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MITF acts as an anti-oxidant transcription factor to regulate mitochondrial biogenesis and redox signaling in retinal pigment epithelial cells

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ABSTRACT

There is increasing evidence that the mechanisms protecting the retinal pigment epithelium (RPE) against oxidative stress are important for preventing retinal degenerative diseases. Little, however, is known about these mechanisms. Here we show that MITF, a transcription factor responsible for RPE development and function, regulates redox signaling by acting through PGC1α, a master regulator of mitochondrial biogenesis. *Mitf* deficiency in mice leads to significantly higher levels of reactive oxygen species (ROS) in both RPE and retina, suggesting that *Mitf* dysfunction might lead to oxidative damage in the RPE and, by extension, in the retina. Furthermore, overexpression of MITF in the human RPE cell line ARPE-19 indicates that MITF up-regulates antioxidant gene expression and mitochondrial biogenesis by regulating PGC1α and protects cells against oxidative stress. Our findings provide new insights into understanding the redox function of MITF in RPE cells and its potential contribution to prevention of RPE-associated retinal degenerations.

1. Introduction

The retinal pigment epithelium (RPE) is a densely pigmented and highly polarized monolayer of cells that plays critical roles in eye development, retinal homeostasis, and vision. RPE cell damage or dysfunction induces photoreceptor dystrophy, retinal diseases, and blindness. In addition to its structural role, RPE cells absorb scattered light, secrete growth factors, phagocytose shed photoreceptor outer segments, participate in the visual cycle, function as a blood-retinal barrier by controlling nutrient and metabolite transport between the choriocapillaris and the neural retina, and provide anti-oxidant functions (Linsenmeier and Padnick-Silver, 2000; Strauss, 2005; Young and Bok, 1969). Consequently, genetic defects or pathological abnormalities in the RPE may lead to secondary damage of photoreceptors and cause retinal dysfunction. In fact, oxidative stress and metabolic dysregulation of RPE cells play vital roles in the pathogenesis of age-related macular degeneration (AMD), the main cause of visual impairment for elderly individuals in developed countries (Ambati and Fowler, 2012; Beatty et al., 2000; Raymond and Jackson, 1995; Sparrow et al., 2010).

Many studies provide compelling evidence that reactive oxygen species (ROS), which are toxic substances mainly generated under oxidative stress conditions, are crucial in the pathogenesis of a variety of retinal diseases, including AMD, diabetic retinopathy (DR) and retinopathy of prematurity (Eshaq et al., 2014; Kowluru, 2005; Schrag et al., 2013; Tokarz et al., 2013). Because the RPE is highly active metabolically, has a high energy demand, and contains a large number of mitochondria, it contributes high levels of free radicals (Alder and Cringle, 1985; Cai et al., 2000; Kaarniranta et al., 2010) and is the prime target of oxidative damage. Recent research has shown, however, that the RPE has also evolved effective defense mechanisms against such damage. For instance, miR-23a (Lin et al., 2011), MMP-3, MMP-9 (Juuti-Uusitalo et al., 2015), MMP-14, TIMP-2 (Alcazar et al., 2007), p38 MAPK-dependent pathways (Qian et al., 2011), and ERK1/2 signaling (Glotin et al., 2006) have all been shown to participate in the regulation of the response of RPE cells to oxidative stress. It is thought that these antioxidant mechanisms in the RPE are also instrumental to counterbalance the oxidative stress of the metabolically highly active retina (Simó et al., 2010).

The RPE is regulated, both before and after birth, by a variety of transcription factors. Among them is the microphthalmia-associated

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transcription factor (MITF), which is expressed in RPE cells (Bharti et al., 2008, 2012; Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000) but also plays roles in many other cell types (Hou and Pavan, 2008). Human genetic studies have revealed that biallelic *MITF* mutations result in microphthalmia, albinism, coloboma, osteopetrosis, macrocephaly, and deafness (George et al., 2016). Many of these severe phenotypes correspond to those seen in *Mitf* mutant mice (Arnheiter, 2010; Hodgkinson et al., 1993; Nakayama et al., 1998; Steingrímsson et al., 1994). Nevertheless, we still know little about how *Mitf* deficiencies contribute to retinal degeneration. Our previous studies have demonstrated that MITF regulates the expression of genes encoding growth factors and proteins controlling the visual cycle and cell proliferation in the RPE (Ma et al., 2012, 2017a, 2017b; Wen et al., 2016). Our work also suggested that the regulation of visual cycle genes by MITF may be involved in retinal degeneration (Wen et al., 2016).

In the present study, we find that ROS production is dramatically increased in *Mitf-/-* retinas, suggesting that *Mitf* deficiency-associated retinal degeneration may in part be due to oxidative damage in the RPE. We further provide evidence that overexpression of MITF significantly protects ARPE-19 cells against oxidative stress in vitro. Moreover, we find that MITF regulates mitochondrial biogenesis and acts by targeting PGC1α (peroxisome proliferator-activated receptor-gamma coactivator 1 alpha) as it does in melanoma cells (Haq et al., 2013). We conclude that the MITF-PGC1α axis is an important pathway to regulate mitochondrial biogenesis in the RPE.

2. Materials and methods

2.1. Animals

C57BL/6J, *Mitf*$^{mi\text{-}vga-9}$ mutant mice (Hodgkinson et al., 1993), here designated *Mitf-/-* mice, were kept on a C57BL/6J background and bred in the pathogen-free facility of Wenzhou Medical University. None of the mice used in this study contain the rd8 mutation of the Crb1 gene (Mattapallil et al., 2012). All animal care and experimental procedures were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the Wenzhou Medical University Animal Care and Use Committee.

2.2. Histological analysis of wildtype and Mitf-/- retina

Eyeballs from P21 C57BL/6J mice and age-matched *Mitf-/-* mice were fixed at 4 °C for 24 h in a tissue fixative solution (2 parts 37% formaldehyde, 3 parts 100% ethanol, 1 part glacial acetic acid, and 3 parts water). Paraffin-embedded sections were stained with hematoxylin and eosin (HE). After mounting, images were obtained using a Zeiss microscope.

2.3. In situ tissue ROS assay

ROS production was assessed with a GENMED kit (Genmed Scientifics Inc., Shanghai, China) according to the standard protocol. Briefly, the freshly enucleated eyes were embedded in Tissue Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Frozen cross sections (10 μm) were washed and incubated in a light-protected moist chamber at 37 °C for 30 min. After mounting, images were photographed.

2.4. Cell culture and viability

The ARPE-19 cell line was purchased from ATCC and cultured in DMEM/F12 culture medium supplemented with 10% FBS. MITF-overexpressing ARPE-19 cells (ARPE-19 + MITF) were produced as described (Ma et al., 2012). The passage number of ARPE-19 cells used in this work was less than 35. 293T and HeLa cells were cultured in DMEM growth medium with 10% FBS. All cells were incubated in a humidified incubator at 37 °C in 5% CO2.

For cell viability assays, the cells were seeded into 96-well plates at a concentration of 1×10^4 cells per well and grown to full confluency. Oxidative stress was induced by treatment with freshly prepared H_2O_2 or glucose oxidase (GOX) at various concentrations as indicated. After treatment with H_2O_2 or GOX for 6 h, the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) Cell Proliferation, Trypan Blue staining and Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, China) were used to investigate cell proliferation and viability according to standard protocols.

2.5. Reagents and antibodies

H_2O_2 and glucose oxidase (GOX) were purchased from Sigma–Aldrich. Antibodies to COX IV, PGC1α and MITF were purchased from Abcam. The goat anti-mouse IgG alexa Fluor® 488 (Life Technologies, A11029) and goat anti-rabbit IgG alexa Fluor® 594 (Life Technologies, A11037) were purchased from Life Technologies.

2.6. LDH release assay

LDH release was assayed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. In brief, following an 8-h exposure to H_2O_2 or GOX, the culture supernatant (50 μL) containing released lactate dehydrogenase (LDH) was transferred to the wells of a 96-well plate and incubated at 37 °C for 30 min with an equal volume of LDH reaction mixture, wrapped with aluminum foil to avoid light-induced decomposition. Controls (untreated cells, maximum LDH release obtained by lysis of the cells with the Lysis Solution) were included in each plate. LDH release was quantified by measuring the absorbance at 490 nm using an automatic microplate reader. Triplicate runs were performed throughout.

2.7. Measurement of ROS

In situ ROS generation was detected by dihydroethidine (DHE, Beyotime Institute of Biotechnology, China) or MitoSOX Red (Invitrogen, Carlsbad, CA, USA). The DHE assay was performed following the manufacturer's protocol. Briefly, after treatment with 0.65 mmol/L H_2O_2 for 4 h, cells were rinsed once with DMEM/F12 medium and incubated with 5 μM DHE in a light-protected humidified chamber at 37 °C for 30 min, washed, and analyzed using a fluorescence microscope equipped with filters allowing excitation at a wavelength of 325 nm and emission at a wavelength of 610 nm.

2.8. In situ and flow cytometry for Annexin V-PE binding to phosphatidylserine

For detection of cell death, Annexin V-PE Apoptosis Detection Kit (Beyotime Institute of Biotechnology) was used according to the manufacturer's protocol. Briefly, following 6 h of H_2O_2 (0.65 mmol/L) treatment, approximately 2×10^6 cells were collected and washed twice with ice-cold PBS, and then incubated for 25 min at room temperature in the dark in a solution containing fluorescein-conjugated Annexin V–PE. The cells were then put on ice in the dark, and the percentage of apoptotic cells and necrotic cells was determined by FACS analysis. For in situ analysis, equal numbers (5×10^5) of cells were seeded into 6-well plates and treated for 6 h with H_2O_2 (0.65 mmol/L). The cells were then washed twice with ice-cold PBS and incubated as mentioned above. Images were obtained using a Zeiss microscope.

2.9. Real-time PCR, western blotting and immunostaining

The methods for real-time PCR, western blotting and immunostaining were as described previously (Ma et al., 2012, 2017). PCR primer sequences are shown in Supplemental Table 1.