



# Alpha-melanocyte stimulating hormone protects retinal pigment epithelium cells from oxidative stress through activation of melanocortin 1 receptor–Akt–mTOR signaling



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## ABSTRACT

Patients with age related macular degeneration (AMD) will develop vision loss in the center of the visual field. Reactive oxygen species (ROS)-mediated retinal pigment epithelium (RPE) cell apoptosis is an important contributor of AMD. In this study, we explored the pro-survival effect of  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) on oxidative stressed RPE cells. We found that  $\alpha$ -MSH receptor melanocortin 1 receptor (MC1R) was functionally expressed in primary and transformed RPE cells. RPE cells were response to  $\alpha$ -MSH stimulation.  $\alpha$ -MSH activated Akt/mammalian target of rapamycin (mTOR) and Erk1/2 signalings in RPE cells, which were inhibited by MC1R siRNA knockdown.  $\alpha$ -MSH protected RPE cells from hydrogen peroxide ( $H_2O_2$ )-induced apoptosis, an effect that was almost abolished when MC1R was depleted by siRNA.  $\alpha$ -MSH-mediated S6K1 activation and pro-survival effect against  $H_2O_2$  was inhibited by Akt inhibitors (perifosine, MK-2206 and LY294002). Further, mTOR inhibition by rapamycin, or by mTOR siRNA knockdown, diminished  $\alpha$ -MSH's pro-survival effect in RPE cells. Thus, Akt and its downstream mTOR signaling mediates  $\alpha$ -MSH-induced survival in RPE cells. In summary, we have identified a new  $\alpha$ -MSH–MC1R physiologic pathway that reduces  $H_2O_2$ -induced RPE cell damage, and might minimize the risk of developing AMD.

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## 1. Introduction

Age-related macular degeneration (AMD) is a progressive retinal degeneration disease, and is important contributor of blindness in elderly people [1]. The precise pathological causes of AMD are still not fully understood, although sunlight ultraviolet (UV) exposure and reactive oxygen species (ROS) damage have been proposed [2,3].

**Abbreviations:** AMD, age-related macular degeneration; RPE, retinal pigment epithelium;  $\alpha$ -MSH,  $\alpha$ -melanocyte stimulating hormone; mTOR, mammalian target of rapamycin; MC1R, melanocortin 1 receptor;  $H_2O_2$ , hydrogen peroxide.

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Under oxidative stress conditions, highly reactive free radicals including superoxide, hydroxyl radical, and singlet oxygen and hydrogen peroxide ( $H_2O_2$ ) cause retinal pigment epithelium (RPE) cell damage by destroying cellular components through excessively oxidation [2,3]. Clinical trial studies showed a dramatic reduction AMD progression in subjects taking anti-oxidants or zinc-containing supplements [4,5]. Thus, limiting oxidative stress may represent an effective strategy to slow or even reverse AMD. For pre-clinical studies,  $H_2O_2$  has been added to cultured RPE cells to create a cellular model of AMD [6,7]. Our previous study demonstrated that nerve growth factor (NGF) rescued oxidative stressed-RPE cells by restoring pro-survival Akt–mammalian target of rapamycin (mTOR) signaling activation [6].

The melanocortin  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) is an important paracrine/autocrine regulator of cutaneous pigmentation [8,9]. It is also an endogenous cytokine that suppresses the inflammation [10,11]. The above actions of  $\alpha$ -MSH are mediated by binding and activating the melanocortin 1 receptor (MC1R), a  $G_s$  protein-coupled receptor with seven trans-membrane

domains [12]. Recent studies have identified a novel role for  $\alpha$ -MSH as a pro-survival factor [13–15].  $\alpha$ -MSH rescues human melanocytes from DNA damage caused by UV radiation [13–15]. The pro-survival effect of  $\alpha$ -MSH is mediated by the repair of DNA photoproducts and inhibiting ROS generation [13,15], and by activating pro-survival Akt signaling [14].

In light of the broad expression of MC1R by various cell types, and the fact that RPE cells are responsible to  $\alpha$ -MSH [16], we hypothesized that  $\alpha$ -MSH could exert a protective effect against oxidative stress in RPE cells. In this study, we explored the effect of  $\alpha$ -MSH on  $H_2O_2$ -treated RPE cells. We identified a new  $\alpha$ -MSH-mediated pro-survival pathway that reduces  $H_2O_2$ -induced RPE cell damage and may minimize the risk of developing AMD.

## 2. Materials and methods

### 2.1. ARPE-19 and HLECs culture

Human retinal pigment epithelial (RPE) cells (ARPE-19 cell line) were maintained as reported [7]. Human lens epithelial cells (HLECs) were maintained in the same medium as ARPE-19 cells.

### 2.2. Primary mouse RPE cell isolation and culture

As reported [7], C57/B6 mice at age of 3–5 d was anesthesia by 75% alcohol, and the eyeballs in asepsis were taken out and diluted for several times with D-hank's fluid. After soaking in the DMEM/F-12 (Hyclone CO., USA) for 6 h, the eyeballs were taken out and the retinas were striped carefully. 0.125% parynzyme was added to digest for 20 min at 37 °C before adding culture medium (Lanzhou, China) containing blood serum to terminate digestion. Then the supernatant was centrifuged twice at 1000 r/min in the culture medium (80% DMEM/F-12, 20% fetal serum) to produce cell suspension after inoculation into the 75 cm<sup>2</sup> culture flask. Cells were divided and were used for designed experiments.

### 2.3. Reagents and chemicals

$H_2O_2$ ,  $\alpha$ -MSH, insulin and rapamycin were purchased from Sigma (St. Louis, MO). Perifosine, MK-2206 and LY294002 were purchased from Selleck (Shanghai, China). Antibody against MC1R was purchased from Santa Cruz Biotech. (CA, USA). All other phosphorylation and non-phosphorylation kinase antibodies used in this study were obtained from Cell Signaling Tech (Danvers, MA).

### 2.4. Cell survival assay

RPE cell survival was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method as previously reported [7,17].

### 2.5. Western blots

Western blots were performed as previously reported [7,17]. Band intensities in the immunoblots were quantified by densitometry using the ImageJ software.

### 2.6. TUNEL staining

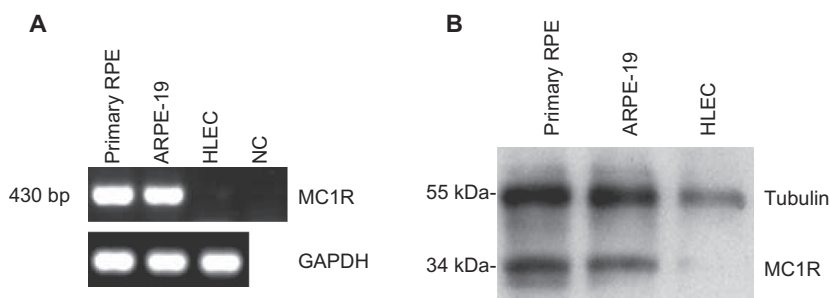
RPE cell apoptosis was detected by the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions [7]. RPE cells were also stained with 4',6'-diamino-2-phenylindole (DAPI, blue fluorescence; Molecular Probes) to visualize the cell nuclei. Apoptosis intensity was determined by TUNEL percentage, which was calculated by the number of TUNEL positive cells divided by the number of DAPI stained cells. At least 1000 total cells in 10 views from 10 repeat wells (1\*100) of each condition were included for counting TUNEL positive cells, and the average was calculated.

### 2.7. RT-PCR

PCR was performed in a thermocycler (Astec, Tokyo, Japan) as follows: 94 °C, 5 min; followed by 35 amplification cycles (94 °C for 30 s; 60 °C for 1 min; 72 °C for 1 min). The sequence of the primers for MC1R are follows: the upper strand, GTG AGT CTG GTG GAG AAT GTG; and the lower strand, TTT TGT GGA GCT GGG CAA TGC (for both human and mice) [18]. For GAPDH: F:GAAGTGAAGGTCGGAGTC, R:GAAGATGGTGATGGGATTTC. The PCR products were electrophoresed in 1.2% agarose gel containing ethidium bromide.

### 2.8. RNA interference (RNAi)

As reported [19], two custom SMART pool<sup>®</sup> RNAi duplexes targeting human MC1R (MC1R siRNA-1 and MC1R siRNA-2) were chemically synthesized by Dharmacon Research (Lafayette, CO). siRNA against mTOR was purchased from Cell Signaling Tech (Shanghai, China). 50 × 10<sup>4</sup> ARPE-19 cells were seeded with 60–70% confluence. For RNAi transfection, 12  $\mu$ l of FuGene6 (Roche Diagnostics, Indianapolis, IN) was diluted in 88  $\mu$ l of DMEM for 5 min at room temperature. Then 10  $\mu$ l of 20  $\mu$ M double-stranded siRNA or the scramble siRNA was mixed with DMEM containing FuGene6 and incubated for 30 min at room temperature. The transfection complex was then added to the well containing 1 ml medium, with the final siRNA concentration of 200 nM. Transfection would take 48 h. Afterwards, target protein expression was determined by Western blot.



**Fig. 1.** MC1R mRNA and protein expression in primary cultured RPE cells and ARPE-19 cells. (A) RNA was extracted from primary PRE cells, ARPE-19 cells and HLECs, and subjected to PCR amplification, using primers specific for MC1R. (B) Protein expressions of MC1R and tubulin in above cells. Experiments were repeated three times to insure consistency of results.

### 2.9. Statistical analysis

All data were normalized to control values of each assay, and were presented as mean  $\pm$  standard deviation (SD). Data were analyzed by one-way ANOVA followed by a Scheffe's *f*-test by using SPSS software (SPSS Inc., Chicago, IL, USA). Significance was chosen as  $p < 0.05$ .

## 3. Results

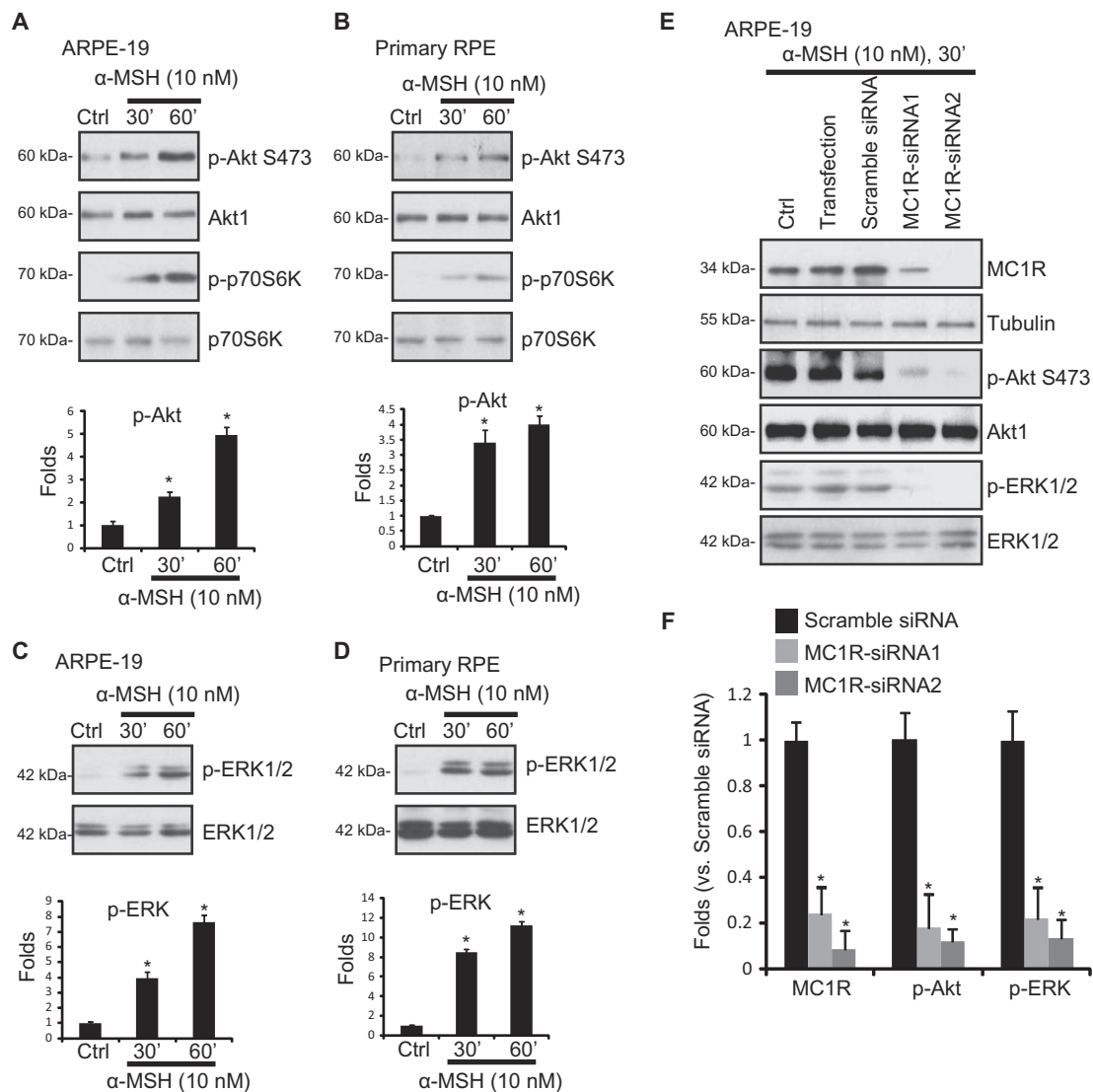
### 3.1. $\alpha$ -MSH receptor MC1R is expressed in cultured RPE cells

MC1R is predominantly expressed in melanocytes, where it is known for its classic role in regulating skin pigmentation [12,14,18]. MC1R has also been reported to be expressed in other tissues and cells, including the pituitary and leukocytes, mast cells and pro-monocytes [20], indicating its putative physiological roles yet to be unveiled. Previous studies have demonstrated that RPE cells are responsible to  $\alpha$ -MSH [16], and  $\alpha$ -MSH stimulates prostaglandin production in cultured RPE cells [16]. Thus, we first

checked the expression of MC1R, the  $\alpha$ -MSH receptor, in RPE cells. RT-PCR and Western blot results in Fig. 1A and B confirmed MC1R expression in primary RPE cells and transformed RPE cells (ARPE-19 line). Note that we failed to detect any expression of MC1R in human lens epithelial cells (HLECs [21,22]) (Fig. 1A and B). These results confirmed MC1R expression in cultured RPE cells.

### 3.2. MC1R in RPE cells is responsible to $\alpha$ -MSH

Stimulation of MC1R by  $\alpha$ -MSH activates multiple signal pathways including cAMP/PKA, PI3K/Akt/mTOR, ERK/mitogen-activated protein kinase (MAPK) [23] and phospholipase C $\gamma$  (PLC $\gamma$ ) pathway [8,24–27]. Of which, activation of PI3K/Akt/mTOR pathway is a well established pro-survival signaling [24]. Above results in Fig. 1 confirmed MC1R expression in cultured RPE cells, we then examined whether this receptor was functional. We treated RPE cells (both primary cells and ARPE-19 cells) with  $\alpha$ -MSH and tested the activation of downstream signalings including Akt/mTOR and Erk. Activation of Akt was reflected by Akt phosphorylation at Ser 473, and activation of mTOR was reflected by phosphorylation of



**Fig. 2.** MC1R in RPE cells is responsible to  $\alpha$ -MSH. Primary cultured RPE cells and ARPE-19 cells were either left untreated ("Ctrl") or treated with  $\alpha$ -MSH (10 nM) for indicated time, activation of Akt/mTOR (A and B) and Erk (C and D) was detected by Western blot using indicated antibodies. Akt phosphorylation (Ser 473) and Erk1/2 phosphorylation (Thr 202/Tyr 204) were quantified. ARPE-19 cells transfected with scramble or MC1R siRNAs (MC1R siRNA-1 or -2) (200 nM each) were treated with  $\alpha$ -MSH (10 nM) for 30', activation of Akt and Erk was detected by Western blot (E and F). Experiments were repeated three times to insure consistency of results. \* $p < 0.05$  vs. Ctrl or scramble siRNA group.

S6K1 (Thr 389). Western blot results in Fig. 2A and B demonstrated a significant Akt and mTOR activation in  $\alpha$ -MSH-stimulated RPE cells. Meanwhile, Erk phosphorylation was also increased after  $\alpha$ -MSH treatment in RPE cells (Fig. 2C and D). Importantly, in ARPE-19 cells, knocking-down of MC1R by two non-overlapping siRNAs significantly inhibited MSH-induced Akt and Erk activation (Fig. 2E and F). Thus, MC1R was functionally expressed in cultured RPE cells, and was responsible to  $\alpha$ -MSH.

### 3.3. $\alpha$ -MSH protects RPE cells from oxidative stress through MC1R

Above results demonstrated that MC1R was functionally expressed in the cultured RPE cells, and  $\alpha$ -MSH activated Erk1/2 and Akt/mTOR signalings in RPE cells. We then tested whether  $\alpha$ -MSH was pro-survival in RPE cells against oxidative stress. In both primary and transformed RPE cells,  $H_2O_2$  dose-dependently inhibited cell survival, indicated by a decreased MTT OD. Significantly,  $\alpha$ -MSH pre-administration rescued RPE cells from  $H_2O_2$  (Fig. 3A and B). Meanwhile,  $H_2O_2$ -induced RPE cell apoptosis, determined by TUNEL staining, was also inhibited by  $\alpha$ -MSH (Fig. 3C and D). Significantly,  $\alpha$ -MSH-induced cytoprotective effect was dependent on MC1R, as MC1R siRNA knockdown almost abolished  $\alpha$ -MSH-mediated effect against  $H_2O_2$  in ARPE-19 cells (Fig. 3E). These results confirmed that  $\alpha$ -MSH protects RPE cells against oxidative stress through MC1R.

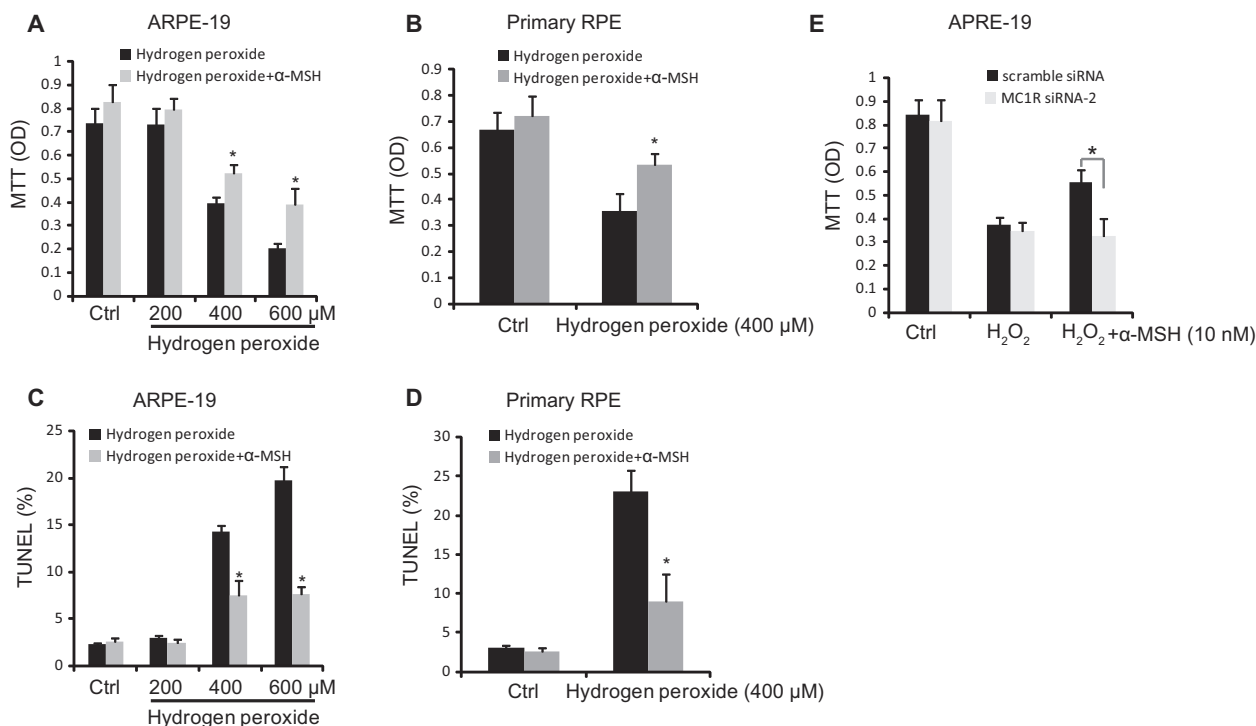
### 3.4. Akt–mTOR signaling mediates $\alpha$ -MSH-induced survival of RPE cells

Akt signaling is known to play a vital role in cell survival [28], thus we tested whether this signal is also important for  $\alpha$ -MSH-mediated RPE cell survival. Results in Fig. 4A once again confirmed Akt activation by  $\alpha$ -MSH, which was not affected by  $H_2O_2$  in RPE cells. Significantly, perifosine [29,30] and MK-2206 [31,32], two specific Akt inhibitors, as well as LY294002 [33], a pan PI3K/Akt/mTOR inhibitor (Fig. 4B), suppressed  $\alpha$ -MSH-mediated survival

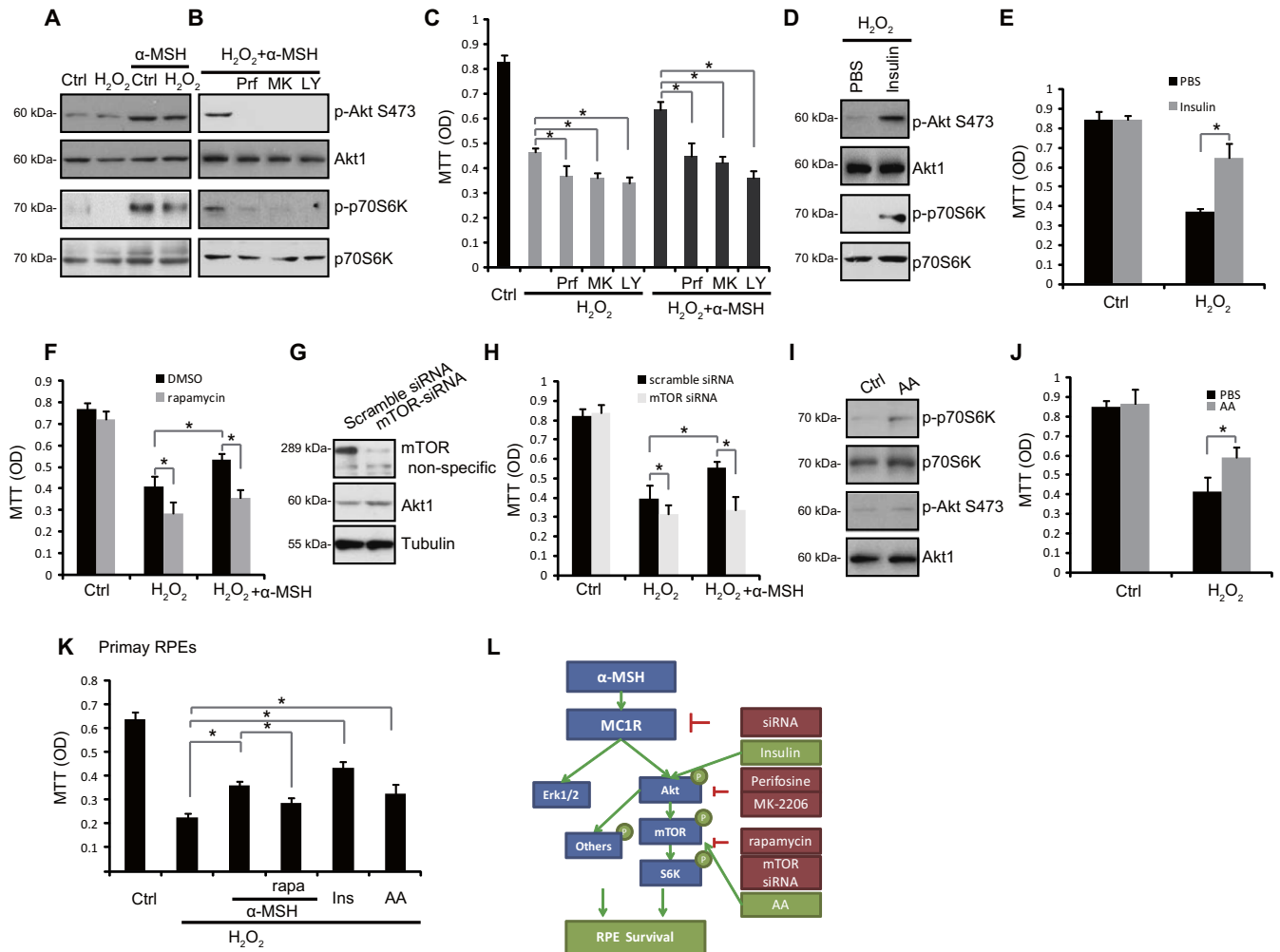
against oxidative stress in RPE cells (Fig. 4C). Reversely, insulin, which activated Akt and S6K1 in RPE cells (Fig. 4D), inhibited  $H_2O_2$ -induced RPE cell death (Fig. 4E). The fact that both perifosine and MK-2206 blocked  $\alpha$ -MSH-induced S6K1 phosphorylation (Fig. 4B) indicates that mTOR activation by  $\alpha$ -MSH is mediated through Akt. Next, we tested whether mTOR was important for  $\alpha$ -MSH-induced survival. In consistent with our previous findings [6], we found that  $H_2O_2$  inhibited mTOR activation (S6K1 phosphorylation) in RPE cells (Fig. 4A). Administration of  $\alpha$ -MSH restored S6K1 phosphorylation in  $H_2O_2$ -treated RPE cells (Fig. 4A). Rapamycin, the mTOR inhibitor, as well as mTOR siRNA knockdown (Fig. 4G), suppressed  $\alpha$ -MSH-mediated pro-survival effect (Fig. 4F and H). On the other hand, amino acids activated mTOR (but not Akt) (Fig. 4I), and protected RPE cells from oxidative stress (Fig. 4J). These results suggested that rapamycin-sensitive mTOR signaling, lying downstream of Akt, mediates  $\alpha$ -MSH-induced survival in RPE cells. It should be noted that Akt inhibitors (perifosine, MK-2206 and LY294002), rapamycin and mTOR knockdown all aggravated  $H_2O_2$ -induced RPE cell death (Fig. 4C, F and H), suggesting that basal Akt–mTOR activation in RPE cells is important for anti-oxidative stress response, while  $\alpha$ -MSH stimulates this pathway to protect cells from  $H_2O_2$ . Interestingly, PD98059, the MEK/Erk inhibitor, had no effect on  $\alpha$ -MSH-mediated survival against  $H_2O_2$  (data not shown). Results in Fig. 4K showed that  $H_2O_2$ -induced reduction of cell viability in primary RPE cells was inhibited by  $\alpha$ -MSH, insulin and amino acids. Rapamycin diminished  $\alpha$ -MSH-mediated pro-survival effect against  $H_2O_2$  in primary RPE cells (Fig. 4K). Together these results suggest that Akt–mTOR signaling mediates  $\alpha$ -MSH-induced pro-survival effect in RPE cells (Fig. 4L).

## 4. Discussion

In the current study, we discovered that MC1R is functionally expressed in primary and transformed RPE cells.  $\alpha$ -MSH activated Akt/mTOR and Erk1/2 MAPK signaling in RPE cells through MC1R.



**Fig. 3.**  $\alpha$ -MSH protects RPE cells from oxidative stress through MC1R. ARPE-19 cells or primary RPE cells were left untreated (Ctrl) or pretreated with  $\alpha$ -MSH (10 nM) for 30 min, followed by indicated  $H_2O_2$  stimulation for 24 h, MTT cell survival was analyzed (A and B), TUNEL staining was utilized to test cell apoptosis (C and D). ARPE-19 cells transfected with scramble or MC1R-2 siRNA (200 nM each) were pretreated with  $\alpha$ -MSH (10 nM) for 30 min, followed by  $H_2O_2$  (400  $\mu$ M) stimulation for 24 h, MTT cell survival was analyzed (E). Experiments were repeated three times to insure consistency of results. \* $p$  < 0.05 vs. group with  $H_2O_2$  stimulation.



**Fig. 4.** Akt-mTOR signaling mediates  $\alpha$ -MSH-induced survival of RPE cells. The effect of  $\alpha$ -MSH (10 nM, 1 h pretreatment) on Akt and S6K1 phosphorylation in ARPE-19 cells treated with or without H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M, 6 h) (A). ARPE-19 cells were pretreated with perifosine (Prf, 1  $\mu$ M), MK-2206 (MK, 0.25  $\mu$ M), or LY294002 (LY, 1  $\mu$ M) for 1 h, followed by H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M), or H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M) +  $\alpha$ -MSH (10 nM) stimulation, cells were further cultured for 6 h, Akt and S6K1 phosphorylation was tested (B), cell survival was also examined after 24 h (C). The effect of insulin (200 nM, 1 h pretreatment) on H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M)-induced Akt and S6K1 phosphorylation (after 2 h, D) and cell viability (after 24 h, E) were shown. ARPE-19 cells were pretreated with rapamycin (200 nM) for 1 h, followed by H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M), or H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M) +  $\alpha$ -MSH (10 nM) stimulation, cells were further cultured for 24 h, cell viability was examined (F). ARPE-19 cells transfected with scramble or mTOR siRNA (200 nM each) were pretreated with  $\alpha$ -MSH (10 nM) for 30 min, followed by H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M) stimulation for 24 h, MTT cell survival was analyzed (H). Expressions of mTOR, Akt and tubulin in above cells were also shown (G). After serum withdrawal overnight, ARPE-19 cells were deprived of amino acids (AA) for 2 h, and then stimulated with double the concentration of amino acids (AA) present in DMEM, cells were further treated with H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M), Akt and S6K1 phosphorylation was tested 2 h after H<sub>2</sub>O<sub>2</sub> stimulation (I), and RPE cell viability (after 24 h) was also shown (J). Primary cultured RPE cells were stimulated with H<sub>2</sub>O<sub>2</sub> (400  $\mu$ M) in the presence or absence of insulin (200 nM), amino acids (AA, see above),  $\alpha$ -MSH (10 nM)/or plus rapamycin (200 nM) for 24 h, and cell survival was analyzed by MTT assay (K). (N) The proposed signaling pathway of this study:  $\alpha$ -MSH activates Erk and Akt-mTOR signaling through MC1R in both transformed and primary RPE cells. Akt-mTOR signaling mediates RPE cell survival by  $\alpha$ -MSH (green arrows stand for "activate" or "promote", while red bars stand for "inhibit"). Experiments were repeated three times to insure consistency of results. \* $p$  < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$\alpha$ -MSH inhibited H<sub>2</sub>O<sub>2</sub>-induced RPE cell death, an effect that was almost reversed by Akt and/or mTOR inhibition (Fig. 4L). While insulin and amino acids exerted similar results as  $\alpha$ -MSH. Thus, we suggest that  $\alpha$ -MSH suppresses H<sub>2</sub>O<sub>2</sub>-induced RPE cell apoptosis through activating MC1R-Akt-mTOR signaling (Fig. 4L).

Recent studies have discovered the new role for  $\alpha$ -MSH as pro-survival agent again UV irradiation in melanocytes [14]. The pro-survival effect of  $\alpha$ -MSH was shown to be independent of their melanogenic effects, and was to involve the activation of Akt pathway [14]. Activated Akt inhibits apoptosis by phosphorylating and inactivating the pro-apoptotic proteins Bad and caspase 9, and Akt activates NF $\kappa$ B that also inhibits apoptosis [34,35]. In the present study, we found that activation of Akt by  $\alpha$ -MSH is also important for its pro-survival effect against H<sub>2</sub>O<sub>2</sub>. Another important downstream target that mediates Akt-mediate survival

is mTOR. mTOR exists in two functionally distinct complexes including mTORC1 complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [36–38]. mTORC1, or the rapamycin sensitive mTOR complex, is composed of mTOR, Raptor, mLST8, and PRAS40, which phosphorylates S6K1 and 4E-BP1 (eIF4E binding protein 1). While mTORC2 is composed of mTOR, Rictor, Sin1, and mLST8, and phosphorylates Akt at Ser 473 [37].

Here we found that  $\alpha$ -MSH-induced pro-survival effect against H<sub>2</sub>O<sub>2</sub> was suppressed by mTORC1 inhibitor rapamycin, or by mTOR knockdown, indicating that rapamycin-sensitive mTOR signaling was required for  $\alpha$ -MSH-mediated survival effect. These results are consistent with our previous studies where we found that NGF-mediated survival was also dependent on Akt-mTORC1 signaling [6]. These results are not surprising, since mTORC1 is proposed as an important survival factor [6,39]. Interestingly, Erk



was not involved in  $\alpha$ -MSH-mediated survival in RPE cells. One explanation might be that, as seen in other studies [40], although  $\alpha$ -MSH activates ERK, its activation is transient and not thought to be mitogenic. As a matter of fact, we failed to find significant Erk activation 2 h after  $\alpha$ -MSH stimulation in RPE cells (data not shown), while Akt/mTORC1 activation lasted for at least 6 h (Fig. 4A). In summary, our results discovered the functional MC1R expression in cultured RPE cells, and its agonist  $\alpha$ -MSH protects RPE cells from oxidative stress probably through activating Akt–mTOR signaling. Since AMD is characterized by a progressive decay of RPE cells at the posterior pole of the eye, and ROS is a major contributor of RPE cell damage in AMD progression, we suggest that  $\alpha$ -MSH might have therapeutical values for AMD patients. Further studies will be focusing on clinical relevance between  $\alpha$ -MSH and AMD.

### Competing interests

The authors declare that they have no competing interests.

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