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The alteration of *MERTK* gene in different passage of retinal pigment epithelium cells

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传代后视网膜色素上皮细胞 *MERTK* 基因表达变化的研究

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摘要

目的:检测传代后的视网膜色素上皮细胞(retinal pigment epithelial, RPE)*MERTK* 基因表达的变化,明确传代的异体 RPE 移植的可行性。

方法:离体培养原代的人视网膜色素上皮细胞,将原代细胞以 1:3 比例进行传代至第 6 代,以 SYBR® Green I real time RT-PCR 方法检测不同传代数的 RPE 细胞 *MERTK* 基因表达的水平,采用 $2^{-\Delta\Delta CT}$ 方法计算定量 PCR 的数值进而比较各代 *MERTK* 基因表达的的变化。

结果:原代及传代的 RPE 细胞均表达 *MERTK* 基因。第二代 RPE 细胞 *MERTK* 基因表达水平与原代相同,第 3~6 代 RPE 细胞 *MERTK* 基因表达水平均明显低于原代。

结论:传代的 RPE 细胞可以作为异体视网膜色素上皮细胞移植的细胞来源,但以第二代的 RPE 细胞为最佳选择。

关键词:视网膜色素上皮; 传代; *MERTK*

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Abstract

• AIM: To exam the alteration of *MERTK* gene in different passages and clarify the feasibility of passaged allograft retinal pigment epithelial (RPE) cells transplantation.

• METHODS: Human primary RPE cells were passaged as ratio 1:3, the level of *MERTK* gene in different passages cells were quantified by SYBR® Green I real time RT-PCR. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments.

• RESULTS: Both primary and passaged RPE cells expressed *MERTK* gene. The level of *MERTK* gene in second passage RPE cells were the same as the primary cells, while in passage 3-6 the level of *MERTK* gene were declined obviously.

• CONCLUSION: The passaged allograft RPE cells could be a source for transplantation, but the second passage RPE cells were the better selected object.

• KEYWORDS: retinal pigment epithelial; passage; *MERTK*

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INTRODUCTION

Retinal pigment epithelial dysfunction is believed to be the main cause of many debilitating retinal diseases of which retinitis pigmentosa (RP) is the most common[1]. In this disease, the retinal pigment epithelial dysfunction leads to photoreceptors damage causing severe vision loss[2]. There are currently no effective treatments, gene-based treatments[2-4] and transplantation of retinal pigment epithelium (RPE) cells[5-7] has been conducted by many researchers in the past 20 years. Though many of the genes have now been identified and their functions elucidated, such as RHO, RP1, *MERTK*[8-11], the efficiency, reliability and safety of gene imported into cells and the ethical issues, gene-based treatments received certain restrictions.

Replacement of retinal pigment epithelium (RPE) cells by transplantation is a potential treatment for some retinal degenerations. This technique aims to restore the subretinal anatomy and reestablish the critical interaction between the retinal pigment epithelium and the photoreceptor[12], which is fundamental to sight. Retinal pigment epithelial transplantation has been performed with two different techniques: retinal pigment epithelial suspension and autologous full-thickness retinal

pigment epithelial-choroid transplantation. Despite the feasibility of RPE suspension technique, the cell source to replace autologous RPE was limited. So enough allograft RPE transplantation as keratoplasty will be the promising treatment. Usually, in order to obtain sufficient number of allograft RPE cells, the primary RPE were amplification by passage, but whether the passaged RPE cells have the same the function as primary cells was unclear. Here, we test the alteration of *MERTK* gene in different passages, which has been known as the phagocytic gene of RPE, to clarify the feasibility of allograft passaged RPE transplantation.

MATERIALS AND METHODS

Materials

Eyeballs All procedures were in accordance with the Association for Research in Vision and Ophthalmology Resolution. Human eyeballs obtained from eye bank in department of Ophthalmology of the First Clinical College of China Medical University. The three eyeballs used for experiments were from 3 distinct donors (age from 24-40 years) who died of trauma or noninfectious disease and dorne the eyeballs to eye bank for clinical and fundamental research.

Methods

Tissue culture According to Xu *et al*[13] eyes from animals were sink by 20mL PBS containing tobramysin 20mg for 20 minutes, and rinsed with PBS (pH 7.4) three times. Then the anterior segments (cornea, iris epithelium, lens), vitreous and the neural retina were removed from the eyes. Eyes from human which had been removed of anterior segments (cornea were used for keratoplasty) were sink by 20mL PBS containing tobramysin 20mg for 20 minutes, and rinsed with PBS (pH7.4) three times. The typsin (0.25% typsin +0.02% EDTA, pH=7.4, Gibco) were added into eye cups and incubated in incubator for 35 minutes at 37℃, then discarded the typsin, added DMEM supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100mg/mL streptomycin (all from Gibco, USA) into eye cups and pipetted continuously for 2 minutes, centrifuged the suspension of RPE cells by 1000rpm for 8 minutes at 4℃. Collected the RPE cells by DMEM+10% FBS and seeded into 35mm^2 dish (Cellstar, Germany). Primary RPE cells were cultured for 5-7 days to confluence. Cultures showed no contamination with fibroblasts or choroidal cells based on a microscopic analysis of cell morphology and immunohistology (RPE showed CK (+) and S-100 (+)). Parts of the confluence primary RPE cells were passaged at rate of 1:3, the other confluence primary RPE cells were collected for next experiment steps. Primary RPE cells were recorded as P_0, the second, third, fourth, fifth and sixth passage as P_1, P_2, P_3, P_4, P_5, respectively.

qRT-PCR

Total cDNA extraction Total RNA was isolated (RNAiso Plus, D9108A, Takara Biotechnology Co., LTD.) according to the manufacturer's protocol. That is, to wash the primary and 2-6 passage RPE cells with PBS (pH=7.4) at 4℃ three times, then treated cells with RNAiso Plus (1mL/dish) for 5 minutes, pipette and collect the suspension into 1.5mL eppendorf tubes. To centrifuge the tubes by 12000rpm for 5 minutes at 4℃, and pipette suspension to a new tube and add 0.2mL chloroform, shake the tubes strongly and state for 5 minutes at room temperature, then centrifuge the tubes by 12000rpm for 5 minutes at 4℃, and pipette the top level supernant about 0.5mL to a new tube, and add 0.5mL isopropanol, shake the tubes strongly and state for 10 minutes at room temperature, then centrifuge the tubes by 12000rpm for 10 minutes at 4℃. The supernant were disarded and precipitation was washed with 1mL 75% alcohol by12000rpm centrifuging for 5 minutes at 4℃. To dilute the RNA precipitation with 10μL RNAase free water. The contents of total RNA was test by ultraviolet spectrophotometer. To calculate the contents of total RNA by formula: $OD_{260}\times40\times$ dilution ratio/1000.

RT To generate total cDNA, reverse transcription was performed according to the manufacturer's protocol (PrimeScript® RT Master Mix, Takara Biotechnology Co., LTD). The reaction volume was 10μL/sample, contained 2μL 5×PrimeScript® RT Master Mix, 0.5ug RNA sample and corresponding volume RNase Free dH2O. The cycle conditions were as follows: 37℃ 15 minutes, 85℃ 5 seconds, then store at 4℃. Reactions were performed using the thermocycler (BIOMETRA T-Gradient Thermoblock).

Real-time RT-PCR Real-time PCR was performed using relative quantification protocol on ABI7500 (Applied Biosystems, USA). The primers used in the QPCR experiment are as follow: for human *MERTK*, 5′-TTTATTCCCGATTGGAGACAGGAC-3′ and 5′-CAG GGCAATATCCACCATGAAC-3′ (77bp); for human β-actin, 5′-TGGCACCCAGCACAA TGAA-3′and5′-CTAAGTC ATAGTCCGCCTAGAAGCA -3′ (186bp) (The primers were designed and composed by Takara Biotechnology Co., LTD). The cDNA was diluted at 1:5 by RNAase free water. The cDNAs were amplified in a 10-μL mixture containing 5μL of SYBR® Premix Ex, 0.2μL *MERTK*/β-actin forward primer (10μmol/L), 0.2μL *MERTK*/β-actin reverse primer (10μmol/L), 0.2μL ROX Reference Dye II (50×) and 1μL cDNA template (diluted at 1:5), according to the manufacturer's protocol (SYBR® Premix Ex Taq™ II (Perfect Real Time), Takara Biotechnology Co., LTD). The cycle conditions were as follows: 95℃ for 1 minute and 95℃ for 30 seconds, followed by 40 cycles of 5 seconds at 95℃ and 34 seconds at 60℃. In addition, a no-template control (ddH_2O) was analyzed for each template. Equation 1[14], as shown below, was applied to calculate the relative expression ratio of the target gene (*MERTK*) in the sample *vs* a control in comparison to a reference gene (β-actin). Equation 1:

Ratio = (Etarget)ΔCTtarget (control-sample)/(Ereference)ΔCTref (control-sample)

ΔCT is the CT (threshold cycle) deviation of the control minus the sample of the target or reference gene transcript. A ratio >2 for mRNA expression of *MERTK* in passaged HRPE cells designated up-regulation, while 0.5≤ratio≤2 for mRNA

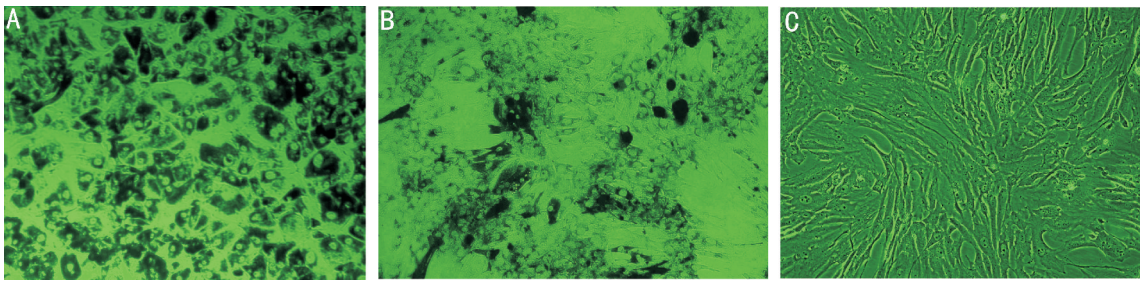

Figure 1 HRPE Cells form of different passages A: P_0 cells(inverted microscope ×100) B: P_1 cells (inverted microscope ×100) C: P_{2-5} cells (inverted microscope ×100).

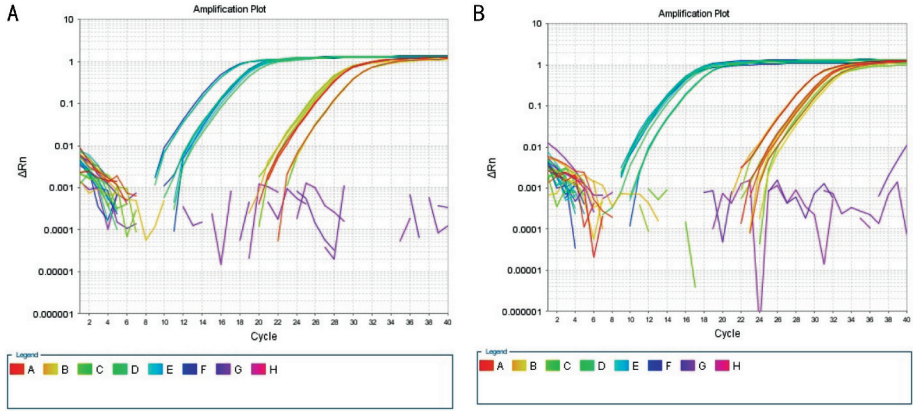

Figure 2 The amplification plot of different passages compared with P_0(β-actin was used as the control gene) A: The amplification plot of P_0, P_1 and P_2; B: The amplification plot of P_0 and P_2- P_5. A-G indicated the different sample: three A, B and C lines indicated three P_0- P_5 sample in each tube; three D, E and F indicated β-actin in each tube; G indicated the negative control (ddH_2O).

Table 1 The expression level of *MERTK* gene in different passage of HRPE

Passage	P_0	P_1	P_2	P_3	P_4	P_5
Ratio	1.0000	0.9108±0.0670	0.0935±0.0458	0.0982±0.0437	0.0627±0.0195	0.0245±0.016
P	—	0.58	<0.05	<0.05	<0.05	<0.05

expression of *MERTK* in passaged HRPE cells designated the same as P_0, a ratio<0.5 for mRNA expression of *MERTK* in passaged HRPE cells designated down-regulation[15,16].

Statistical Analysis The data of *MERTK* gene expression in different passage of HRPE were analyzed by One Way ANOVA, and $P<0.05$ was considered statistically signifcant. All of the test results were analyzed using software SPSS 16.0.

RESULTS

The P_0 cells showed polygon appearance, and a lot of pigment concentrated in the cytoplasm; part of P_1 cells showed polygon appearance and part of P_1 cells showed spindle appearance, less pigment scattered in the cytoplasm; while P_{2-5} cells showed spindle appearance, and none pigment distribute in the cytoplasm.

Real time PCR Compared with P_0 cells, the mRNA level of P_1 almost had no change(the ratio was 0.91, $P=0.58$). While the mRNA level of P_2 decreased significantly, with ratio of about 0.1($P<0.05$). Moreover, the mRNA level of P_{3-5} decreased significantly too, with ratio less than 0.1 ($P<0.05$). (Table 1, Figure 2).

DISCUSSION

The passage cells of RPE were easy to culture, abundant in quantity, and facilitated many sorts of experimental testing, so they were widely used in different kinds of function study of RPE. Furthermore, it was confirmed that the passage cells of RPE expressed the function gene in primary RPE, such as the phagocytic gene *MERTK*, and could phagocyte the outer segments of photoreceptors[11,17]. It was also reported that gene expression changed among 588 known genes of repeated passaged HRPE cells[15], but there were none description of the differences of gene expression among primary and different passage cells so far.

As seen in our research, the morphology of primary and passaged RPE were different: The primary HRPE cells showed polygon appearance, and a lot of pigment concentrated in the cytoplasm; while P_{2-5} cells HRPE cells showed spindle appearance, and none pigment distribute in the cytoplasm. Whether the significant change in morphology among primary and different passage HRPE cells also brought about the alteration in function ?

The results presented here showed that *MERTK* genes expression in P_0- P_5 of HRPE cells changed synchronous with that of morphology, that is, the level of *MERTK* gene expressed in second and primary passage RPE cells were same, while declined significantly in the third passage. In

order to rule out the accident of these changes in gene expression, we repeated the RPE cell culture experiments in eyecups from three different donors, and repeated the gene examination with the same reagent and conditions. The results were completely consistent with before. We further researched the gene expression level of *MERTK* in P_3, P_4 and P_5, and showed that the gene expression level gradually decay as the cell passage increased. Compared with the third passage, the gene expression level of P_3-P_5 weakened inconspicuously.

To avoid of wrong conclusions brought about by the error, firstly, we used SYBR® Premix Ex Taq™ II (Perfect Real Time) to quantify the expression of *MERTK*. The buffer for qPCR could inhibit nonspecific reaction and quantitative target genes accurately. At the same time, to avoid of the error, in the preceding experiments, three separate RNA preparations were made for each passage and carried through the analysis. Therefore, it made sense to treat each sample separately and average the results after the calculation. When replicate PCRs are run on the same sample, it is more appropriate to average CT data before performing calculation. In addition, to minimize the error, statistical data were converted to the linear form by the $2^{-\Delta\Delta CT}$ calculation but not only by presenting the raw CT values[18]. So our results are credible.

As shown in our results, though HRPE cell in vitro expressed the specialfunctional gene, the level of gene expression decreased significantly along with the increasing of passages. This result remind us, although cell number is less than multiple passage cells, the primary or the second passage RPE cells expressed higher level of functional gene, were appropriate for the future RPE transplantation treatment. Whether the change of other functional genes expression of HRPE cells were the same as *MERTK* would reported in our further study.

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