# Function of all-trans retinoic acid observation on similar myopia changes in cultivated rabbit retinal pigment epithelium and relation with myopia relevant factors

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**Abstract**: To observe the role of all-trans retinoic acid (ATRA) during the similar myopia changes of cultured rabbit retinal pigment epithelium (RPE) cells, as well as the variation changes and relationships with myopic correlation factors such as hepatocyte growth factor (HGF) and matrix metalloprateinase-2 (MMP-2). Rabbit RPE cells of primary generation were selected and cultured to fifth generation by subculture. Then the morphology of RPE cells were observed and cell vitality was analyzed by using the Trypan blue reject test. The expressions of HGF and MMP-2 in RPE cells were tested by using an immunobistochemistry method. The HGF concentration in RPE cell culture fluid was detected by applying enzyme-linked immunosorbnent assay (ELISA). As the ATRA concentration enhanced and action time prolonged, the survival rate of RPE cells was reduced, but the expressions of HGF and MMP-2 increased, so did the secretion of HGF. ATRA concentration with no less than 5nM/ml was able to induce the growth inhibition of RPE cells and the decrease in survival rate, which was similar to the changes in RPE cells in myopia. With the actin of ATRA, the expressions of HGF and MMP-2 increased in RPE cells, with more distinct in HGF increase.

**Keywords**: Myopia; all-trans retinoic acid; retinal pigment epithelium; hepatocyte growth factor; matrix metalloprateinase-2.

## INTRODUCTION

In recent years, the myopia researches in experimental animals have been indicated that the material inducing myopia is mainly from retinal neural epithelium. The primary messenger generated by retinal neural epithelium is first acting on retinal pigment epithelium (RPE) cells and uveal cells, which enables the generated next biochemical substances (called second messenger) to act on sclera again, thus further inducing the remodeling of sclera and the extension of eye axial. Certain autocrine or paracrine loop involving cell factors, growth factors and tyrosine kinase receptors may be of vital importance on the pathologic changes of RPE cells. Studies have been shown that all-trans retinoic acid (ATRA) has an inhibiting effect in the proliferation of RPE cells, similar to the pathologic changes of myopia (Dawson and Zhang, 2002). In the growth gene researches of mice eyeball, it has been discovered that the gene making hepatocyte growth factor (HGF) may be closely related to eyeball growth, hence, HGF also may affect the occurrence of myopia (Zhou and Williams, 1999; Lashkari *et al*., 1999). This experiment, from the micro perspective of cells, was to observe the role of ATRA inducing the myopia changes of RPE cells, quantitatively and qualitatively, as well as the variation changes in and relationships with myopic factors such as HGF and matrix metalloprateinase-2 (MMP-2).

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## MATERIAL AND METHODS

### *Main materials and reagents*

Twelve healthy black rabbits in 1-week old, fetal bovine serum (FBS) (Hangzhou Sijiqing Company), high sugar type dulbecco modified eagle medium (DMEM) (America GIBCO), 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (America GIBCO), Trypan blue powder (America sigma), ATRA (America sigma), mouse anti-human polyclone Cytokeratin8 (CK8) (ZSGB-BIO ORIGINE Company), Vimentin (ZSGB-BIO ORIGINE Company), hepatocyte growth factor antibody (ZSGB-BIO ORIGINE Company), MMP-2 antibody (ZSGB-BIO ORIGINE Company), immunohistochemical SABC kit (Wuhan BOSTER Bioengineering, Ltd.) and goat anti-rabbit HGF-ELISA kit (America ADL).

### *Culture in vitro of rabbit RPE cells*

*Primary generation and subculture of rabbit RPE cells*

The reasearch was done in Ophtalmology Department of Zhengzhou First People's Hospital. After the healthy black rabbits in 1-week old were anesthetized, their binoculus were took out under the sterile condition and cut away in annular at 2mm behind corneal limbus, then anterior ocular segment tissues and vitreous body were eliminated. The posterior eye segment was worked into eyecup and fixed, washing eyecup twice by using PBS. 2 mL 0.25% Trypsin/0.02% EDTA was added for digestion 3min, in order to extract the digested retinal neuroepitheliμm layer. Afterwards, about 2ml Trypsin was added for digestion 15-20min at 37$^{o}$ with 5% $CO_2$, at the

same time, DMEM containing FBS was added for terminating digestion. The sucker was blew and beat slightly to make RPE cells fall off. The cell suspension was collected in centrifuge tube, with centrifugation at 1500 r/min for 5min. Then the supernatant was abandoned, PBS was added for rinse once, and DMEM containing 20% FBS was inoculated in 100ml culture bottle, being placed in the cell culture incubator at 37$^{o}$ with 5% $CO_2$, observed after 48 h and exchanged after 72 h with liquid. As the primary generation cell grew closely into fusion, the culture solution was discarded, using PBS for rinsing three times and adding about 1ml 0.25% Trypsin/0.02% EDTA, and then being observed under a microscope. When the cells became round, DMEM containing FBS was added for terminating digestion. The cells were completely fell off into cell suspension with slight blow and beat on bottle walls, with centrifugation at 1500 r/min for 5min. Then the supernatant was abandoned, PBS was added for rinse once, and DMEM containing 20% FBS was added for inoculation. With this method, it could be used in experiments when generating in the 3$^{rd}$-5$^{th}$ generation.

***Rabbit RPE cell detection***

After chemical staining on the third generation of RPE cells and CK8 immune cells, positive reaction in brown yellow was indicated in cytoplasm. After chemical staining on Vimention immune cells, the cytoplasm was negative reaction (fig. 1 and fig. 2).

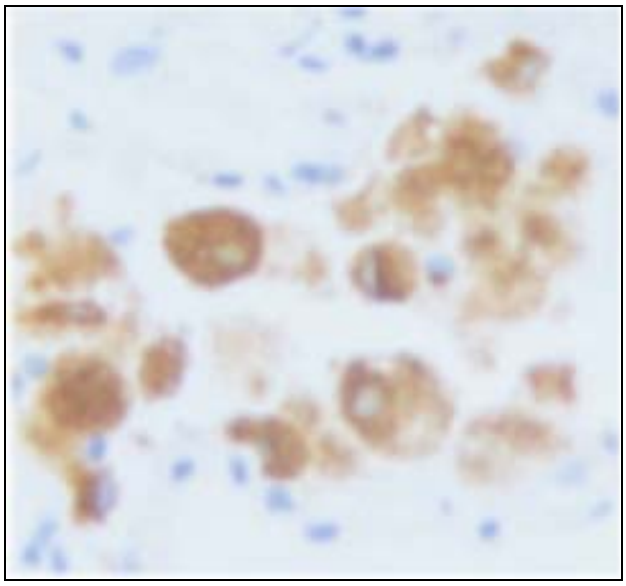

**Fig. 1**: Positive expression of CK8 in cultured RPE cell (640×437)

***Detection on the RPE cell survival rate with different concentration and action time of ATRA by Trypan blue reject test***

When RPE cells were with passage in fifth generation, ATRA with 5nM/mL, 10nM/ml, 20nM/ml was added respectively for cultivation. After 24h, 48h and 72h, pancreatic enzyme was used for digesting into cell suspension. 9 drops of cell suspension were selected and added with 1 drop of 0.4% Trypan blue normal saline solution, then added into the blood counting chamber with a cover slip after mixing. Cell population in four big squares was calculated, in which cells with non-staining were living cells and those staining light blue were dead cells. Cell survival rate (%) = (cell population with non-staining/total cells) ×100%.

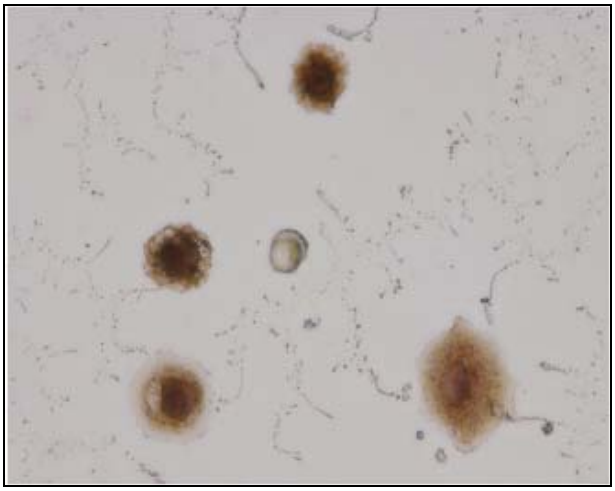

**Fig. 2**: Negative expression of Vimentin in cultured RPE cell (640×437)

***Detection on the expressions of HGF and MMP-2 in RPE cells by immunohistochemistry***

The fifth generation RPE cells in $1\times10^{5}$/ well were cultivated in 24-well plates with coverslips in bottom. After they were cultured for 24h by using DMEM containing 20% FBS, then transferred into DMEM without FBS for culturing 24h, finally transferred into DMEM containing 5nM/mL, 10nM/mL, 20nM/mL ATRA. Absolute ethyl alcohol with the same volume in experimental group was added in negative control group and DMEM was added in blank control group. At 24h, 48 h and 72h after ATRA added, supernatants were resorbed respectively, with PBS for rinsing three times, then 4% paraformaldehyde was used for fixation 20-30min, with PBS for rinsing three times again. 21 pieces of 30% $H_2O$ and 50 pieces of pure methanol were mixed, soaked for 30 min at room temperature. After washed three times with distilled water, 5% BSA confining liquid was dropped and placed for 20 min at room temperature. The residual liquor was cast off, without washing. Proper rabbit IgG was dropped and placed for 1h at 37$^{o}$, then being rinsed by PBS for 2 min in three times. Biotinylated goat anti-rabbit IgG was dropped and placed for 20 min at 20-37$^{o}$, then being rinsed by PBS for 2min with three times in total. SABC reagent was dropped and placed for 20 min at 20-37$^{o}$, then being rinsed by PBS for 5 min with four times in total. Then DAB was developed. 1mL distilled water plus with reagent A, B and C with each 1

drop in DAB color development kit were mixed and placed on the microscope section. It was developed at room temperature. The response time was controlled under microscope. Hematoxylin was used for slight counterstain. 50% glycerinum was used for dehydration, and then sealed. The inverted phase contrast microscope was applied for observation.

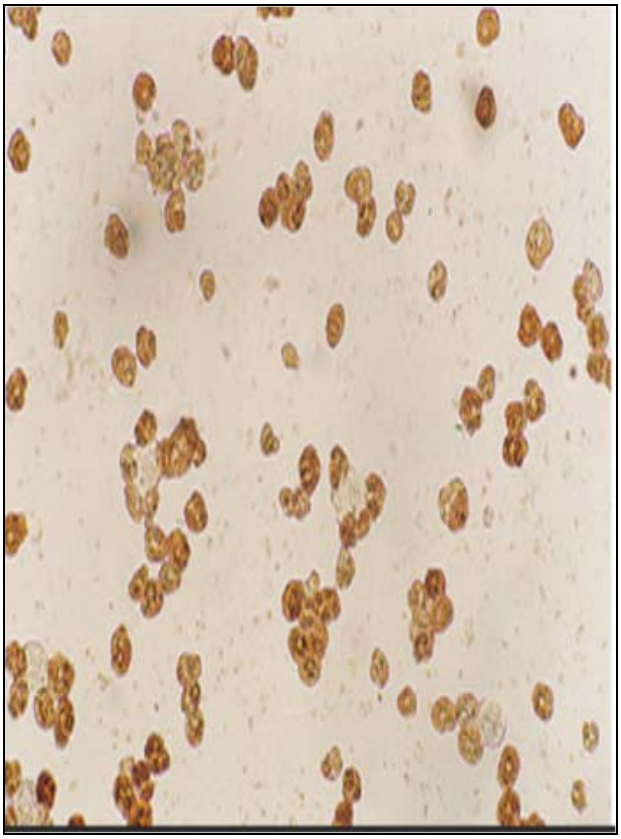

**Fig. 3**: HGF shows the blown staining in RPE cell after 48h (640×437)

***Detection on HGF secretion levels of RPE cells by ELISA***

Cell culture fluids at three concentration and three time points were collected and placed in sterile EP tube. The specimens were stored in refrigerator at -80$^{o}$ for standby application. ELISA plate was took out at room temperature, 50μL standard substances and specimens were added successively into blank micropores with 3 multiple wells being set; 10μL biotin labeling solution was added in specimens; 100μL enzyme labeling solution was added in the wells of both standard substances and specimens, slightly mixing in15 sec; After incubation at 37$^{o}$ for 60 min, the mixed liquor used for incubation was discarded. The plates were washed for 5 times with distilled water, with the still standing in 10-20 sec every time; The residual water drops on absorbent paper were knocked off; Substrate solution A and B with each in 50μL were added in each well, slightly shaking in 5 sec; After incubation for 15 min away from light at 37$^{o}$, 50μL stop buffer was added in each well for terminating reaction; OD value, concentration and curve chart for every well were immediately read in the enzyme-linked immunometric meter with 450 nm wave length.

***Data processing***

SPSS10.0 statistical software was adopted for analysis. X±S was represented for experimental data. One-way analysis of variance was used for numerical variable for treatment and LSD method for comparison among groups; Chi-square test was applied for classified variable; Correlation and regression analysis of straight line was employed for standard curve of ELISA. $P$<0.05 considered statistical significance.

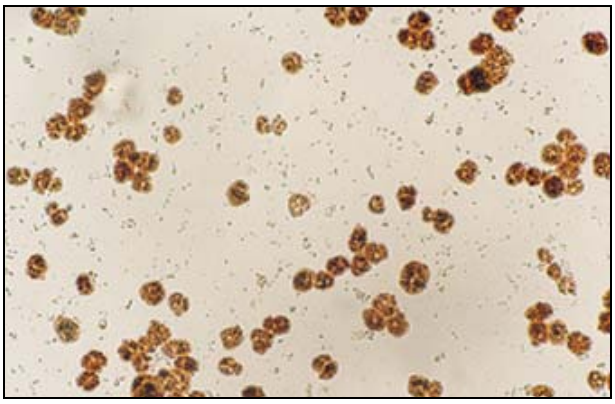

**Fig. 4**: MMP-2 shows the blown staining in RPE cell after 48h (640×437)

## RESULTS

***Morphological observation***

ATRA in 5nM/mL, 10nM/mL and 20nM/mL equally was able to inhibit the proliferation of RPE cells, with flat cell enlargement, decrease in protuberance and dispersion of pigment. After 24h, 48h and 72h of ATRA action, cells were presented with enlargement, flatness, decrease in protuberance, partial division and weakening in activity.

***Trypan blue reject test***

Trypan blue reject test suggested that after 24 h of ATRA action in 5nM/mL, 10nM/mL and 20nM/mL, the cell survival rates were 99.50%, 91.67% and 87.44% ($P$= 0.001), respectively; those after 48 h were 99.02%, 88.17% and 80.44% ($P$=0.0001), respectively; those after 72h were 98.44%, 86.94% and 71.08% ($P$=0.0001), respectively.

***Immunohistochemical method***

Detection on the expressions of HGF and MMP-2 by immunohistochemical method suggested that the positive expressions of HGF and MMP-2 in RPE cells were enhanced with the increase of ATRA concentration and also enhanced with the extension of action time. In the same concentration and action time, the positive

**Table 1**: The secretion of HGF in different concentration and action time of ATRA groups (X±S, nM/mL)

| Group | Concentration of ATRA | The secretion of HGF | | |
|---|---|---|---|---|
| | | 24 h | 48 h | 72 h |
| Control | 0 | 2.60±0.09 | 2.60±0.19 | 2.60±0.18 |
| ATRA | 5 | 2.77±0.16 | 2.89±0.06 | 2.95±0.23 |
| | 10 | 3.17±0.08 | 3.21±0.47 | 3.27±0.40 |
| | 20 | 3.27±0.39 | 3.30±0.17 | 3.47±0.17 |
| *F* | | 17.245 | 4.357 | 13.266 |
| *P* | | 0.001 | 0.043 | 0.002 |

expression of HGF was significant than that of MMP-2 (fig. 3, fig. 4).

***Detection on HGF secretion volume by ELISA***

The HGF volume secreted by RPE cells was augmented with the increase of ATRA concentration and comparisons between any two groups showed that there was statistically significant difference ($P<0.05$); The length of ARTTA action time did not much affect the volume of HGF volume secreted by RPE cells and comparisons between any two groups showed that there was no statistically significant difference ($P>0.05$) (table 1).

## DISCUSSION

The originating factors and subsequent controlling factors for experimental myopia eyeball growth are located in the specific spots of retina. Retina itself can recognize external objects to image them on its plane (Schaeffel and Diether, 1999), afterwards to release the messenger molecules in order to control the growth rate of its underlying tissues (Hu and Steven, 2000). Second messenger that promotes the occurrence of myopia is able to restrain the growth of scleral fibroblast cells and synthesis of extra cellular matrix, or degrade the extra cellular matrix to induce scleral weakness and myopia. Second messengers found by studies are involving in various growth factors (such as TGF-β, HGF, bFGF, INF, PDGF, EGF and $MMP_S$), dopamine, acetylcholine and ATRA. Among known myopia messengers, bFGF and TGF-β have been deeply studied in recent years, while HGF affecting eyeball growth was rarely studied. This study observed that on the basis of RPE cell myopic pathologic changes induced by ATRA, the expressions of HGF and MMP-2 were increased and the increase of HGF was more significant, hence, it was speculated that HGF may affect the changes of MMP-2.

The synthesized ATRA in retinal cells was also called retinoic acid, the most active metabolite in vitamin A. ATRA not only is related to the visual physiology of retina, which can regulate retinal photoreaction, but also is able to directly combine with nuclear receptor so as to participate in the transcription of different target gene, which can induce certain growth factors and their receptors expression, thus further inducing cell differentiation at specific environment (Kishi, 1994). All these enable ATRA to possess the conditions of being a messenger, which attracts myopia researchers' attention in recent years. The changes in visual environment induce the changes of messenger substances in retina such as dopamine, acetylcholine and retinoic acid (RA). The changes of those messenger substances are regarded as signals to regulate the secretion of choroid RA, and choroid RA further acts on retina and controls retina growth. The previous studies have confirmed (Lv *et al*., 2006) that the changes of retina RARβ caused by form-deprivation are earlier than the changes of diopter and eye axial. During the acceleration of myopia eyeball growth, the retinal RA content increases, while eyeball growth is suppressed, the retinal RA content reduces. At present, there are numerous reports confirming that ATRA can induce the growth inhibition of various cells, such as tumor cells. This study also confirmed that ATRA concentration in no less than 5 nM/ml was able to induce the growth inhibition of RPE cells and the decrease in survival rate, similar to the changes of myopia RPE cells.

HGF is a cell factor with various bioactivities. It not only can promote the division, movement and differentiation of cells, but also has a crucial relationships with the physiological equilibrium, damage repair and related diseases of eye tissues, for example, it can promote the proliferation of corneal epithelial cell, corneal endothelial cell, pigment epithelial cell and lens epithelial cells, and HGF in lacrimal gland can promote the division and differentiation of corneal epithelial cell. At normal condition, HGF is generated by mesenchymal cells and it receptor Met is expressed in epithelial cells. HGF is referred to epithelial-mesenchymal conversion process (He *et al*., 1998), which plays important role in the occurrence and development of myopia. RPE cells are located between sense retina layers and vascular choroid layer, the connection in epithelial-mesenchyma, secreting various cell factors and playing importance role in the self-stabilization and visual function of retina; RPE cells participate the occurrence and development of various diseases in eyes. Recent studies have considered that RPE cells can express HGF and secret its receptor c-met (Han *et al*., 2006) at the same time. Our studies also confirmed that HGF in rabbit RPE cells cultured *in vitro* showed expression on protein level, suggesting that HGF was able to regulate the function of RPE cells by paracrine and (or) autocrine.

MMPs and TIMPs are extensively existing in RPE-choroid, palying vital role in the epimatrix damage of retinal cells, angiogenesis and fibrosis during the vitreoretinopathy (Leu *et al*., 2002). Form-deprivation myopia inducing the expression imbalance of sclera MMP-2/TIMP-2 in posterior pole can promote the epimatrix of sclera cells to remould, thus inducing excessive extension of eye axial and forming axial myopia. This study confirmed that in rabbit RPE cells, MMP-2 expression showed positive correlation with HGF expression, consistent with the reported regulation effect of HGF on MMPs and TIMPs, which suggested that MMP-2 also had a crucial role in the study of myopia RPE cells.

## CONCLUSION

It was concluded that ATRA concentration in no less than 5nM/ml had inhibition effect in RPE cells, inducing the representation of flat cell enlargement, decrease in protuberance, dispersion of pigment, partial division and weakening in activity, which provided models for changes of RPE cells occurring active compensation for myopia. ATRA in a certain concentration had the ability to induce the increase of the expressions of myopia-related factors like HGF and MMP-2 in ROE cells, with more significant increase for HGF. Therefore, it was speculated that the increase of HGF may promote the increase of MMP-2, further helping the latter to promote the occurrence and development of myopia.

## REFERENCES

Dawson MI and Zhang XK (2002). Discovery and design of retinoic acid receptor and retinoid X receptor class- and subtype-selective synthetic analogs of all-trans-retinoic acid and 9-cis-retinoic acid. *Curr. Med. Chem.,* **9**(6): 623-637.

Hu YN and Steven A (2000). McCormick. Role of RPE-Choroid axis on the occurrence of myopia. *CJO&O,* **2**(4): 197-200.

He PM, He S and Garner JA *et al* (1998). Retinal pigment epithelial cells secrete and respond to hepatocyte growth factor. *Biol. Biop. Res. Comm.,* **249**(1): 253-257.

Han W, Yap MK and Wang J *et al* (2006). Family-based association analysis of hepatocyte growth factor (HGF) gene polymorphisms in high myopia. *Invest Ophth. Vis. Sci.,* **47**(6): 2291-2299.

Kishi H, Mishima HK and Yamashita U (1994). Growth regulation of retinal pigment epithelial cell *in vitro*. *Curr. Eye. Res.,* **13**(9): 661-668.

Lashkari K, Rabimi N and Kazlauskas A (1999). Hepatocyte growth factor receptor in human RPE cells: implication in proliferative vitreoretinopathy. *Invest Ophth. Vis. Sci.,* **40**(1): 149-156.

Lv Y, Zhang JS and Yan PS (2006). RARβ expression in form deprivation myopia in guinea pig eye. *COR,* **24**(4): 411-414.

Leu ST, Batni S, Radeke MJ, Johnson LV, Znderson DH and Clegg DO (2002). Drusen are cold spots for proteolysis: Expression of Matrix Metalloproteinases and Their Tissue Inhibitor Proteins in Age-related Macu1ar Degeneration. *Exp. Eye. Res.,* **74**(1): 141-154.

Schaeffel F and Diether S (1999). The growing eye: An autofocus system that works on very poor images. *Vision Res.,* **39**(9): 1585-1589.

Zhou G and Williams RW (1999). Eye1 and Eye2: Gene loci that modulate eye size, lens weight, and retinal area in the mouse. *Invest Ophth. Vis. Sci.,* **40**(5): 817-825.