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MMP-2 participates in the sclera of guinea pig with form-deprivation myopia via IGF-1/STAT3 pathway

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Abstract. – OBJECTIVE: To investigate the expression changes of MMP-2 (matrix metalloproteinases-2) mediated by IGF-1 (insulin-like growth factors-1) STAT3 (signal transducer and activator of transcription 3) pathway in the sclera of the form-deprivation myopia guinea pigs.

MATERIALS AND METHODS: Twenty-four three-week-old guinea pigs were randomly divided into 4 groups: group A (Control), B, C and D. Guinea pigs in group A were sacrificed after 21 days without any special treatment. Guinea pigs in group B were sacrificed 7 days after receiving stitch in the right eye. Guinea pigs in group C were sacrificed 14 days after receiving stitch in the right eye. Guinea pigs in group D were sacrificed 21 days after receiving stitch in the right eye. Eyeball refraction and axial length of guinea pigs were measured before sacrifice. Eyeballs of guinea pigs were enucleated after sacrifice. The expressions of IGF-1, STAT3 and MMP-2 in scleral tissue were detected by Western blot.

RESULTS: Axial length extension and myopia appeared in the right eye of guinea pigs in group B. The expressions of IGF-1, STAT3 and MMP-2 in the sclera significantly increased after 7 days of occlusion compared with that in control group A ($p<0.05$). In the right eye of group C, the axial prolongation and myopia formation appeared after 14-day occlusion. The expressions of IGF-1, STAT3 and MMP-2 in sclera significantly increased compared with that in group A ($p<0.05$). In the right eye of group D, the axial extension and myopia formation occurred. IGF-1, STAT3 and MMP-2 in scleral significantly upregulated 21 days after occlusion ($p<0.05$). Furthermore, at different stages of deprivation, protein expressions of MMP-2 and IGF-1 in sclera were positively correlated ($r = 0.962$, $p<0.01$).

CONCLUSIONS: Form-deprivation of guinea pigs lead to increased expressions of IGF-1, STAT3 and MMP-2 in the sclera and myopia of guinea pigs. The expressions of IGF-1, STAT3 and MMP-2 increased progressively over the time of deprivation. Additionally, overexpression of MMP-2 mediated by IGF-1/STAT3 pathway in sclera might promote the formation of myopia.

Key Words:
Form-deprivation myopia, Sclera, Matrix metalloproteinase-2, Insulin-like growth factor-1.

Introduction

Myopia is a common eye disease with a high incidence worldwide. In Australia, the incidence of myopia among 12 to 17-year-old students is 42.7-59.1%[1,2]. In addition to its high incidence, it also severely threatens physical health of myopia patients in different stages. Mild to moderate stage of myopia could affect quality of life. Additionally, complications of severe myopia such as retinal detachment and macular hemorrhage could potentially lead to blindness. Therefore, it is crucial to find out an effective way to prevent myopia.

The formalization of animal or human eyes depends on the coordination and precise control of different parts of the eye. Appropriate visual stimulation in early stage is very important to the normal growth and formalization of the eyeball. During the neonatal development, disturbed vision-dependent feed-back by form deprivation might cause the axial length of eye prolong, thus leading to myopia, namely form-deprivation myopia (FDM). In 1977, Wiesel et al[3(6)] successfully established animal model of form-deprivation myopia[4-6]. The thinning of the sclera is an important feature after the form-deprivation myopia, which is mainly due to the remodeling of the scleral extracellular matrix, especially the dynamic remodeling of the posterior scleral tissue. This remodeling has been considered as a result of imbalance between the scleral extracellular matrix (ECM) synthesis and degradation, in which the matrix metalloproteinases (MMPs) play a crucial role. Given that type I collagen can degrade human scleral collagen, matrix metalloproteinase 2 (MMP-2) has attracted the attention

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of researchers among kinds of MMPs[7]. MMPs are enzymes that are widely involved in the degradation of extracellular matrix in animals and plants. They can degrade almost all extracellular matrix components except for polysaccharides and play an important role in embryonic development and tissue plasticity. Among them, MMP-2 is capable of degrading various kinds of collagen composition[8]. Extracellular matrix degradation and sclera remodeling in the formation of deprivation myopia depend on MMP-2 secreted by posterior pole scleral fibroblasts[9]. Signal transducer and activator of transcription-3 (STAT3), an upstream factor of MMP-2, plays an important role in the regulation of MMP-2 expression. Zhang et al[10] found that STAT3 can mediate extravascular fibroblast migration via regulating MMP-2 expression. This finding indicated that MMP2 regulated by STAT3 signaling pathway may be one of the mechanisms leading to remodeling of the fibrous tissue in posterior pole of the sclera.

Various myopia-related growth factors such as insulin-like growth factor 1 (IGF1) can induce activation of STAT3 signaling in guinea pig scleral fibroblasts cultured *in vitro*[11]. In recent years, research focused on the mechanism of eyeball growth and regulation as well as the role of IGF-1 in eyeball growth. Insulin-like growth factor (IGF), as a molecular signal, is regarded to play an important role in maintaining and controlling cell growth, proliferation, differentiation, maturation and regeneration. The system consists of two polypeptide growth factors (IGF-I and IGF-II), IGF receptors (IGF-IR and IGF-IIR), insulin-like growth factor binding protein (IGFBP) and IGF protease[12]. Among them, insulin-like growth factors 1 (IGF-1) can promote cell proliferation, differentiation, maturation as well as suppress cell apoptosis. Additionally, IGF-1 can also promote growth and anabolism, decrease blood sugar and regulate immune system via mediating various growth hormones. It has been reported that there is a significant correlation between the IGF-1 gene and many eye diseases, such as diabetic retinopathy (DR), retinopathy of prematurity (ROP) and age-related macular degeneration (AMD)[13-17]. Some scholars[18-20] confirmed that IGF-1 gene activation plays an important role in the development of human myopia.

The primary purpose of this study was to investigate the changes of IGF-1/STAT3 pathway along with the expression of MMP-2 in sclera of the form-deprivation myopia guinea pigs. Our results could provide a theoretical basis for further elucidating the molecular mechanism of myopia, and provide a new molecular target for myopia therapy.

Materials and Methods

Experimental Animals and Groups

A total of 24 three-week-old weaning guinea pigs, male or female, without eye disease and congenital myopia, were collected. This work was approved by the Animal Ethics Committee of Affiliated Hospital of Weifang Medical University Animal Center. Guinea pigs were housed in an experimental conditions with natural circadian rhythm and free access to drinking water at 22-28°C. All guinea pigs were randomly divided into 4 groups: group A, group B, group C and group D. Group A was considered as blank control group without intervention. Animals were sacrificed after feeding 21 days. In group B, the translucent mask was stitched to the right eye for 7 days. Next, members in group B were sacrificed. Guinea pigs in group C were covered for 14 days. The eye patch was sewn to the right eye for 14 days before sacrifice. In group D, the members were covered for 21 days before sacrifice. In each group, the right eye was used as an occluded eye, and the left eye as a self-control eye.

Animal Model Establishment and Data Collection

All the guinea pigs in treatment group were treated with 3% pentobarbital sodium intraperitoneal anesthesia followed by translucent eye mask fixed in the right eye. For diopter test, 0.15% tropicamide eye drops were used to paralyze ciliary muscle with dripping every five min for three times. After the pupil was fully dilated, refractive diopter was measured under streak retinoscopes. After anesthesia with ketamine, ocular surface anesthesia was performed with 1% tetracaine eye drops. Then the axial length was measured by A-mode ultrasonoscope.

Collecting Scleral Tissue

Diopter and axial length of guinea pigs were measured after covering the right eye for 7, 14, and 21 days, respectively. Guinea pigs were then sacrificed by cervical dislocation. The equatorial parts of the eyes were cut and then the eyeballs were cut off circularly to remove the anterior segment, vitreous body, retina and choroid, subsequently. Part of the sclera was fixed in 10% neu-

tral formaldehyde. The other part was stored in liquid nitrogen in the cryopreservation tube.

Hematoxylin-Eosin (HE) Staining in Scleral Tissue

The scleral specimens were fixed in neutral formalin for 24 h. Then the sections were routinely dehydrated, dipped in wax and embedded into four-micrometer scleral vertical sections. After HE staining, double-blind reading was performed under the optical microscope with 10 × 40 magnification.

Scleral MMP-2 and IGF-1 Expression Detected by Western Blot

Kidney tissues (0.1 g ± 0.05 g) were collected for protein extraction. The amount of sample was fixed. The primary antibodies (anti-STAT3 antibody, anti-MMP2 antibody, anti-IGF-1 antibody, Abcam, Cambridge, MA, USA) were added after conventional electrophoresis. Then the membrane bands were incubated overnight at 4°C. The next day the bands were incubated in the HRP(horseradish peroxidase) -labeled secondary antibody (Cell Signaling Technology, Danvers, MA, USA, goat anti-rabbit IgG, dilution: 1:5000) after being washed at room temperature for 2 h. The bands were developed by enhanced chemiluminescence (ECL) imaging (Shanghai Biyuntian Biotechnology, Shanghai, China). The integral optical density (IOD) value of each band was measured with a gel imaging analysis system, with β-actin as an internal reference. The relative expression of protein was calculated by the ratio of IOD in each band to β-actin IOD.

Statistical Analysis

Statistical product and service solutions software package 17.0 (SPSS Inc., Chicago, IL, USA) were employed for the statistical analysis. Paired *t*-test was used in the data group along with one-way analysis of variance (ANOVA) among groups. Results were expressed as mean ± standard deviation. Least Significant Difference (LSD) was used as the post hoc test to identify significance between groups. The relationship between the expressions of IGF-1 and MMP-2 were analyzed by Pearson's correlation analysis $p<0.05$ was considered as statistically significant.

Results

Comparison of Diopter and Axial Length of Each Group

We compared diopter and axial length of guinea pigs among groups. The results showed that the axial length and refraction of right eyes in groups B, C, and D significantly increased at the 7th day, 14th d, and 21st d, compared with the left eye of their own and the right eye of group A, respectively ($p<0.05$) (Table I).

Eye Pathology of Groups of Guinea Pigs

In control group, the thickness of sclera of guinea pigs was normal. Collagen fibers were arranged neatly, normalized and uniform in diameter with extracellular matrix evenly distributed. The sclera of guinea pigs after special treatment for 7 and 14 days were significantly thinned, disordered, broken and separated with the collagen fibers distributed sparsely. And the diameter of which also decreased dramatically. In addition, we also found that gap between the fibers and extracellular matrix significantly increased. The guinea pigs in 21 days group showed more significant changes than those of the previous groups (Figure 1).

Table I. Comparison of diopter and axial length of each group (mean ± standard deviation).

		7 d		14 d		21 d	
Group		Diopter (mm)	Axis oculi (mm)	Diopter (mm)	Axis oculi (mm)	Diopter (mm)	Axis oculi (mm)
A	Right eye	2.55±1.10	7.65±0.25	2.51±0.09	7.85±0.06	2.56±0.09	7.86±0.08
	Left eye	2.46±0.11	7.73±0.14	2.48±0.13	7.77±0.09	2.59±0.13	7.74±0.18
B	Right eye	-1.39±0.14*#	8.19±0.07*#				
	Left eye	2.44±0.09	7.66±0.09				
C	Right eye	-1.63±0.11*#	8.15±0.07*#	-2.63±0.23*#	8.35±0.08*#		
	Left eye	2.52±0.09	7.76±0.11	2.37±0.09	7.59±0.14		
D	Right eye	-1.72±0.09*#	8.17±0.03*#	-2.41±0.09*#	8.41±0.09*#	-5.59±0.12*#	8.78±0.13*#
	Left eye	2.42±0.10	7.69±0.11	2.51±0.10	7.84±0.06	2.60±0.13	7.57±0.19

*: Compared with the control group A, the difference was statistically significant, p<0.05; #: Compared with their own left eye, the difference was statistically significant.

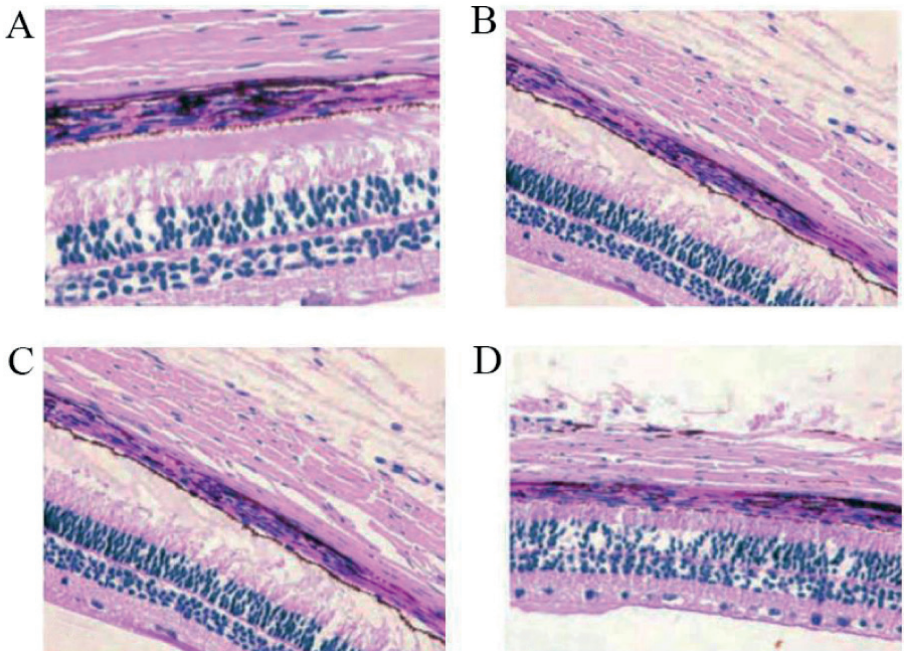

Figure 1. Histopathological changes in the covered eye of guinea pigs in each group. ***A,*** In control group, the thickness of sclera of guinea pigs was normal, the collagen fibers were arranged neatly, normalized, uniform in diameter and extracellular matrix little and equally distributed in. ***B-C,*** The sclera of guinea pigs in 7 and 14 days group were significantly thinned, the collagen fibers were distributed sparsely and disordered, broken and separated. ***D,*** The sclera of guinea pig further was thinned after 21 days of masking with collagen fibers fractured and separated, the interspaces between fibers increased and the extracellular matrix increased (400×).

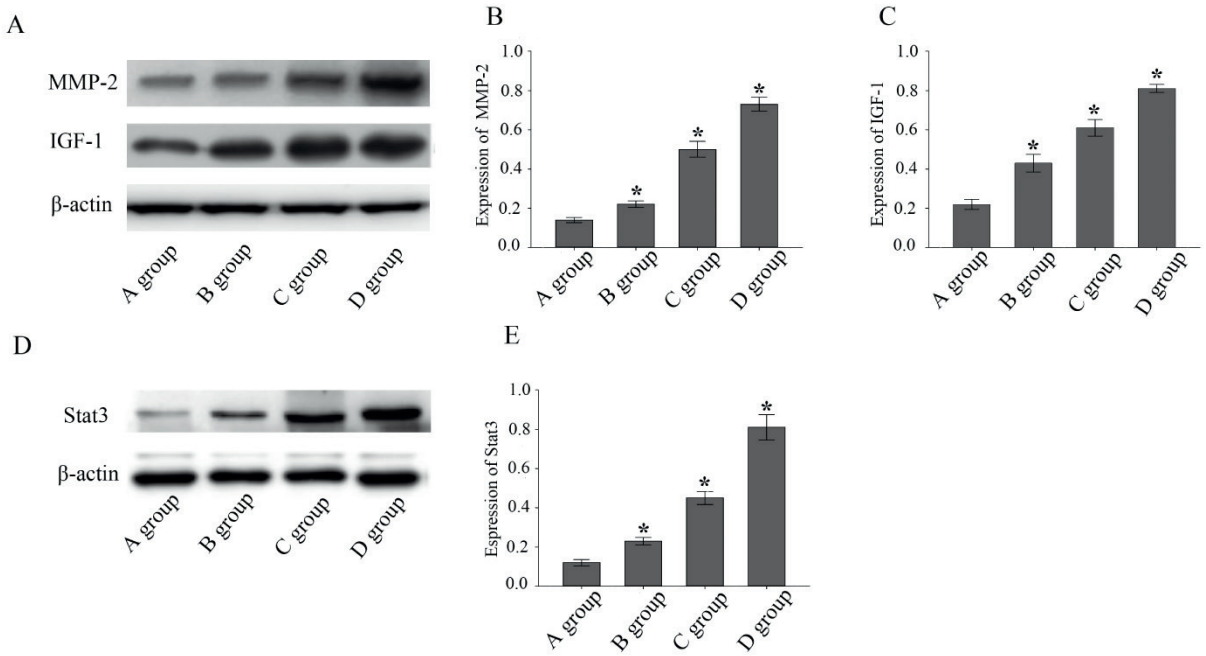

Figure 2. Comparison of IGF-1, STAT3 and MMP-2 expressions in scleral tissue between guinea pigs in each group. ***A,*** After 7^d, 14^d, and 21^d, the expression of MMP-2 in scleral tissue of guinea pig covered eyes (right eye) was significantly higher than that of the control group. ***B,*** After 7^d, 14^d, and 21^d, the expression of IGF-1 in scleral tissue of guinea pigs covered eyes (right eye) was significantly higher than that of the control group. ***C,*** After 7^d, 14^d, 21^d, the expression of STAT3 in scleral tissue of guinea pig covered eyes (right eye) was significantly higher than that of the control group. *: Compared with the control group A, the difference was statistically significant, $p<0.05$.

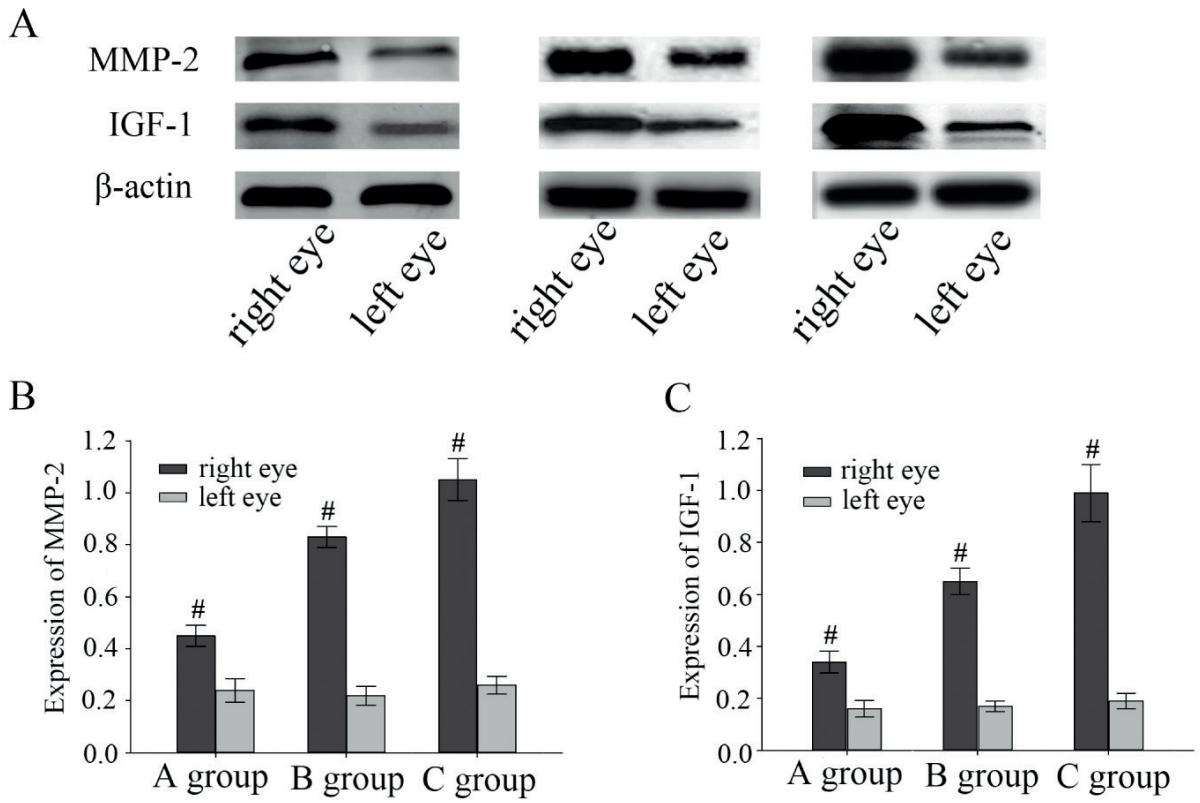

Figure 3. Comparison of IGF-1 and MMP-2 expressions in sclera of covered eyes of guinea pigs and that of their own left eye with the same cover time. ***A,*** In the same cover time, the expression of MMP-2 in scleral of the covered eyes (right eyes) was significantly higher than that of the left eyes. ***B,*** IGF-1 expression in the scleral tissue was significantly increased in the guinea pig covered (right eye) eyes compared with the left eye at the same cover time. #: Compared with the expression of sclera in left eye, the difference was statistically significant ($p<0.05$).

MMP-2 and IGF-1 Expression Comparison in Sclera of Each Group

Western blot results showed lower expressions of IGF-1, STAT3 and MMP-2 in the control group than those of treatment group. With the prolongation of the occlusion time, expressions of above three proteins in the treatment groups at the 7th d, 14th d, and 21st d increased gradually (Figure 2). Meanwhile, we also observed expression changes of IGF-1 and MMP-2 between the right eye and the left eye of the same guinea pig in the experimental group. We found that, the expressions of IGF-1 and MMP-2 in the scleral tissue of the covered eye were significantly higher than those in the left eye ($p<0.05$ Figure 3).

Correlation Between MMP-2 and IGF-2 Expression in Sclera of Guinea Pigs with Form-Deprivation Myopia

Correlation analysis showed that the protein expressions of MMP-2 and IGF-1 in scleral were strongly correlated (R=0.962, $p<0.01$) at different stages of form deprivation. The above results indicated that both of them were closely associated with formation of deprivation myopia. These two proteins may jointly promote the increase of diopter and ocular axis elongation, eventually leading to myopia (Figure 4).

Discussion

In our study, the monocular form deprivation myopia models in guinea pigs were successfully established. With the prolongation of occlusion time, the degree of myopia of the occluded eyes increased. The differences of diopter and axial length between covered eyes and control eyes were statistically significant, indicating that form deprivation could give rise to axis extension. The myopia caused by form-deprivation is axial myopia, suggesting that we successfully established form deprivation myopia model in guinea pig.

The current researches have shown that the form deprivation regulates the growth of adja-

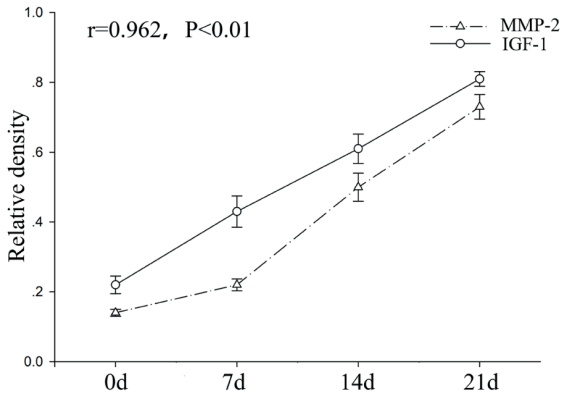

Figure 4. Correlation diagram of protein expressions of MMP-2 and IGF-1 in sclera at different occlusion time points. With the extension of time, the expression of MMP-2 and IGF-1 in the sclera of guinea pigs showed an upward trend with a strong correlation. $p<0.01$.

cent sclera mainly through the local retinal mechanism and can lead to the expression changes of many neurotransmitters and growth factors in the retina. As a first-class messenger, these neurotransmitters and growth factors act on the retinal pigment epithelium cells and choroid to produce secondary messengers, thus affecting the synthesis or degradation of the extracellular material (ECM). As a result, sclera is reshaped and the ocular axis prolonged, thereafter leading to myopia at last[21-23]. Collagen in mammal sclera accounts for about 90% of sclera. Among, type I collagen accounts for most of the total sclera collagen. Abnormal regulatory in sclera would lead to dysfunctions of eye growth and development process, eventually resulting in incompatibility between axis growth and refractive condition, and even refractive errors[24,25].

STAT3, one of the members of the STATs family, is involved in a series of physiological changes such as cell proliferation, differentiation and cell cycle[26,27]. STAT3 monomer itself in the cytoplasm is inactive. The activation of STAT3 mainly depends on the JAK tyrosine kinase[28]. Therefore, tyrosine in STAT3 molecule is phosphorylated by JAK to form an active dimer. The dimer subsequently translocates into the nucleus and binds to DNA, further leading to the STAT3 signal transduction. STAT3 mediates the signal transduction of many cytokines and growth factors to the nucleus, thereby affecting the transcription of target genes and regulates the function of cells. MMP-2 is one of the downstream target genes of STAT3 signal transduction pathway[29]. MMP-2 is an important gene that regulates sclera remodeling after myopia and plays an important role in the occurrence and development of myopia[30]. The balance of MMP-2 expression plays an essential role in the extracellular matrix metabolism of the sclera. Jones et al[31] conducted a study on the correlation between MMP-2 and myopia in 1996. They found that the activity of myofibrillar gelatinase A markedly increased in the form-deprivation myopia. Rada et al[32] also found that MMP-2 activity in the posterior sclera of form-deprivation myopia model was significantly higher than that of control eyes.

IGF-1 is considered to participate in various biological processes, including cell proliferation, differentiation, apoptosis, blood sugar maintenance and immune function regulation. Meanwhile, IGF-1 is widely expressed in eyes and involved in the development of various ophthalmic diseases[33,34]. Functionally, the mRNA expression of IGF-1R was detected in the posterior sclera of chicks. With the growth of the eyeball being dramatically accelerated, mRNA expression of IGF-1R increased significantly in the posterior pole sclera of the covered eye with the occlusion time prolonging. The mRNA level of IGF-1 receptor in the sclera of the posterior pole of the eye was significantly higher than that of the control eyes after masking, whereas the level of IGF-1R began to decline after de-masking[35]. Penha et al[36] showed that IGF-1 injection into the glass of chicken can lead to diopter change and axial extension, resulting in the changes of the shape of the eye. In addition to animal experiments, Metlapally et al[37] also found a genetic relationship between high degree of myopia and IGF1.

Semi-quantitative analysis of Western blot showed that MMP-2 was expressed in scleral tissue of both experimental and control eye, while the expression level in control eye was relatively lower. With extension of treatment time, the expressions of MMP-2 and IGF-1 in the sclera of right eyes gradually increased on the 7th, 14th, and 21st day after masking, respectively. Above results suggested that MMP-2 and IGF-1 might be involved in the formation of form-deprivation myopia. Correlation analysis showed that the expressions of MMP-2 and IGF-1 in scleral tissue were strongly correlated ($r=0.962$, $p<0.01$) at different stages of deprivation, indicating that both of them are involved in the formation of FDM.

Based on those results, we suggested that there is an interaction between MMP-2 and IGF-1 during the development of form-deprived myopia. Kenney et al[38] found that inhibiting the IGF-1

pathway down-regulates the expression of MMP-2 in the sclera of guinea pigs and reduces the remodeling of the sclera. Therefore, IGF-1 may be an upstream regulating molecule of MMP-2. Evidence also demonstrated that overexpression of MMP-2 and IGF-1 during myopia formation might be responsible for scleral remodeling. However, the specific signaling pathway that regulates scleral remodeling is still not fully elucidated. The mechanism of MMP-2 in the formation and development of myopia remains to be further studied. It has been found that various biological factors can regulate MMP-2 expression. Further researches will be needed to clarify the pathogenesis of myopia and to develop new highly selective MMP-2 inhibitors.

Conclusions

We observed that form-deprivation of guinea pigs can enhance the expressions of GF-1, STAT3 and MMP-2 in the sclera and cause myopia in guinea pigs. The expressions of IGF-1, STAT3 and MMP-2 increased progressively with prolonged time of deprivation. Additionally, overexpression of MMP-2 mediated by IGF-1/STAT3 pathway in sclera may promote the formation of myopia.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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