

## Investigation of Melanogenic Factors Gene Expression in Human Adult and Neonate Retinal Pigment Epithelium Cell Cultures

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### Abstract

Retinal pigment epithelium is responsible for maintaining the structural integrity of the retina by an efficient defense against free radicals, photo-oxidative exposure and light energy. For this purpose the main RPE line of defense is melanosomes. Melanin content of retinal pigment epithelium cells in adults and neonates reveals remarkable variations. In the current study we compared melanogenic factors gene expression levels in human adult and neonate RPE cells in culture. RPE cells from adult and neonate human eye globes were isolated and then cultured in DMEM: F12 (1:1) containing 10% FBS. Expression of RPE65 and Cytokeratin 8/18 markers in isolated cells was confirmed by immunocytochemistry. To evaluate the responsible factors in the pathway of melanin biosynthesis, gene expression of orthodenticle homeobox 2, microphthalmia-associated transcription factor A and H as prominent transcription factors, tyrosinase and dopachrome tautomerase as effective enzymes in the melanin biosynthetic pathway were examined in human neonate derived RPE cells compared to human adult derived RPE cells in culture. According to the Real-Time RT-PCR data, gene expression of MITF-H, OTX2, TYR and DCT in RPE cells of the neonates showed a significant increase compared to the adults. With increasing passage number, gene expression of MITF-H, OTX2, TYR and DCT showed remarkable decline. According to the role of OTX2 and MITF-H as the main transcription factor effectors on the TYR and DCT, restoration of OTX2 and MITF-H gene expression may be retain melanin content in RPE cells.

**Keywords:** Human RPE cells, Melanogenic transcription factors, Enzymes

### Introduction

Retinal pigment epithelium (RPE) is a single layer of post-mitotic pigmented cells; RPE layer has key roles in the pathophysiology of several diseases of the eye and vision (Cai et al., 2009; Nussenblatt and Ferris III, 2007; Pfeffer and Philp, 2014). A prominent distinction characteristic of RPE cells is the pigment melanin. Melanin plays an important role in protecting the retina by absorbing radiation and scavenging free radicals and reactive oxygen species. Melanogenesis, is an enzymatic process catalyzed by tyrosinase (TYR), TYR-related protein 1 (Typr1) and dopachrome tautomerase (DCT) that convert tyrosine to melanin pigments. It is presumed that RPE melanogenesis is just prenatally, since TYR was detected only in human early stage embryos and was not detectable after gestation stopped (Schraermeyer et al., 2006; Boulton, 2014; Pfeffer and Philp, 2014). With age

melanin content of RPE cells and its protective properties reduced and resulting in risk of degenerative retinal diseases such as AMD and RP (Boulton 2014; Le et al., 2014; Zarbin et al., 2014). Differentiated RPE cells do not divide and remain functional throughout the life of an individual. However cultured human RPE cells can be grown in large quantities and used in biochemical and functional assays or transplantation studies (Maminishkis et al., 2006; Klimanskaya et al., 2008; Hu and Bok, 2001). Nevertheless, the value of cultured RPE cells depends on its ability to retain functional and genetic characteristics of the native tissue (Strunnikova et al., 2010). Retinal pigment epithelium cell lines do not show features typical of a functional RPE, such as pigmentation and expression of specific markers (Aruta et al., 2011) and deficiency of an *in vitro* system recapitulating all the features of *in vivo* RPE cells has been one of

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the limitations in molecular and functional studies of the RPE. Orthodenticle homeobox 2 (OTX2) is a transcription factor expressed not only at the earliest stage of the eye morphogenesis (Simeone et al., 1992) but also in postnatal RPE and adult eye (Baas et al., 2000). Otx2 controls the expression of several groups of genes involved in RPE-specific functions, especially melanogenesis, pH regulation, retinol metabolism, and metal concentration (Takeda et al., 2003; Housset et al., 2013). Microphthalmia-associated transcription factor (MITF) consists of many isoforms with different N-termini. A, H and D are the main isoforms of this gene in RPE cells (Bharti et al., 2008; Capowski et al., 2014). Many transcriptional targets of MITF suggest its involvement in multiple aspects of melanogenesis (Levy et al., 2006) by regulating the expression of TYR and DCT (Cheli et al., 2010; Ludwig et al., 2004; Raviv et al. 2014). Moreover MITF has been linked to control of gene expressions involved in survival, cell cycle control, and cell morphology (Johnson et al., 2011). Because of the principal impression of MITF-A, MITF-H, OTX2, TYR and DCT in melanin biosynthesis and the key roles of RPE cells in visual function, they have been evaluated in the current study to distinguish their differential gene expression in different cell culture passages and between adult and neonate human RPE cells.

## Materials and Methods

### Cell Culture and Sample Preparation

RPE cells were isolated from neonatal and adult human cadaver globes provided by central eye bank of Iran within 24 hours of death and cultured in DMEM: F12 (1:1) supplemented with 10% FBS. After cultures reached to 80-90% confluency, the cells were passaged. RPE cells' RNA was extracted and was reverse transcribed using cDNA synthesis kit and subjected to amplification by Real-Time PCR.

### Cell Identification

Cell cultures were evaluated by means of morphology and expression of characteristic molecular markers. All the cultures were considered under light microscope in terms of morphology. Then RPE cells were cultured on coverslips in 24-well plate. Coverslips were fixed by  $-10^{\circ}\text{C}$  methanol for 10 minutes. The cells were made permeable using Teriton x-100 (0.25%) and cultures were blocked in 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Specific mouse anti-human cytokeratine 8/18

monoclonal antibody (1:1000; Santa Cruz, USA) for epithelial characteristics confirmation of isolated cells and rabbit anti-human RPE65 polyclonal antibody (1:100; Santa Cruz, USA) against the specific protein of RPE microsomal membranes were utilized to confirm the RPE characteristics of isolated cells. Fluorescein isothiocyanate (FITC) conjugated antibodies (goat anti-mouse IgG, goat anti-rabbit IgG, Santa Cruz, USA) against the above-mentioned antibodies were diluted (1:400) and used to detect immunoreactivity of cultures to primary antibodies. After final washing, the slides were incubated with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (1.5 mg/ml, Santa Cruz, USA) for 10 minutes in order to stain nuclear DNA. The slides were examined by Axiophot Zeiss fluorescence microscope (Zeiss, Germany) equipped with a 460 nm filter for DAPI dye and a 520 nm filter for FITC conjugated antibodies.

### Real-time RT-PCR

Total RNA was extracted from RPE cells in each examined passage (between the first and the 7th passages) by RNA extraction kit (Qiagen, Germany). Concentration and purity of the isolated RNA were determined using spectrophotometric analysis and the integrity of the RNA was verified by means of electrophoresis in an agarose gel followed by ethidium bromide staining. Reverse transcription reaction was performed with oligo dT primers and a superscript reverse transcriptase kit (Promega, USA). Then quantitative real-time RT-PCR was performed by a SYBR Green QPCR master mix (Roche, USA). PCR parameters were initial denaturation (one cycle at  $95^{\circ}\text{C}$  for 10 minutes); denaturation, amplification and quantification for 40 cycles at  $95^{\circ}\text{C}$  for 30 seconds,  $52-60^{\circ}\text{C}$  for 17 seconds, and  $72^{\circ}\text{C}$  for 25 seconds; melting curve,  $65^{\circ}\text{C}$ , with the temperature gradually increased ( $0.5^{\circ}\text{C}$ ) to  $95^{\circ}\text{C}$ . mRNA expression was normalized to the levels of GAPDH mRNA, and the changes were calculated according to standard curve and efficiency (E) for each primer. Sequences of primers used for real-time PCR were shown in table 1.

### Statistical Analysis

3 neonate globs and 2 adult globs were included in this study. The real-time RT-PCR analysis was performed at least in 3 independent experiments. Each sample was run and examined in duplicate. Differences between groups (different passages in neonates and adults) were analyzed using the t-test.  $P < 0.05$  was considered statistically significant.

Table 1. Primer Sequences

Sequence definition	Sense primer	Anti-sense primer
MITF-A	AAGTCGGGGAGGAGTTTCAT	CGTAGCAAGATGCGTGATGT
MITF-H	TTCAGATGTTTCATGCCATGCTC	GCGTAGCAAGATGCGTGATG
OTX2		QT00213129
TYR		QT00080815
DCT		QT00033523
GAPDH		QT01192646

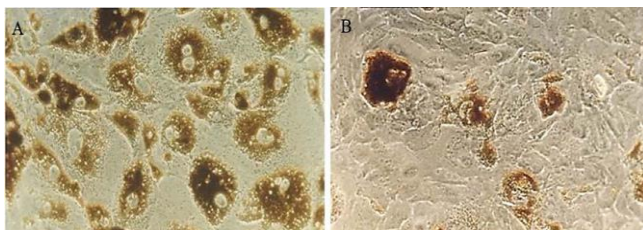
\*Sequence of primers for MITF-A and MITF-H have been shown.

For OTX2, TYR, DCT and GAPDH predisigned primers were bought with the presented cat. numbers.

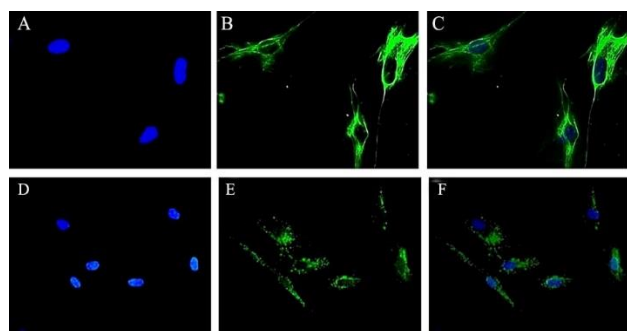
## Results

### Isolated Cells Exhibited Specific Markers of RPE Cells

In terms of morphology, confluent monolayers of RPE cell cultures exhibited epithelial morphology and heavy pigmentation (Figure. 1). ICC revealed that the isolated cells expressed both cytokeratin 8/18 and RPE65, confirming their identity as RPE cells (Figure. 2).



**Figure 1.** Human cultured RPE cells grew as a monolayer of cuboidal epithelial cells arranged in a regular hexagonal pattern (magnification: (A) 10x40, (B) 10x20).



**Figure 2.** Human cultured RPE cells subjected to ICC with cytokeratin 8/18 and RPE65 antibodies. (A) Nuclei stained blue with DAPI. (B) The RPE cells stained positively for the FITC-conjugated cytokeratin 8/18 antibody (green). (C) Merged image (FITC-labeled cytokeratin 8/18 and DAPI) (magnification: 10x40). (D) DAPI-stained RPE cell nuclei (blue). (E) The RPE cells stained positively for the RPE65 antibody (green). (F) Merged image (FITC-labeled RPE65 and DAPI) (magnification: 10x20).

### Neonate RPE Cells Revealed Different Patterns of Melanin Biosynthesis Responsible Factors

MITF-A gene expression did not alter at different passages of neonate samples (Figure. 3A). MITF-H decreased at passage 7 in neonate RPE cells when compared to, specially, passage 1 (Figure. 3B). OTX2 significantly was decreased in passages 4-7 of neonate samples compared to the other passages and specially passage 1 and 2 (Figure. 3C). TYR gene expression decreased at passages 4-7 in neonate samples compared to the other passages and specially passage 1 (Figure. 3D).

DCT was remarkably decreased at passages 2-7 compared to the passage 1 of neonate samples (Figure. 3E).

### Adult RPE Cells did not Show Significant Variance of Melanogenic Factors Gene Expressions

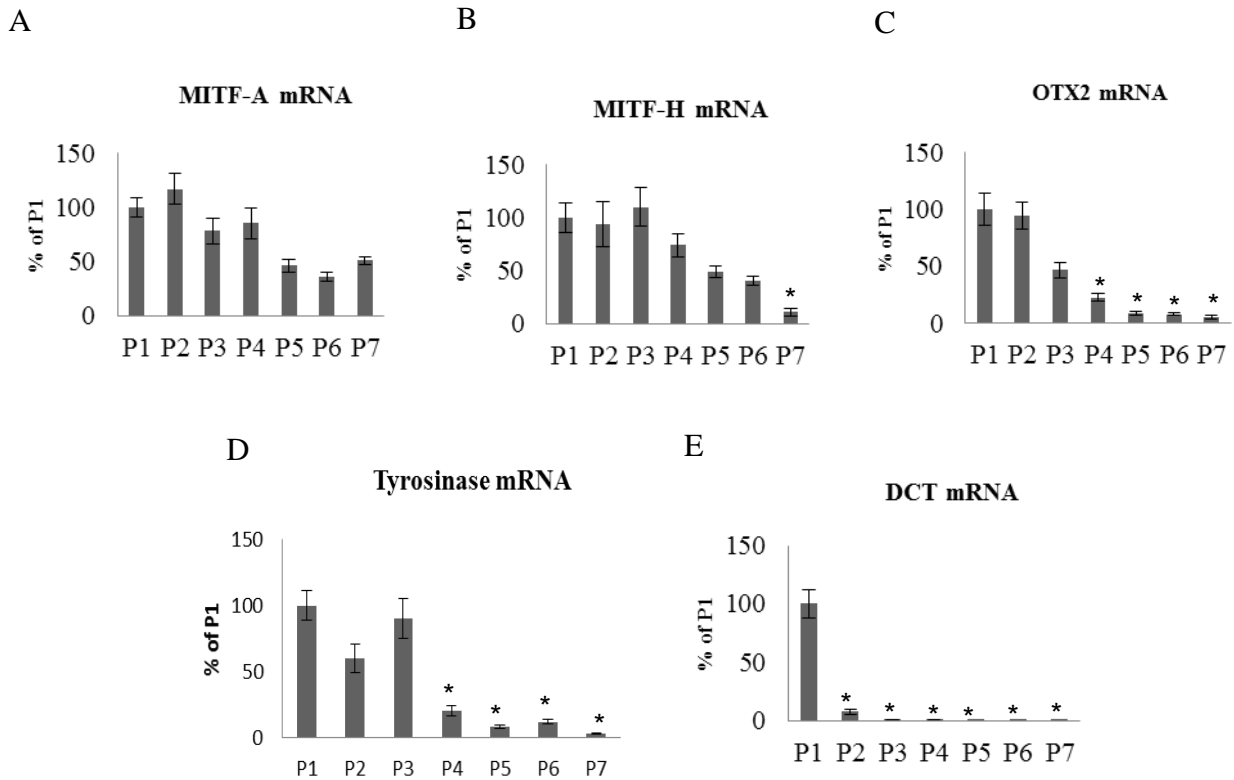
MITF-A (Figure. 4A), MITF-H (Figure. 4B), OTX2 (Figure. 4C), TYR (Figure. 4D) and DCT (Figure. 4E) gene expression did not alter at different passages of adult samples.

### Cultured Neonate and Adult RPE Cells Manifest Significant Variance of Melanogenic Factors

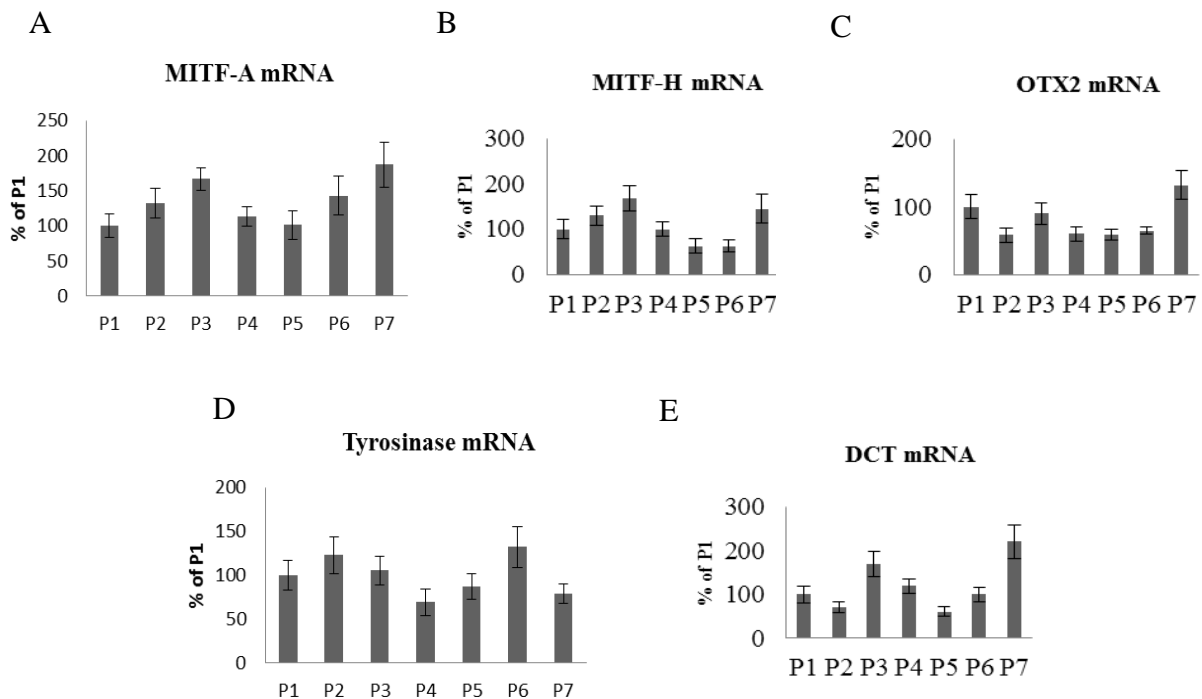
Neonate gene expression of MITF-A at different passages was not significantly different in adults RPE cell cultures (Figure. 5A).

MITF-H (Figure. 5B), OTX2 (Figure. 5C) and TYR (Figure. 5D) gene expression were significantly increased at passages 1, 2 and 3 of neonates compared to the adults.

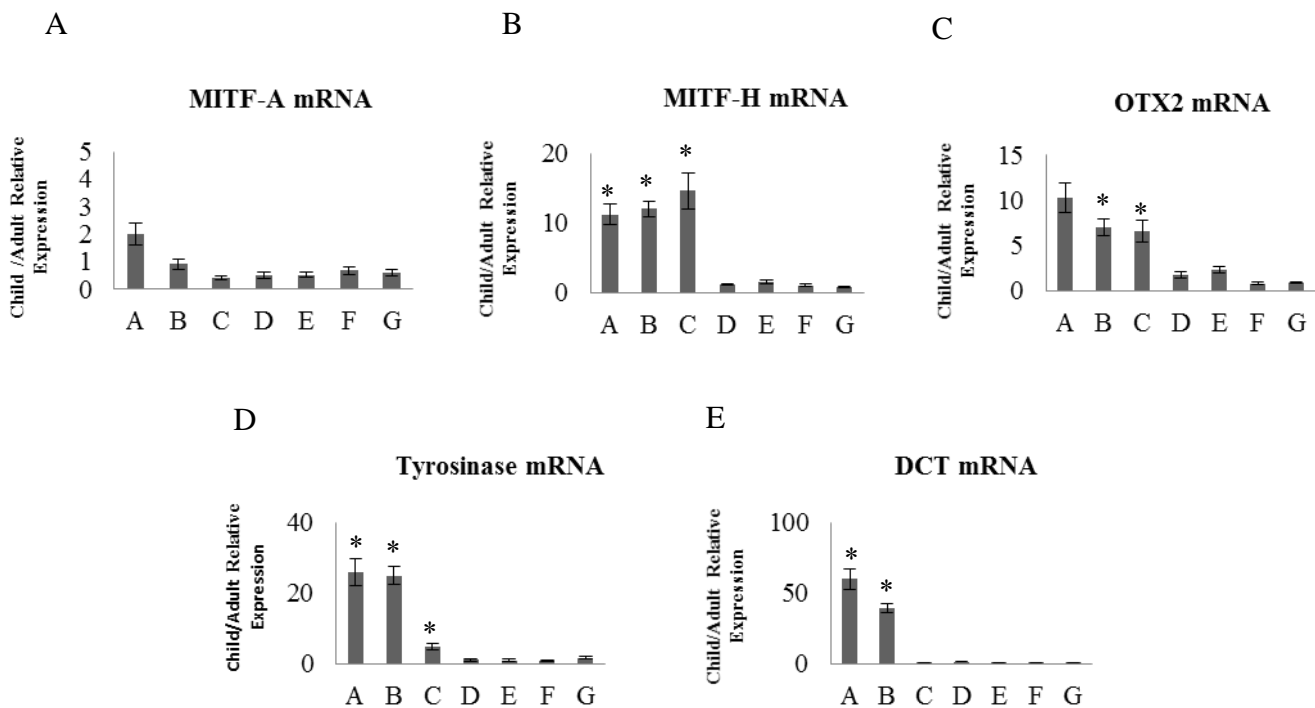
DCT remarkably increased at passages 1 and 2 of neonates compared to the adults RPE cultures (Figure. 5E).



**Figure 3.** Quantitative real-time RT-PCR analysis of MITF-A, MITF-H, OTX2, TYR and DCT in passages 1-7 of cultured neonate RPE cells. RPE cell preparation and RNA extraction were performed as described in methods. Relative gene expression was determined by quantitative real-time PCR. mRNA levels were normalized to GAPDH and presented as percentages of control values. (B) MITF-H at passage 7, (C) OTX2 at passages 4-7, (D) TYR at passages 4-7 and (E) DCT at passages 2-7 decreased compared to the other passages specially regarding passage 1. \*P < 0.05



**Figure 4.** Quantitative real-time RT-PCR analysis of MITF-A, MITF-H, OTX2, TYR and DCT in passages 1-7 of cultured adult RPE cells. RPE cell preparation and RNA extraction were performed as described in methods. Relative gene expression was determined by quantitative real-time PCR. mRNA levels were normalized to GAPDH and presented as percentages of control values. Gene expression of (A) MITF-A, (B) MITF-H, (C) OTX2, (D) TYR and (E) DCT did not alter in different passages of adult samples. \*P < 0.05.



**Figure 5.** Comparison of MITF-A, MITF-H, OTX2, TYR and DCT gene expression in passages 1-7 of cultured neonate RPE cells compared to adult RPE cells. (B) MITF-H, (C) OTX2 and (D) TYR gene expression were significantly increased at passages 1, 2 and 3 of neonates compared to the adult samples, while (E) DCT remarkably increased at passages 1 and 2 of neonates compared to the adult samples. \*P < 0.05.

## Discussion

In the present study we declared that gene expression of MITF-H in higher passages of neonate samples significantly decreased and subsequently gene expression of TYR and specially DCT enzymes was reduced. As a result, overexpression of MITF-H in RPE cell cultures could be a solution to retain the features of RPE in its *in vivo* circumstance and growing native RPE cells in large quantities for studying their biology, biochemical features and functional properties. OTX2 is another transcription factor which plays important functions in the melanin synthesis. OTX2 was identified in nucleus of RPE cells. It in collaboration with MITF, or alone, regulates expression of principal RPE cell genes. Overexpression of OTX2 in neural retina induces pigmentation phenotype, this factor binds to the promoter of TYR and DCT and activates their expression (Takeda et al., 2003; Martínez-Morales et al., 2003; Reinisalo et al., 2012). Our results showed gene expression of OTX2 significantly decreased at earlier passages of neonate samples and this decrease was prominently more than MITF-A and MITF-H. Gene expression

of TYR and DCT decreased at early passages too. Expression of TYR and DCT is presumably primarily under regulation of OTX2 more than two other aforementioned transcription factors. Human RPE cells contain a large amount of melanin, which is produced during the prenatal period but polymerization of melanin continues to occur in melanosomes until turning to approximately two years old, when the RPE contains only mature melanosomes (Boulton and Dayhaw-Barker, 2001; Boulton, 2014). No melanin production could be demonstrated *in vitro* and *in vivo* status of RPE cells after birth. Humans have a substantial amount of melanin in their RPE cells. Melanin content of RPE cells decreases with increasing age and sharply reduces their protective working and may be responsible for the occurrence of various retinal diseases like AMD, the leading cause of irreversible blindness among the elderly in industrialized nations (Panda-Jonas et al., 1996). Melanin content in cultured RPE cells also decreased rapidly pursuant to cell division. In normal culture conditions, RPE cells lose many traits that are important for the proper



functioning of vision, including bearing melanin. Melanin content of cultured RPE cells decreases with increasing passage number, so that the cells in the higher passages have a little melanin content (Lu et al., 2007; Pfeffer and Philp, 2014).

Some reports showed that adult human RPE cells cultured with Ca<sup>++</sup>-switch method (low Ca<sup>++</sup>) expressed TYR and produced melanin (Rak et al., 2006). Expression of various genes in RPE cells, including TYR and its enzymatic activity are induced in cultured human adult RPE by phagocytosis of ROS (Julien et al., 2007; Chowers et al., 2004).

Our Results showed among MITF-A, MITF-H and OTX2 transcription factors, gene expression of MITF-H and OTX2 was significantly dissimilar between different passages of neonate and adult RPE cells in culture.

For researches who study RPE cells biology MITF have been the center of attention because it is involved not only in the induction of pigmentation but also in regulating cell proliferation, structure of the cytoskeleton and cell differentiation. It is called as one of the key elements in RPE cell behaviors (Tsukiji et al., 2009). MITF affects gene expression of DCT and TYR enzymes positively and its reduction will negatively affect gene expression of mentioned enzymes and therefore melanin content (Yasumoto et al., 1997).

RPE cells display prominent role in defense against free radicals and photo-oxidative exposure and light energy. Melanosomes are the key players in RPE cells preservative role. Our data showed variation of OTX2 and MITF-H transcription factors gene expression especially OTX2 is similar to melanogenic enzymes gene expression and proposed restoration of MITF-H and especially OTX2 gene expression in adult RPE cells may culminate to retain melanin content of the cells by recovering key enzymes in melanin synthesis pathway, e.g. TYR and DCT. Quantitative studies on the expression of various transcription factors and enzymes, relevant to the melanogenesis, combined to the rate of melanin production, may provide the *in vitro* models for studying melanogenesis of human RPE cells and could be used in experiments/purposes that require natively pigmented RPE cells.

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