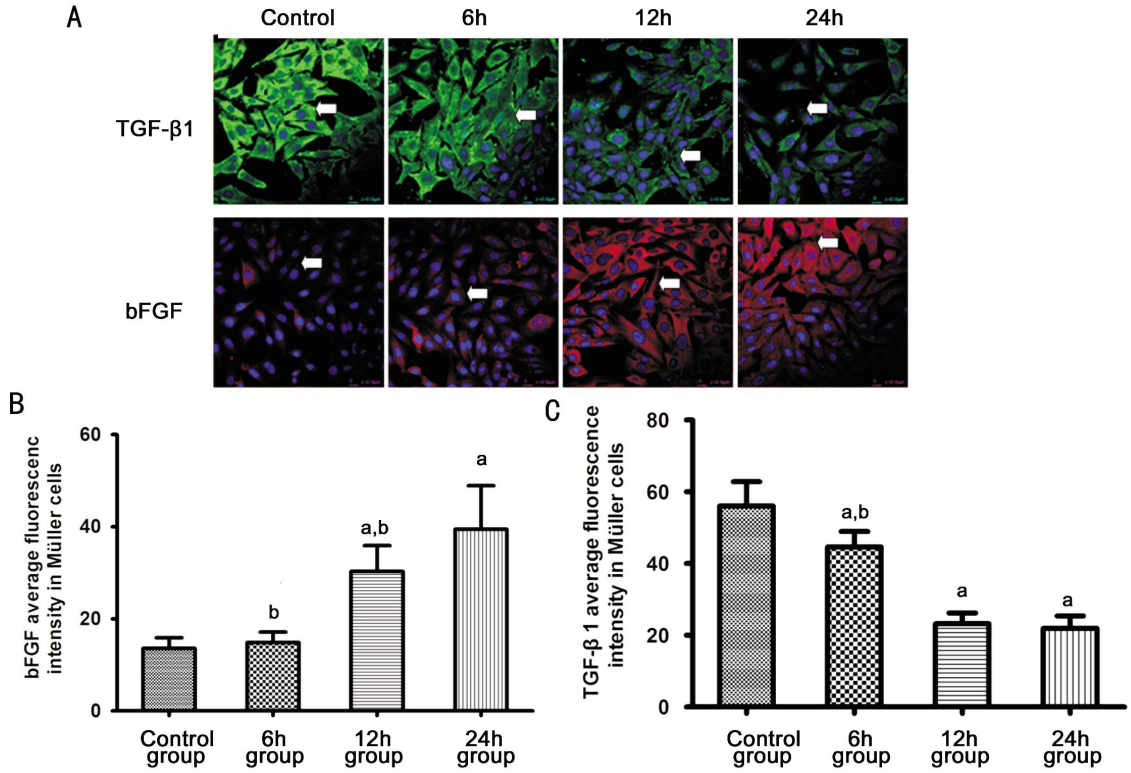

Figure 4 bFGF and TGF-β1 fluorescence intensity signals in Müller cells (×630) A: The fluorescence intensity signals of bFGF and TGF-β1; B: The extension of illumination time, bFGF fluorescence intensity signals were significantly enhanced in all three experimental groups compared with the control group; C: The fluorescence intensity signals of TGF-β1 was reduced. [a]P <0.001 *vs* control group; [b]P <0.001 *vs* 24h group.

Myopia induced by monochromatic light is a branch of myopia research. Wang *et al* [19] randomly distributed 48 guinea pigs into white-light (control), green-light (530 nm), and blue-light (480 nm) groups and found that those in the green-light group had a somewhat myopic refractive status. Expressions of retinal melanopsin were significantly lower in the green-light group. Conversely, expressions of melatonin (MT1) receptor in the retina and sclera were significantly higher. Liu *et al* [20] found that red light (610 nm monochromatic light) could promote guinea pig eye growth and myopia occurrence, whereas blue light (430 nm monochromatic light) had the opposite effect. VIP (vasoactive intestinal peptide), a potential signal molecule, may be involved in the regulation of monochromatic lights on eye growth by affecting scleral metabolism and morphological structure. Zheng *et al* [21] exposed human embryonic retinal pigment epithelial (RPE) cells to different wavelength monochromatic light. The irradiation of different wave1ength lights could affect RPE cell growth and proliferation as well as the secretion of hepatocyte growth factor, bFGF, and TGF-β in RPE cells *in vitro*.

In our previous study, we found no obvious effects on cell growth or cell cycle after 530 nm monochromatic light culturing of rat retinal Müller cells with 125 lx or 250 lx light intensity. In contrast, cell growth was inhibited and the cytokine (*e.g.* bFGF, TGF-β, T helper, and inducible nitrous oxide synthase) expressions in Müller cells were downregulated if the light intensity exceeded 250 lx. A 500 lx light intensity might cause cellular damage [11]. Müller cells, the major glial cells in the retina, are widely distributed among various cell bodies and fibers in the retina and are essential for the formation of the neuron-glia regulatory network among the three layers of retinal nerve cells. Müller cells also play important roles in the physiological and pathological processes in the retina [9]. Ambient light reaches the retina through refractive media and the retinal to be accepted by photoreceptor cells. Müller cells have regular and parallel arrangements so that the light reaches the photoreceptors through the retina [22]. Müller cells are important sources of cytokines that are produced in and secreted by the in myopic retina, and these cells modulate cytokine expression to participate in the development of myopia. In mammals myopia models, bFGF and TGF-β mRNA and protein expression levels were changed significantly in Müller cells[11,23].

In this study, Müller cells were cultured *in vitro* using a 530 nm monochromatic light with 250 lx light intensity for various illumination times, and the cytokine expression changed in a time-dependent manner. Also, bFGF expressions in the light-treated groups were higher than that in the control group and the light-induced upregulation was positively correlated with illumination time. TGF-β1

expression was inhibited by light, and the relationship between TGF-β1 expression and illumination time was in contrast to that of bFGF. These results indicated that bFGF and TGF-β might serve as a "blocker" and a "growth" signal on scleral tissue, respectively. In addition, bFGF and TGF-β might participate in scleral remodeling synergistically or antagonistically. Thus, we speculate that in the process of myopia development induced by monochromatic light, abnormal visual signals may affect the retinal Müller cells and disrupt the homeostasis between bFGF and TGF-β as well as the balance between MMP and TIMP, which may lead to scleral remodeling.

This study showed that bFGF and TGF-β1 expression changed in a time-dependent manner in Müller cells exposed to a 530 nm monochromatic light with 250 lx illumination intensity. This was a preliminary investigation into the relationship between TGF-β1 and bFGF expression in Müller cells and myopia. In the visual system, photoreceptor cells directly accept light illumination, but Müller cells are not photoreceptors. Experiments *in vitro* found that monochromatic light could affect of cytokine expression in Müller cells at various illumination times. However, whether the same results are obtained by experiments *in vivo* requires further research.

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