

# Obacunone protects retinal pigment epithelium cells from ultra-violet radiation-induced oxidative injury

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## ABSTRACT

Ultra-violet (UV) radiation (UVR) causes significant oxidative injury to retinal pigment epithelium (RPE) cells. Obacunone is a highly oxygenated triterpenoid limonoid compound with various pharmacological properties. Its potential effect in RPE cells has not been studied thus far. Here in ARPE-19 cells and primary murine RPE cells, obacunone potently inhibited UVR-induced reactive oxygen species accumulation, mitochondrial depolarization, lipid peroxidation and single strand DNA accumulation. UVR-induced RPE cell death and apoptosis were largely alleviated by obacunone. Obacunone activated Nrf2 signaling cascade in RPE cells, causing Keap1-Nrf2 disassociation, Nrf2 protein stabilization and nuclear translocation. It promoted transcription and expression of antioxidant responsive element-dependent genes. Nrf2 silencing or CRISPR/Cas9-induced Nrf2 knockout almost reversed obacunone-induced RPE cytoprotection against UVR. Forced activation of Nrf2 cascade, by Keap1 knockout, similarly protected RPE cells from UVR. Importantly, obacunone failed to offer further RPE cytoprotection against UVR in Keap1-knockout cells. *In vivo*, intravitreal injection of obacunone largely inhibited light-induced retinal damage. Collectively, obacunone protects RPE cells from UVR-induced oxidative injury through activation of Nrf2 signaling cascade.

## INTRODUCTION

Age-related macular degeneration (AMD) is one primary reason for vision loss and blindness among elderly people [1–3]. In the United States, AMD is detected in over eight million people [3]. For the vast majority of AMD patients with the “dry” or atrophic form, there is no effective therapy [1–3]. It is therefore urgent to explore novel and more efficient anti-AMD strategies.

In the pathology of AMD, Ultra-violet (UV) radiation (UVR) could induce substantial oxidative injury to human retina [1, 2, 4]. Sustained and intensive UVR will exert significant retinal damage, particularly in aged persons [5, 6]. Sunlight UV is divided into three segments based on the spectrum, including UVA,

UVB, and UVC. Of which, UVC (below 286 nm) is filtered by the earth's ozone layer. Most UVB (286–320 nm) is blocked by human cornea and lens (< 295 nm). UVA (320–400 nm) is the most eye-damaging UV spectrum, which transmits from human crystalline lens to retina [5, 6]. Melanin will efficiently absorb harmful UV in retina. However, melanin levels will drop significantly in aged people [5, 6]. Wearing sunglasses that block UV (<400 nm) could efficiently protect human retina from light damage [5]. Contact lenses can offer additional retinal protection by absorbing significant amounts of UVR [5].

Retinal pigment epithelium (RPE) is located between Bruch's membrane and photoreceptors in human retina. It transports nutrients to photoreceptors from

the choroid, and also transports wasters from the photoreceptors to the choroid [7–9]. Additionally, RPE cells are important for phagocytosis of photoreceptor outer membranes, maintaining blood-retina barrier, and polarized secretion of vascular endothelial growth factor (VEGF) [10, 11]. Among AMD patients, RPE atrophy, photoreceptors and Bruch's membrane dysfunction are commonly detected [7–9].

Studies have shown that short wavelength UVR, UVA2 (320-340 nm) and certain UVB (300-320 nm), induced significant oxidative injury in RPE cells. This will lead to impaired vision or even permanent blindness [5, 6, 12]. UVR induces profound reactive oxygen species (ROS) production and oxidative stress, causing lipid peroxidation and DNA damage, and eventually RPE cell death and apoptosis [13–19]. Conversely, antioxidants and ROS scavengers can efficiently protect RPE cells from UVR [14, 20–25].

We have previously shown that MIND4-17, a nuclear-factor-E2-related factor 2 (Nrf2) activator, protected RPE cells from UVR-induced oxidative stress [17]. Furthermore, PF-06409577 activated AMP-activated protein kinase (AMPK) signaling to inhibit UVR-induced RPE cell apoptosis [26].

Nrf2 cascade is a vital cellular endogenous antioxidant mechanism [27–30]. Without stimulation, Nrf2 binds Kelch-like ECH-associated protein 1 (Keap1), then ubiquitinated by a Cul3-based E3 ligase and degraded [27–30]. Activated Nrf2, as a transcription factor, will bind antioxidant responsive elements (ARE) to promote transcription and expression of antioxidant genes and phase II detoxification enzymes [27–30]. Nrf2-ARE dependent genes include *NAD(P)H quinone oxidoreductase 1 (NQO1)*, *heme oxygenase 1 (HO-1)* and several other important antioxidant genes [27–30]. Forced activation of Nrf2 signaling cascade, using genetic strategies or pharmacological agents, can efficiently protect RPE cells and other retinal cells from UVR and other oxidative insults [17, 26].

Obacunone is a highly oxygenated triterpenoid limonoid compound mainly found in the *Rutaceae* family plants (citrus and others). Obacunone has displayed multiple biological and pharmacological actions, including antioxidant [31], anti-inflammatory [32], anti-cancer [33], anti-obesity [34] and neuroprotective [35] activities. Its potential effect in RPE cells has not been studied thus far. The results of this study showed that obacunone activated Nrf2 cascade to protect RPE cells from UVR-induced oxidative injury.

## RESULTS

### Obacunone attenuates UVR-induced cytotoxicity in ARPE-19 cells and murine RPE cells

Obacunone has not been studied in retinal cells. The established ARPE-19 human RPE cells (from Dr. Jiang [36]) were treated with obacunone from 1-50  $\mu\text{M}$ . The concentrations were determined based on the previous studies [37, 38] and our preliminary results. As demonstrated, obacunone treatment (1-50  $\mu\text{M}$ ) for 48h was safe for ARPE-19 cells, as it did not induce significant viability reduction (Figure 1A) and LDH release (Figure 1B). In line with the previous findings [18, 19, 36], UV radiation (UVR, 30  $\text{mJ}/\text{cm}^2$ ) induced profound cytotoxicity in ARPE-19 cells, causing significant viability reduction (Figure 1A) and medium LDH release (Figure 1B). Significantly, pre-treatment with obacunone, at 10-50  $\mu\text{M}$ , largely inhibited UVR-induced cytotoxicity in ARPE-19 cells (Figure 1A, 2B). Obacunone-induced RPE cytoprotection was dose-dependent, and ineffective at low concentrations (1-5  $\mu\text{M}$ ) (Figure 1A, 2B). Since 25  $\mu\text{M}$  of obacunone effectively protected ARPE-19 cells from UVR (Figure 1A, 2B), this concentration was selected for further analyses.

In primary murine RPE cells, UVR led to potent viability reduction (Figure 1C) and cell death (Figure 1D), which were largely inhibited by obacunone (25  $\mu\text{M}$ ) as well (Figure 1C, 1D). Obacunone single treatment failed to induce death in murine RPE cells (Figure 1C, 1D). To mimic oxidative injury, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) treatment (400  $\mu\text{M}$ , for 48h) induced profound cell death in ARPE-19 cells (Figure 1E) and murine RPE cells (Figure 1F), which were largely attenuated with obacunone (25  $\mu\text{M}$ ) pretreatment (Figure 1E, 1F). These results showed that obacunone alleviated UVR-induced cytotoxicity in ARPE-19 cells and murine RPE cells.

### Obacunone attenuates UVR-induced apoptosis in ARPE-19 cells and primary murine RPE cells

UVR can induce apoptosis activation in ARPE-19 cells [18, 19]. Results in Figure 1 demonstrated that obacunone potently inhibited UVR-induced cytotoxicity in RPE cells, we next tested its effect on cell apoptosis. As shown UVR significantly increased caspase-3 (Figure 2A) and caspase-9 (Figure 2B) activity in ARPE-19 cells, which was largely inhibited by pre-treatment with obacunone (25  $\mu\text{M}$ ) (Figure 2A, 2B). Furthermore, significant apoptosis activation was detected in UVR-treated ARPE-19 cells, evidenced by increases in Annexin V-positive cell percentage (Figure 2C) and nuclear TUNEL staining (Figure 2D). In

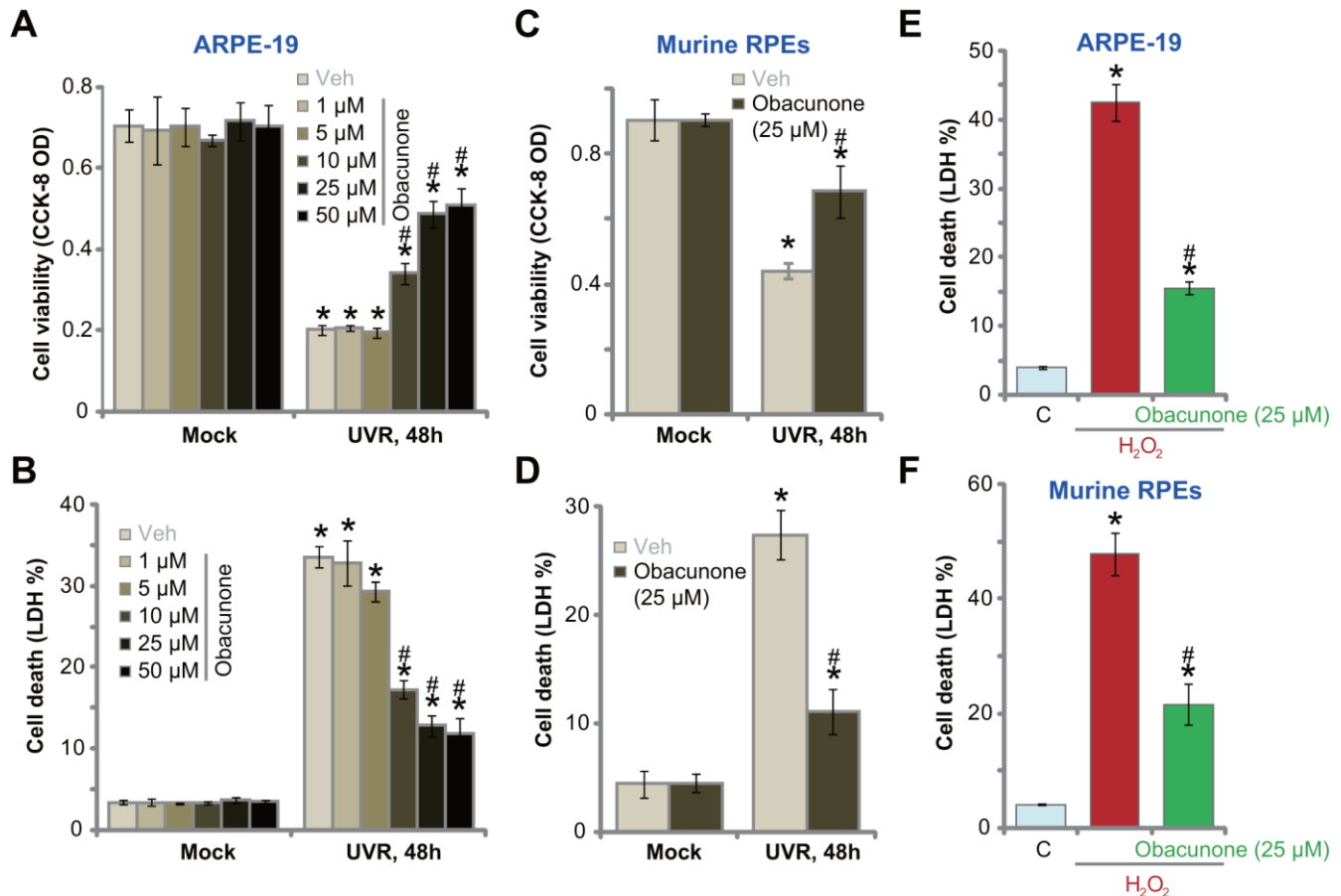
ARPE-19 cells, obacunone (25  $\mu\text{M}$ ) pretreatment potently inhibited UVR-induced apoptosis activation, decreasing ratios of Annexin V- and TUNEL-positive cells (Figure 2C, 2D).

TUNEL staining results, Figure 2E, confirmed that UVR-induced cell apoptosis in primary murine RPE cells was also largely attenuated by obacunone pretreatment. Obacunone single treatment failed to provoke cell apoptosis in ARPE-19 cells (Figure 2A–2D) and murine RPE cells (Figure 2E). These results showed that obacunone inhibited UVR-induced apoptosis in ARPE-19 cells and primary murine RPE cells.

### Obacunone ameliorates UVR-induced oxidative injury in ARPE-19 cells and primary murine RPE cells

UVR induces significant ROS production and oxidative injury to RPE cells, a key mechanism mediating cell

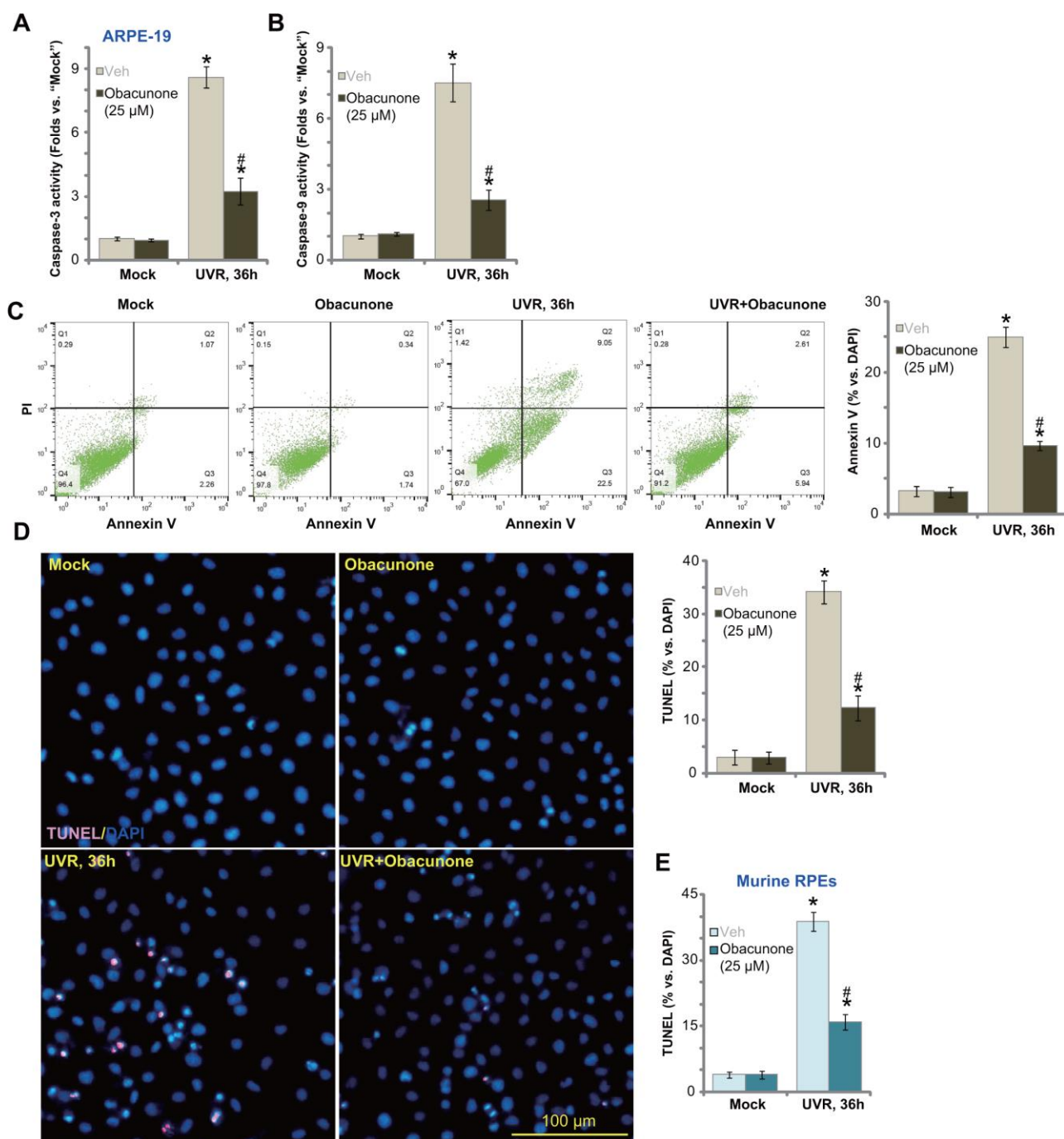
death [18, 19, 36]. Conversely, ROS scavengers or antioxidants can protect RPEs from UVR-induced cytotoxicity [18, 19, 36]. Obacunone could activate Nrf2 signaling cascade to fight against oxidative injury [38], we therefore tested whether obacunone can inhibit UVR-induced oxidative injury in RPE cells. Applying a CellROX dye assay [39, 40], we found that UVR induced significant ROS production in ARPE-19 cells (Figure 3A), which was accompanied by mitochondrial depolarization (JC-1 green monomers accumulation, Figure 3B). Obacunone pretreatment potently attenuated UVR-induced oxidative injury by suppressing CellROX intensity increase (Figure 3A) and mitochondrial depolarization (Figure 3B) in ARPE-19 cells. Further confirming oxidative injury, UVR induced significant lipid peroxidation (Figure 3C) and single strand DNA (ssDNA) accumulation (Figure 3D) in ARPE-19 cells, which were largely inhibited by obacunone as well (Figure 3C, 3D).



**Figure 1. Obacunone attenuates UVR-induced cytotoxicity in RPE cells.** ARPE-19 cells (A, B, E) or the primary murine RPE cells (C, D, F) were pretreated for 60 min with applied concentration of obacunone, cells were then subjected to UV radiation (UVR, UVA<sub>2</sub>+B, 30 mJ/cm<sup>2</sup>) or H<sub>2</sub>O<sub>2</sub> (400  $\mu\text{M}$ ), and were further cultured for 48h; Cell viability was tested by CCK-8 assay (A, C), with cell death examined by LDH release in the conditional medium (B, D, E, F); Data were presented as mean  $\pm$  SD (n=5). “Mock” stands for no UVR stimulation. “Veh” stands for the vehicle control. “C” stands for untreated control. \**p* < 0.05 vs. “Mock”/“C” treatment. #*p* < 0.05 vs. UVR/ H<sub>2</sub>O<sub>2</sub> only treatment. Experiments were repeated three times, with similar results obtained.

In the primary murine RPE cells UVR similarly induced ROS production (CellROX intensity increase, Figure 3E) and mitochondrial depolarization (JC-1 green monomers accumulation, Figure 3F). With obacunone pretreatment, UVR-induced oxidative

injury was significantly attenuated (Figure 3E, 3F). Obacunone single treatment failed to induce oxidative injury in RPE cells (Figure 3A–3F). Therefore, obacunone potently inhibited UVR-induced oxidative injury in RPE cells.



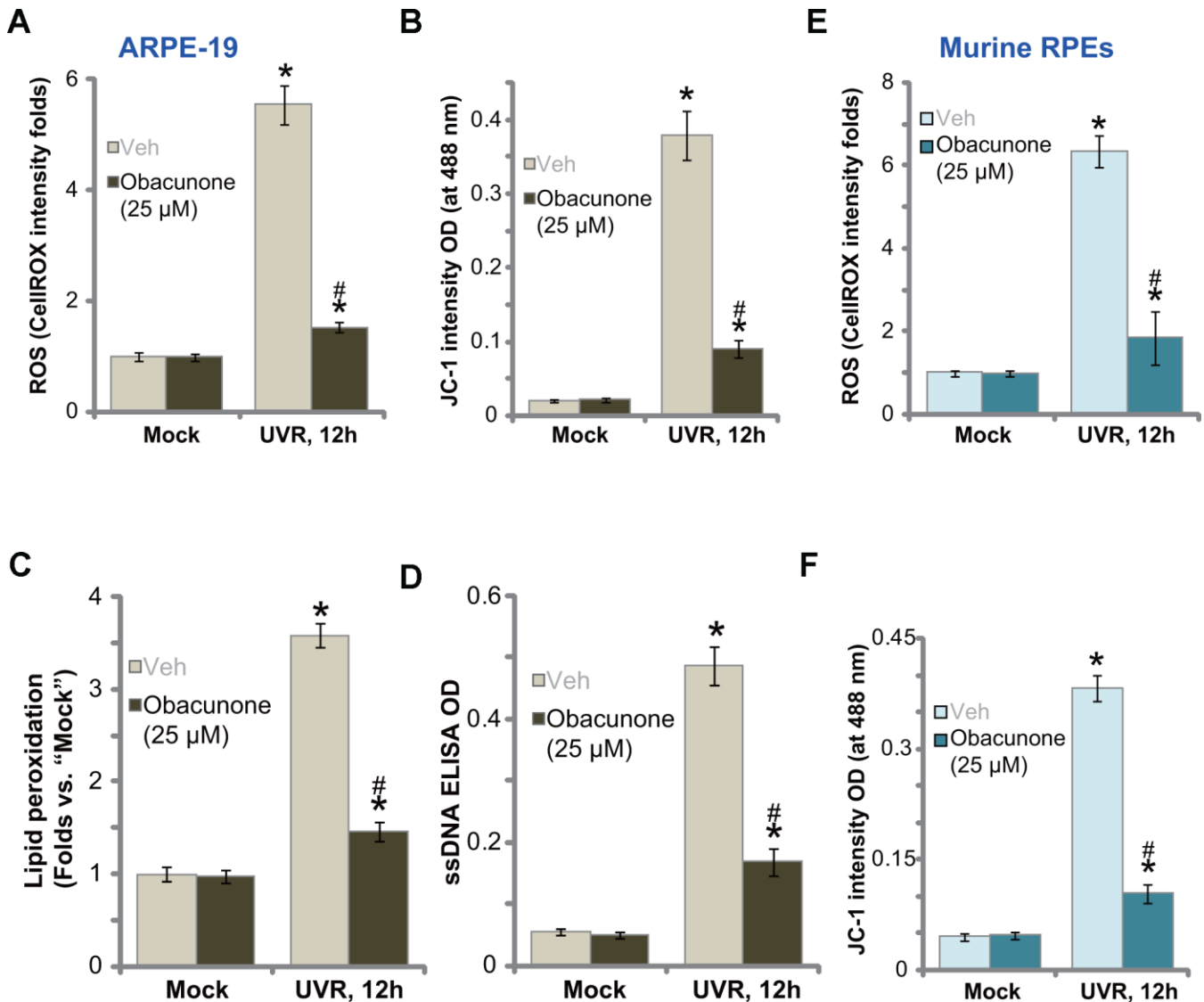
**Figure 2. Obacunone attenuates UVR-induced apoptosis in RPE cells.** ARPE-19 cells (A–D) or primary murine RPE cells (E) were pretreated for 60 min with obacunone (25 μM), cells were then subjected to UV radiation (UVR, UVA<sub>2</sub>+B, 30 mJ/cm<sup>2</sup>), and further cultured for 36h, relative caspase-3/-9 activity (A, B) and apoptosis activation (C–E) were tested by the listed assays. Data were presented as mean ± SD (n=5). "Mock" stands for no UVR stimulation. "Veh" stands for the vehicle control. \**p* < 0.05 vs. "Mock" treatment. #*p* < 0.05 vs. "UVR" only treatment (with vehicle pretreatment). Experiments were repeated three times, with similar results obtained. Bar= 100 μm (D).



### Obacunone activates Nrf2 signaling cascade in RPE cells

Forced activation of Nrf2 signaling can protect RPE cells from UVR-induced oxidative injury and cell apoptosis [17]. Since obacunone inhibited UVR-induced oxidative stress (Figure 3), we tested its potential effect on Nrf2 cascade in RPE cells. By applying a co-immunoprecipitation (Co-IP) assay, we showed that in ARPE-19 cells Keap1 immunoprecipitation with Nrf2 was disrupted with obacunone

treatment (Figure 4A), resulting in Nrf2 protein stabilization and accumulation in cytosol fraction (Figure 4B). Keap1 protein levels were however unchanged (Figure 4B). Nrf2 protein also translocated to cell nuclei following obacunone treatment, evidenced by increased Nrf2 protein in the nuclear fraction lysates (Figure 4C). Importantly, obacunone treatment significantly increased ARE luciferase activity in ARPE-19 cells (Figure 4D) and promoted expression of ARE-dependent genes, including *HO1* and *NQO1* (Figure 4E). The protein expression of HO1 and

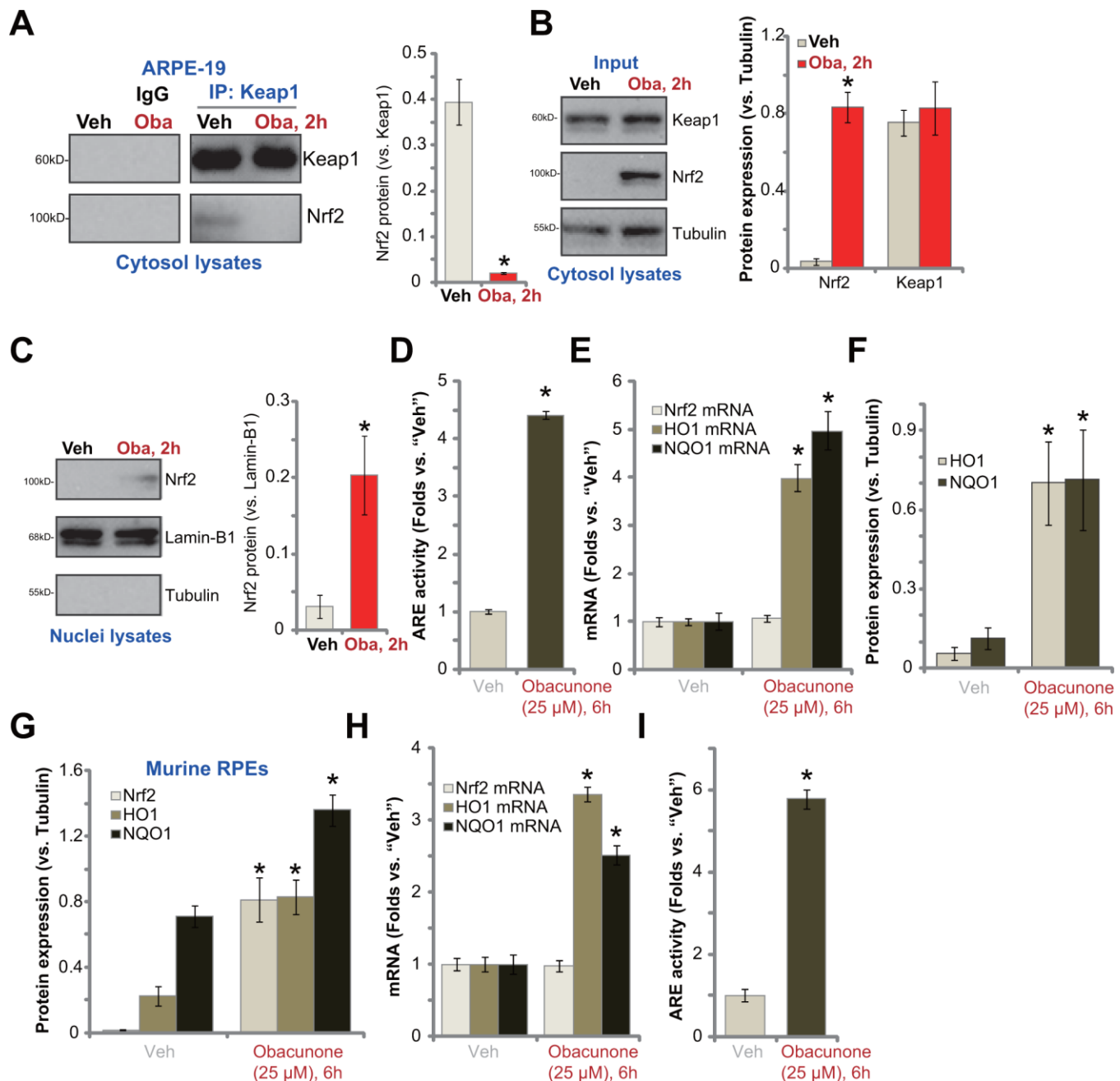


**Figure 3. Obacunone ameliorates UVR-induced oxidative injury in RPE cells.** ARPE-19 cells (A–D) or the primary murine RPE cells (E and F) were pretreated for 60 min with obacunone (25  $\mu$ M), cells were then subjected to UV radiation (UVR, UVA<sub>2</sub>+B, 30 mJ/cm<sup>2</sup>), and were further cultured for 12h, ROS production (CellROX intensity, A, E), mitochondrial depolarization (JC-1 green monomers fluorescence intensity, B and F), lipid peroxidation (measuring relative TBAR activity, C) and DNA damage (measuring ssDNA contents, D) were tested, and results were quantified. Data were presented as mean  $\pm$  SD (n=5). “Mock” stands for no UVR stimulation. “Veh” stands for the vehicle control. \* $p$  < 0.05 vs. “Mock” treatment. # $p$  < 0.05 vs. “UVR” only treatment (with vehicle pretreatment). Experiments were repeated three times, with similar results obtained.

NQO1 was upregulated as well following obacunone treatment (Quantified results in Figure 4F), with *Nrf2* mRNA expression unchanged (Figure 4E). Therefore, in ARPE-19 cells obacunone induced Keap1-Nrf2 disassociation, causing Nrf2 protein accumulation,

nuclear translocation and expression of ARE-dependent genes.

In the primary murine RPE cells, obacunone treatment similarly induced Nrf2 protein accumulation (Figure 4G),



**Figure 4. Obacunone activates Nrf2 signaling cascade in RPE cells.** ARPE-19 cells (A–F) or the primary murine RPE cells (G–I) were treated with obacunone (“Oba”, 25 μM), cells were further cultured for applied time periods, the co-immunoprecipitation (Co-IP) assay was performed to test Keap1-Nrf2 association (A); Expression of listed proteins (in cytosol lysates and nuclear lysates) and mRNAs were tested by Western blotting (B, C, F, G) and qPCR (E, H) assays, and results were quantified; The relative ARE luciferase activity was tested as well (D, I). Expression of listed proteins was quantified, normalized to the loading control, and expressed as mean ± SD (n=5) (A–C, F, G). Data were presented as mean ± SD (n=5). “Veh” stands for the vehicle control. \**p* < 0.05 vs. “Veh” treatment. Experiments were repeated three times, with similar results obtained.

expression of ARE-dependent genes (*HO1* and *NQO1*) (Figure 4G, 4H) and elevation of ARE luciferase activity (Figure 4I). It did not alter *Nrf2* mRNA expression (Figure 4H). Taken together, obacunone activated Nrf2 signaling cascade in RPE cells.

### **Obacunone-induced RPE cytoprotection against UVR is abolished with Nrf2 silencing or knockout**

If Nrf2 cascade activation is required for obacunone-induced RPE cell protection against UVR, Nrf2 depletion should reverse obacunone-induced actions. To test this hypothesis, ARPE-19 cells were transfected with Nrf2 shRNA lentivirus. Stable cells were established with selection by puromycin: sh-Nrf2 cells. Alternatively, a CRISPR/Cas9-Nrf2-KO construct was transduced to ARPE-19 cells. FACS sorting and puromycin selection were applied to establish stable cells: ko-Nrf2 cells. As compared to the control cells with scramble shRNA plus CRISPR/Cas9 control vector (“sh-c+Cas9-C”), *Nrf2* mRNA levels were dramatically decreased in sh-Nrf2 cells and ko-Nrf2 cells (Figure 5A). Consequently, obacunone-induced Nrf2 protein stabilization was abolished (protein expression results were quantified in Figure 5B). Furthermore, obacunone-induced mRNA (Figure 5A) and protein (Figure 5B) expression of HO1 and NQO1 was blocked by Nrf2 shRNA or KO. In sh-Nrf2 cells and ko-Nrf2 cells adding obacunone failed to increase ARE activity (Figure 5C).

As compared to the control cells, UVR-induced viability (CCK-8 OD) reduction (Figure 5D) and cell death (measuring medium LDH release, Figure 5E) were intensified in sh-Nrf2 and ko-Nrf2 ARPE-19 cells. Importantly, obacunone was ineffective against UVR in ARPE-19 cells with Nrf2 shRNA or KO (Figure 5D, 5E). Therefore, obacunone-induced ARPE-19 cytoprotection against UVR was reversed with Nrf2 shRNA or KO. These results suggested that activation of Nrf2 cascade is required for obacunone-induced actions.

In primary murine RPE cells, transfection of Nrf2 siRNA largely inhibited obacunone-induced Nrf2 protein stabilization (protein expression results were quantified in Figure 5F) and expression of HO1 (Figure 5F). With Nrf2 silencing, obacunone-induced RPE cytoprotection against UVR was compromised (Figure 5G, 5H). UVR-induced viability reduction (Figure 5G) and cell death (Figure 5H) were only slightly inhibited by obacunone pretreatment in Nrf2-silenced murine RPE cells.

### **Keap1 depletion mimics obacunone-induced RPE cytoprotection against UVR**

Obacunone induced Keap1-Nrf2 disassociation and Nrf2 cascade activation, inhibiting UVR-induced

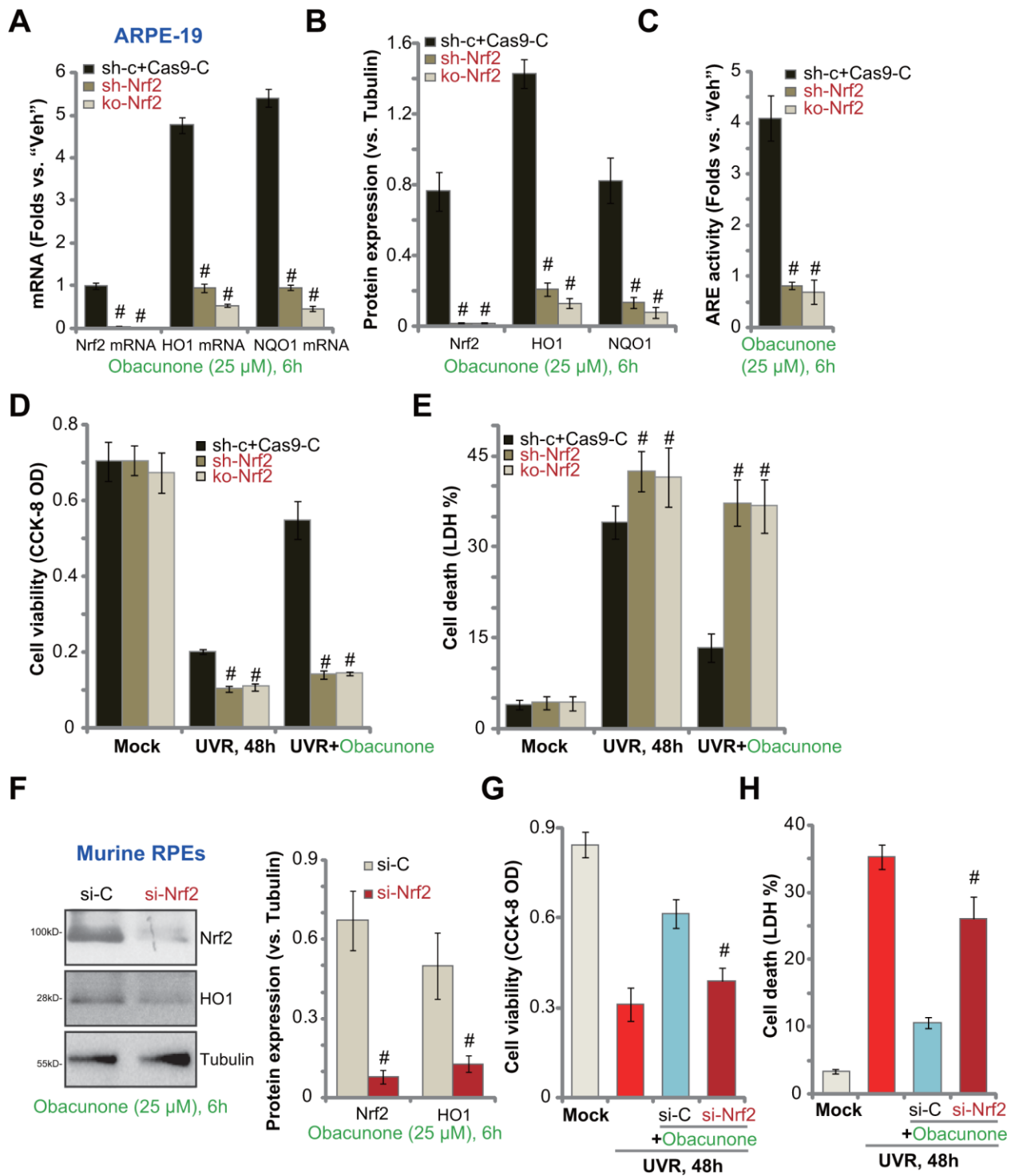
oxidative injury and apoptosis in RPE cells. Therefore, Keap1 depletion should mimic obacunone-induced actions, activating Nrf2 signaling and protecting RPE cells from UVR. To test this hypothesis, a CRISPR/Cas9-Keap1 KO construct was transduced to ARPE-19 cells. Stable cells were established with FACS sorting and puromycin selection: “ko-Keap1” cells (Figure 6A). With Keap1 KO, Nrf2 protein was stabilized and accumulated (Figure 6A). HO1 and NQO1 mRNA and protein expression was significantly elevated (Figure 6A, 6B). *Nrf2* mRNA level was again unchanged (Figure 6B) but ARE activity increased (Figure 6C). Therefore, CRISPR/Cas9-induced Keap1 KO activated Nrf2 cascade in ARPE-19 cells.

The ko-Keap1 ARPE-19 cells were protected from UVR, and presented with decreased viability reduction (Figure 6D) and cell death (Figure 6E), as compared to the control cells. Significantly, in ko-Keap1 RPE cells, obacunone treatment failed to further increase Nrf2 cascade activation (Figure 6A–6C), nor did it offer further cytoprotection against UVR (Figure 6D, 6E). Thus, obacunone was invalid against UVR in Keap1-KO cells with Nrf2 signaling pre-activated. These results further support that activation of Nrf2 cascade is required for obacunone-induced RPE cytoprotection against UVR.

### **Obacunone protects mouse retina from light-induced damage**

At last, the potential effect of obacunone on mouse retina *in vivo* was studied. Using a previously-described light-induced retinal injury mouse model ([15, 21]), we showed that electroretinography (ERG) dysfunction reached peak 24h after the light exposure (Figure 7A, 7B) [41]. ERG b-wave amplitudes were tested from the trough of the a-wave to the peak of the b-wave, with the amplitudes of the a-wave measured from the initial baseline [15, 21, 41]. Significantly, intravitreal injection of obacunone (2.5 mg/kg body weight, 1h pre-treatment before light damage) potentially alleviated light-induced reductions of both a- and b-wave amplitudes (Figure 7A, 7B). These results imply that obacunone protected mouse retina from light-induced damage.

Retinal tissues were collected and tested. As shown, 24h after light injury, levels of cleaved-caspase-3 and cleaved-poly (ADP-ribose) polymerase (PARP) (Figure 7C) as well as the caspase-3 activity (Figure 7D) were significantly increased in mouse retinal tissues. Importantly, intravitreal injection of obacunone potentially inhibited apoptosis activation in the light-injured retinal tissues (Figure 7C, 7D).



**Figure 5. Obacunone-induced RPE cytoprotection against UVR is abolished with Nrf2 silencing or knockout.** Stable ARPE-19 cells with Nrf2 shRNA ("sh-Nrf2") or the CRISPR/Cas9-Nrf2-KO construct ("ko-Nrf2"), as well as the control cells ("sh-c+Cas9-C"), were treated with obacunone (25 μM), cells were further cultured for 6h, expression of listed genes was shown (A, B), with ARE activity tested (C). Alternatively, cells were pretreated with obacunone (25 μM) for 1h, followed by UVR and cultured for another 48h, cell viability and death were tested by CCK-8 (D) and medium LDH release (E) assays, respectively. The primary murine RPE cells were transfected with Nrf2 siRNA ("si-Nrf2", 500 nM) or the scramble control siRNA ("si-C", 500 nM), after 48h cells were treated with obacunone (25 μM) for another 6h, expression of listed proteins was shown (F). The murine RPE cells were pre-treated with obacunone (25 μM) for 1h, followed by UVR for another 48h, cell viability (G) and death (H) were tested. Expression of listed proteins, normalized to the loading control, and expressed as mean ± SD (n=5) (B, F). Data were presented as mean ± SD (n=5). #  $p < 0.05$  vs. "sh-c+Cas9-C" cells (B-E). #  $p < 0.05$  vs. "si-C" cells (G, H). Experiments were repeated three times, with similar results obtained.



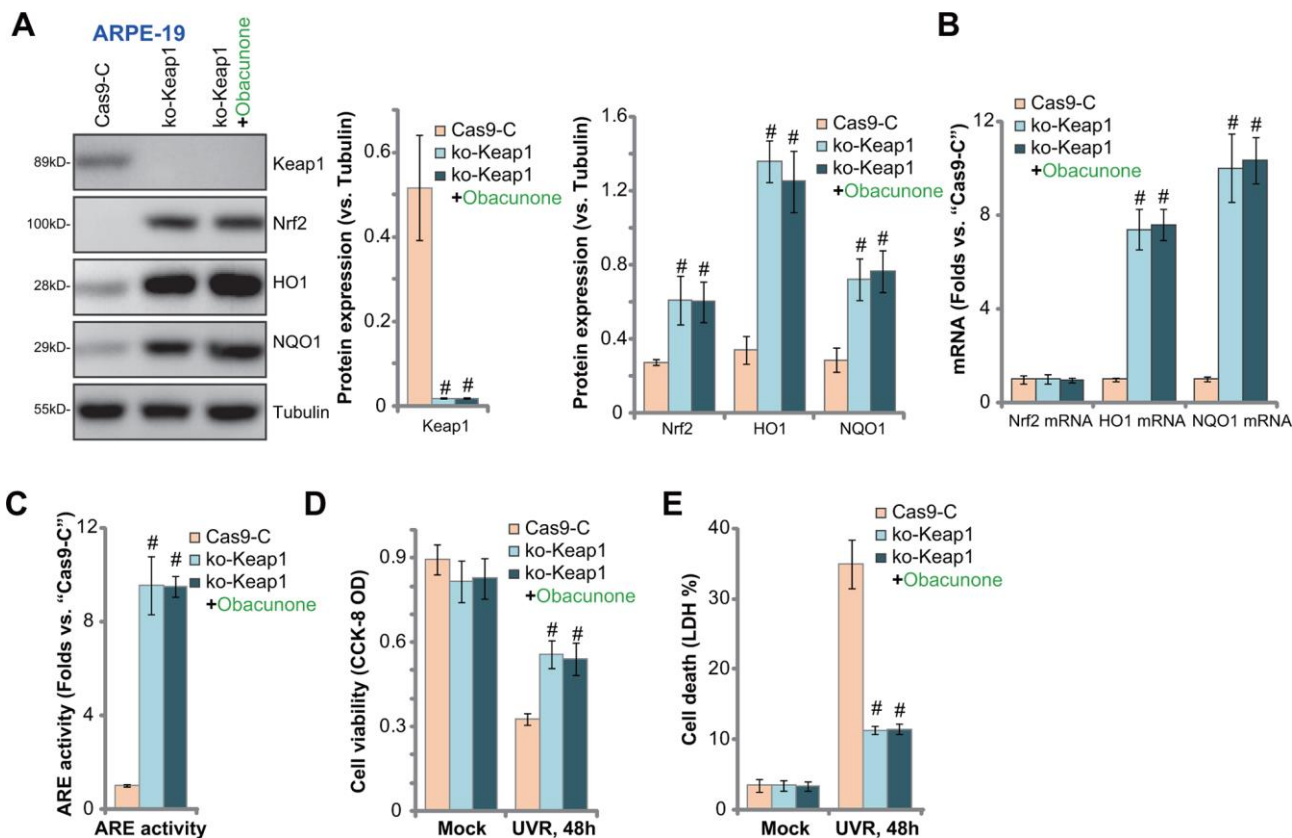
## DISCUSSION

Studies have demonstrated that excessive and long-term UVR will induce significant oxidative injury and cytotoxicity in RPE cells [24, 25]. It is a major contributor of retinal degeneration in AMD pathology [1, 2, 4]. Conversely, antioxidants and ROS scavengers can protect RPE cells from UVR-induced cell injury [1, 2, 4]. Obacunone has displayed cytoprotective actions. For instance, Yoon et al., have shown that obacunone protected endothelial cells and restored endothelial function in ApoE-null mice [42]. Jeong et al., demonstrated that obacunone attenuated glutamate-induced oxidative injury in murine hippocampal neurons [35]. Furthermore, Zhou et al., reported obacunone inhibited high glucose-induced oxidative damage in renal tubular epithelial cells [38].

The results of the current study reported RPE cytoprotective activity of obacunone. Obacunone

potently inhibited ROS accumulation, mitochondrial depolarization, lipid peroxidation and ssDNA accumulation in UVR-treated RPE cells. Furthermore, UVR-induced cell death and apoptosis were alleviated by obacunone. Therefore, obacunone exerted significant RPE cell protective activity by suppressing UVR-induced oxidative injury and apoptosis in RPE cells.

Our group and others have shown that activation of Nrf2 signaling can protect RPE cells from UVR [16, 17, 19, 36]. MIND4-17, a compound that activated Nrf2 signaling through blocking association with Keap1, protected RPE cells from UVR [17]. Recently, Chen et al., showed that by activation of Nrf2 signaling MIND4-17 protected retinal cells from high glucose-induced oxidative injury [36]. Tang et al., demonstrated that ginsenoside Rh3 activated Nrf2 signaling and protected RPE cells from UVR [19]. Ginsenoside Rh3 induced microRNA-141 expression, causing downregulation of Keap1 in RPE cells [19]. Interestingly, in RPE cells,

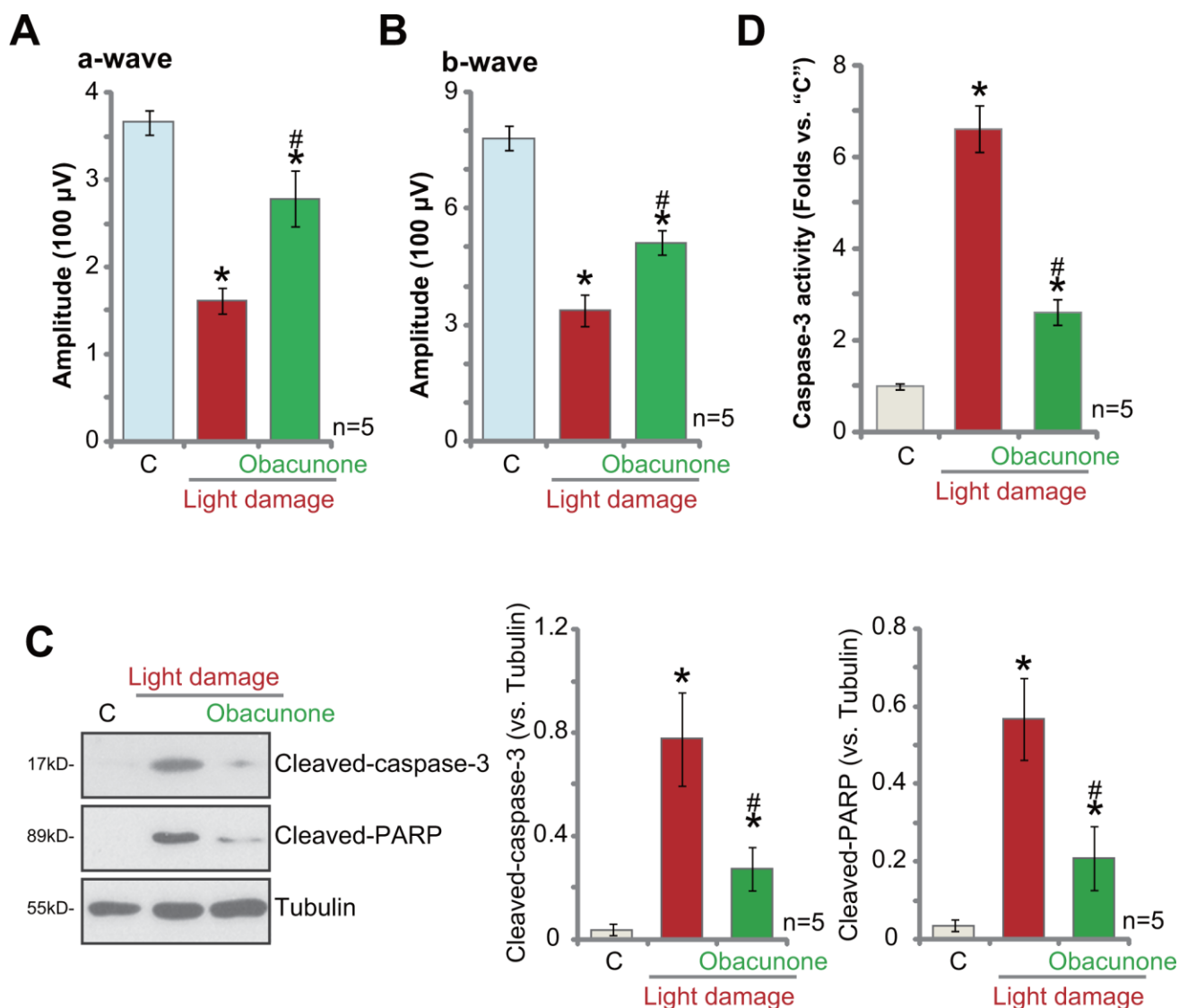


**Figure 6. Keap1 depletion mimics obacunone-induced RPE cytoprotection against UVR.** Stable ARPE-19 cells with the CRISPR/Cas9-Keap1-KO construct ("ko-Keap1") or the empty vector ("Cas9-C") were treated with or without obacunone (25  $\mu$ M), cells were further cultured for 6h, expression of listed genes was shown (A, B), with ARE activity tested (C). Alternatively, cells were pretreated with obacunone (25  $\mu$ M) for 1h, followed by UVR for another 48h, cell viability and death were tested by CCK-8 (D) and medium LDH release (E) assays, respectively. Expression of listed proteins was quantified, normalized to the loading control, and expressed as mean  $\pm$  SD (n=5) (A). Data were presented as mean  $\pm$  SD (n=5). #  $p$  < 0.05 vs. "Cas9-C" cells. Experiments were repeated three times, with similar results obtained.

forced overexpression of microRNA-141 activated Nrf2 signaling, inhibiting UVR-induced oxidative injury [16]. These results clearly indicated that forced activation of Nrf2 signaling cascade, using pharmacological or genetic methods, can protect RPE cells from UVR-induced oxidative injury.

Xu et al., have shown that obacunone can activate Nrf2 signaling in human cancer cells and immune cells, possibly by decreasing Nrf2 ubiquitination and increasing its stability [31]. In the present study we showed that obacunone activated Nrf2 signaling cascade in RPE cells, causing Keap1-Nrf2

disassociation, Nrf2 protein stabilization, cytosol accumulation and translocation to cell nuclei. Afterwards, it promoted transcription and expression of ARE-dependent genes (*NQO1* and *HO1*). Activation of Nrf2 cascade is required for obacunone-induced actions. As Nrf2 silencing or KO completely abolished obacunone-induced RPE cytoprotection against UVR. Furthermore, forced activation of Nrf2, through Keap1 KO, protected RPE cells from UVR-induced cell death. Importantly, obacunone was ineffective against UVR in Keap1-KO RPE cells. Together, obacunone inhibited UVR-induced oxidative injury in RPE cells through activation of Nrf2 signaling cascade.



**Figure 7. Obacunone protects mouse retina from light-induced damage.** Twenty-four hours after the light exposure in mice retina, with or without obacunone intravitreal injection (2.5 mg/kg body weight, 1h pre-treatment), ERG was recorded, quantified amplitudes of a- and b-waves were presented (A, B). Retinal tissues were collected and expression of listed proteins was shown (C); The relative caspase-3 activity was tested as well (D). Data were expressed as mean  $\pm$  standard deviation (SD, n=5). \*  $p < 0.05$  vs. undamaged control mice ("C"). #  $p < 0.05$  vs. "Light damage" only group (no obacunone administration).

Nrf2 can be used as a drug target for prevention and therapy of AMD and other retinal diseases related to oxidative stress. Agents that can inhibit or even reverse the development of geographic atrophy and prevent progression from atrophic to the “wet” form of AMD would be beneficial for patients. Here we found that intravitreal injection of obacunone largely inhibited light-induced retinal damage in mice. However, the current experimental results from cultured RPE cells and light-induced retinal injury mouse model could not be directly translated to human. Thus the efficacy and safety of obacunone against AMD and other retinal degeneration diseases will require further characterizations. Testing this compound with other administration routes, including oral administration or intravenous injection, in the mice model is certainly needed. Follow-up studies are required as well to test the pharmacokinetics and toxicology of this compound, using at the intravitreal route or other routes.

## **MATERIALS AND METHODS**

### **Reagents and chemicals**

Obacunone, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), neomycin and puromycin were purchased from Sigma-Aldrich (St Louis, Mo). Antibodies for HO1 (#70081), NQO1 (#3187), Nrf2 (#12721), Keap1 (#8047), Tubulin (#2125) and Lamin B1 (#13435) were provided by Cell Signaling Tech (Shanghai, China). All cell culture reagents were from Gibco Co. (Suzhou, China).

### **Cell culture**

Cultures of the established human ARPE-19 cells were described early [17]. Primary murine RPE cells were provided by Dr. Jiang [21, 43], maintained under the previously-described protocol [21, 43]. This study is approved by the Institutional Animal Care and Use Committee (IACUC) and Ethics Committee of Nanjing Medical University.

### **UV radiation**

UV radiation (UVR, UVA2+B, 30 mJ/cm<sup>2</sup>) procedure was described early [26]. RPE cells were exposed to UVR for a total of 5 min. After the applied UVR procedure, RPE cells were maintained in basic medium for applied time periods.

### **Cell viability and cell death assays**

For testing cell viability, RPE cells were initially seeded into 96-well plates at 3,000 cells per well. After UVR, a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories,

Kumamoto, Japan) was utilized to test cell viability [17], with the CCK-8 optic density (OD) tested at 450 nm [44]. For cell death assays, RPE cells were initially seeded into six-well plates at 100,000 cells per well. Lactate dehydrogenase (LDH) release in the medium was tested by a commercial available kit (Takara, Tokyo, Japan), which was normalized to total LDH contents [17].

### **Annexin V FACS**

As described early [17], RPE cells were initially seeded into six-well plates at 150,000 cells per well. Following the applied treatment, cells were harvested, washed and resuspended in binding buffer with 1 μL of Annexin V-FITC and 1 μL of propidium iodide (PI) (Thermo-Fisher Invitrogen, Shanghai, China). After incubation for 15 min under the dark, cells were sorted by flow cytometry via CellQuest software (BD Biosciences, Shanghai, China). Annexin V ratio was recorded as a quantitative indicator of apoptosis intensity [43, 45].

### **TUNEL assay**

As described early [17], RPE cells were initially seeded into 12-well plates at 50,000 cells per well. After the applied treatments, RPE cells were stained with TUNEL and DAPI fluorescence dyes (Sigma). From ten random fluorescence images containing at least 1,000 cells per treatment, the TUNEL ratio (vs. DAPI staining) was calculated [46].

### **ROS assay**

RPE cells were initially seeded into 12-well plates at 50,000 cells per well. Following the applied treatments, RPE cells were incubated with 10 μM of CellROX for 30 min under the dark. The CellROX intensity was tested by a fluorescence spectrofluorometer (Molecular Devices, San Jose, CA).

### **Caspase-3/-9 activity**

RPE cells were initially seeded into 12-well plates at 50,000 cells per well. Following the applied treatments, 30 μg of cytosolic proteins per treatment were incubated with the caspase assay buffer [47] and the AFC-conjugated caspase-3/-9 substrate (Merck Millipore). AFC fluorescence was examined by a Fluoroskan Ascent FL machine.

### **Single strand DNA (ssDNA) assay**

RPE cells were initially seeded into six-well plates at 200,000 cells per well. Following the applied treatment,

the ssDNA contents were tested by an ApoStrand™ ELISA apoptosis detection kit (BIOMOL International, Plymouth Meeting, PA).

### Mitochondrial depolarization

JC-1 fluorescence dye can aggregate to form green monomers in cells with mitochondrial depolarization [48]. RPE cells were initially seeded into 12-well plates at 50,000 cells per well. After the applied treatments, RPE cells were stained with JC-1 (10 µg/mL, Sigma) and its green intensity was examined at 488 nm using a fluorescence spectrofluorometer (Molecular Devices, San Jose, CA).

### Western blotting

The detailed protocols for the Western blotting assays were described previously [17, 49]. The same set of lysate samples were run in sister SDS-PAGE gels to test different proteins. For data quantification, the intensity (total gray) of each band was quantified using the ImageJ software (NIH), normalized to each loading control.

Co-Immunoprecipitation (Co-IP). Following the applied treatment a total of 800 µg protein lysates from APRE-19 cells were pre-cleared and an anti-Keap1 antibody was added. Keap1-bound proteins were captured with protein IgA/G beads and tested by Western blotting assays.

### Quantitative real-time PCR (qPCR)

The total cellular RNA was extracted using the TRIzol reagents (Thermo-Fisher Invitrogen), quantified and reversely transcribed [17]. Protocols for qPCR, using a SYBR Premix Ex Taq™ kit under the ABI-7700 fast PCR system (Takara Biotechnology, Japan), were described previously [17]. mRNA primers for human and murine *HO-1*, *NQO1*, *Nrf2* and *GAPDH* were described early [17, 50]. We employed a  $2^{-\Delta\Delta Ct}$  method to calculate relative expression of targeted mRNA, with *GAPDH* as the reference gene and internal control.

### ARE reporter assay

RPE cells were initially seeded into six-well plates at 100,000 cells per well, transfected with the ARE-inducible firefly luciferase vector (from Dr. Jiang at Nanjing Medical University [51]). Following the applied treatment total cell lysates (30 µg per treatment) were subjected to ARE-reporter luciferase activity assay under a luminescence machine.

### Lipid peroxidation

RPE cells were initially seeded into six-well plates at 100,000 cells per well. Following the applied treatment,

the thiobarbituric acid reactive substances (TBAR) activity, indicating lipid peroxidation, was examined using the previously-described protocol [17].

### Nrf2 shRNA

RPE cells were initially seeded into six-well plates at 150,000 cells per well. Nrf2 shRNA lentiviral particles (sc-37030-V, Santa Cruz Biotech, Shanghai, China) or the scramble control shRNA lentiviral particles (sc-108080, Santa Cruz Biotech) were added for 24h. Afterwards, puromycin (5.0 µg/mL) was added to select stable cells. Nrf2 knockdown was verified by Western blotting and qPCR assays.

### Nrf2 siRNA

The murine Nrf2 siRNA (sc-37049) and the scramble control siRNA were purchased from Santa Cruz Biotech. Murine RPE cells were cultured into six well tissue culture plates at 60% confluence, siRNA was utilized at 500 nM using Lipofectamine 2000 for transfection (last for 48h). Nrf2 silencing in primary murine RPE cells was verified by Western blotting.

### CRISPR-Cas9-mediated gene knockout

From Dr. Liu at Jiangsu University [52], the lentiviral CRISPR-Cas9-Keap1-KO construct and the lentiviral CRISPR-Cas9-Nrf2-KO were obtained, each with GFP and puromycin selection gene. Each construct was transfected to cultured ARPE-19 cells (in complete medium with polybrene). Transfected ARPE-19 cells (GFP positive) were sorted by FACS, and monoclonal cells were further selected by puromycin to establish the stable cells. Keap1 KO or Nrf2 KO in the stable cells was verified by qPCR and Western blotting assays.

### Light damage to murine retinas

As reported [19] the BALB/c mice were provided by the Experimental Animal Center of CAS Shanghai (Shanghai, China). The detailed procedures of light exposure to mice retina were described early [15, 21]. Briefly, in each mice following the pupillary dilation retinas was exposed to 5000 lux of white fluorescent light [41]. One hour before light exposure obacunone (at 2.5 mg/kg body weight) was intravitreally injected to the right eye. For electroretinography (ERG) b-wave amplitudes were tested from the trough of the a-wave to the peak of the b-wave, with the amplitudes of the a-wave measured from the initial baseline [15, 21, 41]. The animal studies were performed according to the IACUC standards with ethical approval by Nanjing Medical University.

## Statistics

Results were presented as the mean  $\pm$  standard deviation (SD). One-way ANOVA was employed to examine the significant differences between groups using SPSS 23.0 (SPSS, Chicago, CA). A two-tailed unpaired T test (Microsoft Excel 2007) was applied to test significance between two treatment groups. Values of  $p < 0.05$  were considered statistically significant.

## Editorial note

<sup>&</sup>This corresponding author has a verified history of publications using a personal email address for correspondence.

## AUTHOR CONTRIBUTIONS

All authors carried out the experiments, participated in the design of the study and performed the statistical analysis, participated in its design and coordination and helped to draft the manuscript.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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