Gene expression analysis

To assess the effects of CDDO-Im (obtained from Mochida Pharmaceuticals) on NC-induced RGC death, we initially administered different doses to three groups of mice for 3 days, via gavage: 1, 3, or 10 μmol/kg body weight (dissolved in 10% dimethylsulfoxide, 10% Cremophor-EL, PBS). However, CDDO-Im at these doses did not result in an increase in the expression of Nrf2-regulated cytoprotective genes, such as *Nqo1*, *Ho-1*, *Gclc*, *Gclm*, *Gsta4*, and *Txnrd* (data not shown). Therefore, we added a new group of mice, treated with CDDO-Im at 30 μmol/kg body weight. All groups were administered CDDO-Im or vehicle via gavage, and were killed 6 h after the last dose. We then removed the retinas and immediately placed them in RNA later for RNA purification and gene expression measurements, as described above.

Statistical analysis

All data were expressed as mean ± SD. The values were processed for statistical analysis, (Mann–Whitney *U*-test) followed by a two-tailed Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

The expression of mRNA and retinal reactive oxygen species increase after NC

RT-PCR analysis revealed that *Nrf2* (588 bp) and *Keap1* (340 bp) were expressed in the retinas of WT mice, but that in the retinas of Nrf2 KO mice, Nrf2 mRNA had been completely eliminated (Fig. 1a). To confirm antioxidative effects in the mouse retina after NC, 8-OHdG and 4-HNE were measured using the immunostaining technique. In DNA, 8-OHdG is a well-known marker for oxidative stress-induced damage. To assess cellular DNA damage, we performed IHC for the anti-8-OHdG antibody. Cells positive for 8-OHdG were observed in the ganglion cell layer (GCL) 7 days after NC, while almost none were seen in retinas without NC (Fig. 1b). We next performed IHC for the 4-HNE antibody, which is a by-product of lipid peroxidation. Cells positive for 4-HNE were observed in the GCL of the retina after NC, but almost none were seen in

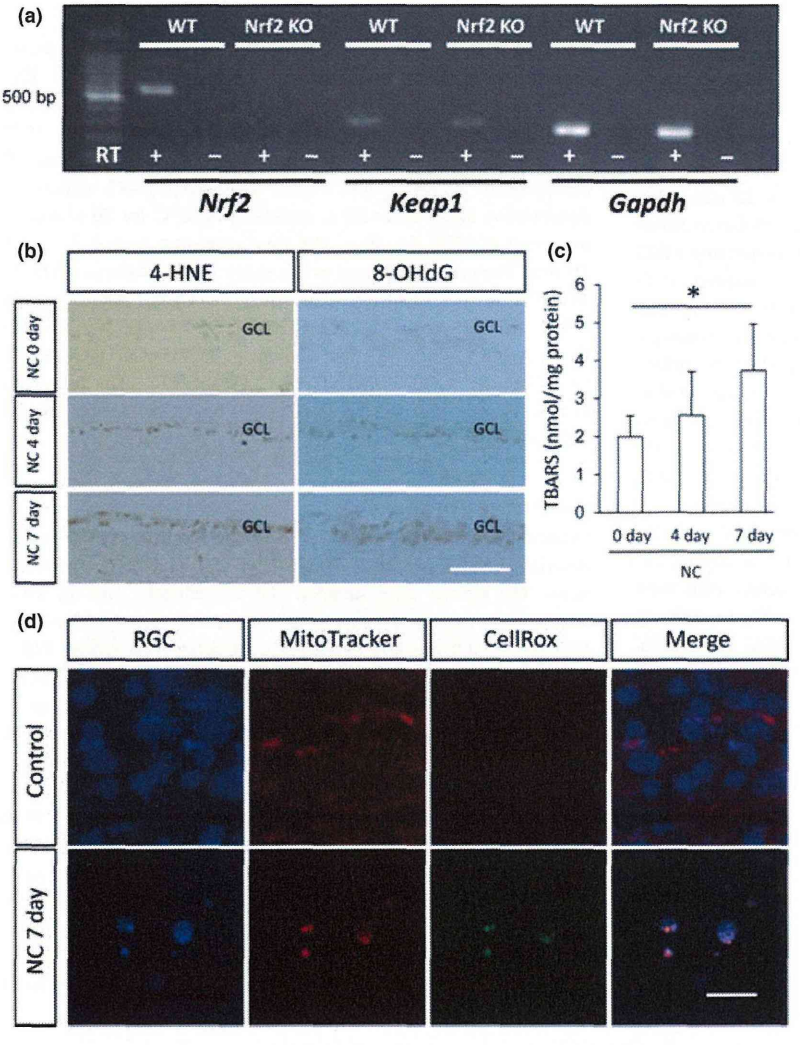

Fig. 1 (a) RT-PCR of the retina. RT-PCR analysis confirmed the presence and absence of *Nrf2* mRNA in the retina of wild-type and Nrf2 deficient mice, respectively. (b) Representative immunohistochemistry for 8-OHdG and 4-HNE-modified proteins in the retina after nerve crush. (c) Thiobarbituric acid reactive substances values of retina ($n = 6$ in each group). *$p < 0.05$. (d) Confocal images of reactive oxygen species and mitochondria. Blue, Fluoro-gold labeled retinal ganglion cell, red, Mito Tracker, green, CellRox. Scale bar 20 μm.

the GCL of retinas without NC (Fig. 1b). To confirm the overproduction of reactive oxygen species (ROS) in the retinas after NC, we checked TBARS values. The TBARS level was assessed in the retinas after NC as an index of lipid peroxidation. Levels were significantly higher 7 days after NC ($p < 0.05$; Fig. 1c). Compared to the control group, however, no significant change was observed in the levels of TBARS 4 days after NC. Moreover, we investigated the expression of ROS in the mitochondria of the RGCs after NC (Fig. 1d). To evaluate ROS production, we used CellROX Deep Green reagent. ROS positive cells (green) were not observed in RGCs without NC. However, at 7 days after NC, a significant number of ROS positive cells were present in the RGCs. Some RGCs, both with and without NC, were also stained with MitoTracker Red dye, a commercially available mitochondrial indicator. Co-localization of the ROS positive cells with MitoTracker was seen in RGCs 7 days after NC.

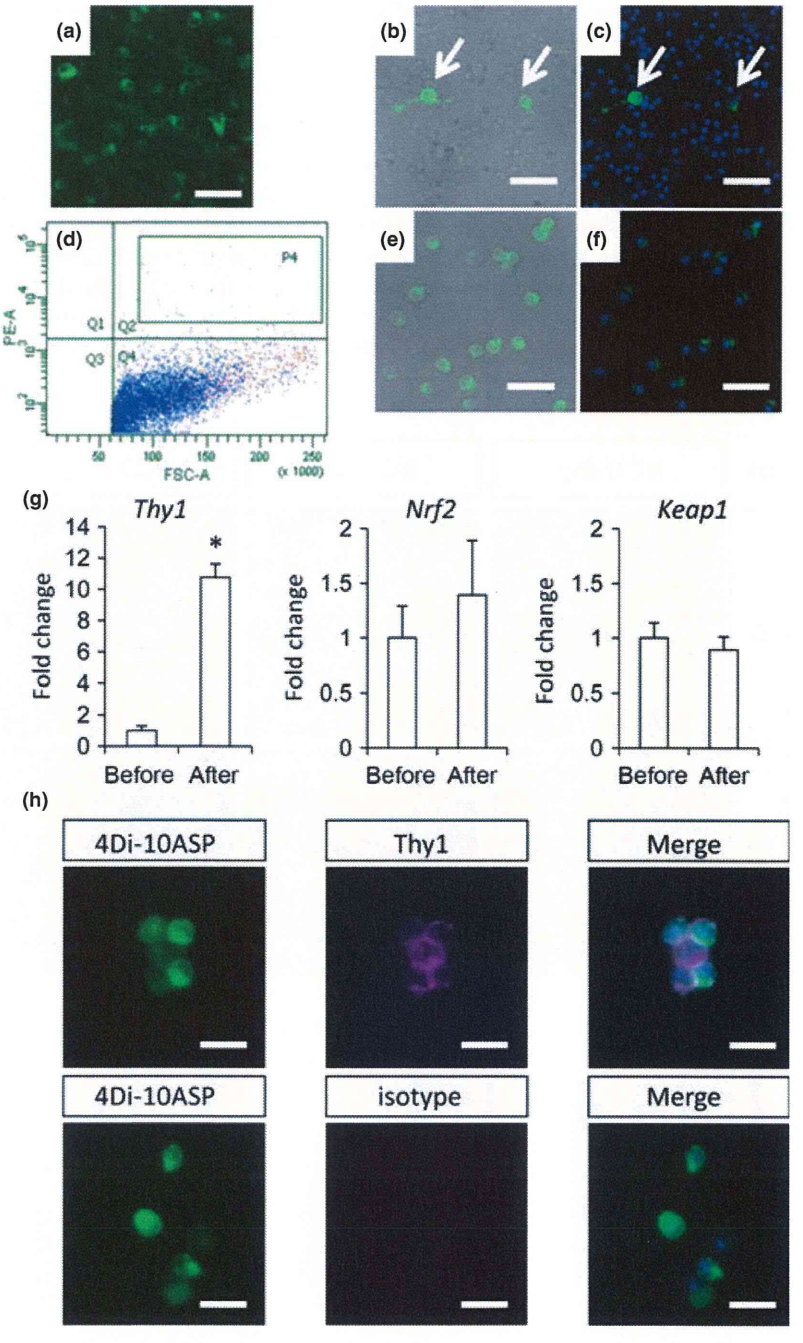

Fig. 2 Retinal ganglion cell (RGC) purification using fluorescence-activated cell sorting (FACS) from dissociated retinas retrogradely labeled with *N*-4-[4-didecylaminostryryl]-*N*-methyl-pyridinium iodide (4Di-10ASP). (a) Representative photograph of a flat-mount retina. The RGCs were labeled with 4Di-10ASP (green). (b, c) Representative photographs of dissociated retinal cells before sorting. (d) Analysis of retinal cells by FACS. (e, f) Representative photographs of large cells with high fluorescence selected for sorting. Scale bar 50 μm. (g) quantitative RT-PCR results showing the quality of cell purification. We assessed the relative abundance of *Thy1*, the marker gene for RGCs. *Nrf2* and *Keap1* were also detected in the RGCs. *$p < 0.05$. (h) Representative immunocytochemistry photographs of RGCs after sorting. Thy1 was mainly expressed in sorted RGCs. Nuclei were labeled with DAPI (blue). Green represents 4Di-10ASP labeled RGCs. Purple represents Thy1. Scale bar 20 μm.

Isolation of RGCs by fluorescence-activated cell sorting

Because RGCs represent only a small percentage of the total cell population in the retina, it was necessary to purify these cells to investigate changes in gene expression in them. The ratio of 4Di-10ASP$^+$ RGCs in a sample of dissociated retinal cells before sorting was only 0.2% (Fig. 2b and c). High purification was achieved using FACS, taking advantage of the fact that RGCs are the only cells in the retina that are marked when 4Di-10ASP is injected into the superior colliculus. RGCs were retrogradely labeled with 4Di-10ASP 7 days after NC (Fig. 2a). FACS analysis demonstrated that before sorting, the population of large and highly fluorescent cells in the dissociated retina was relatively small (Fig. 2d). Large cells with high fluorescence were selected for sorting. After sorting, the proportion of these cells had increased (Figs. 2e and f). The data showed that the ratio of 4Di-10ASP$^+$ RGCs to 4′-6-diamino-2-phenylindole (DAPI$^+$) cells following cell sorting was 96.4%, a high level of RGC purity. In addition, we used QPCR to assess the relative abundance of *Thy1*, the marker gene for RGCs, in the purified cells. We found *Thy1* to be over 10 times more abundant than before sorting (Fig. 2g). Finally, we examined the expression of *Nrf2* and *Keap1* in the purified RGCs with QPCR, and found that they were detectable (Fig. 2g). To confirm the number of collected cells from the flowcytometry analysis, we stained the cells with an RGC marker, such as Thy1 (Fig. 2h). Thy1 was detected in the sorted RGCs, but not in the isotype controls. This immunocytochemistry result clearly indicated that the collected cells were RGCs.

The role of Nrf2 in RGC death following NC

To investigate the neuroprotective effect of Nrf2 after NC, we first performed NC on WT and Nrf2 KO mice, and then assessed the density of surviving RGCs 4 and 7 days later (Fig. 3a and b). Before undergoing NC, there was no significant difference in the density of FG-labeled RGCs in WT (3815 ± 430 cells/mm^2) and Nrf2 KO mice (3846 ± 468 cells/mm^2). Four days following NC, there was still no significant difference in the density of FG-labeled RGCs in WT (3022 ± 473 cells/mm^2) and Nrf2 KO mice (2503 ± 634 cells/mm^2). However, 7 days following NC, the density of FG-labeled RGCs had decreased significantly in the Nrf2 KO mice (313 ± 123 cells/mm^2) compared to the WT mice (692 ± 88 cells/mm^2, $p < 0.01$).

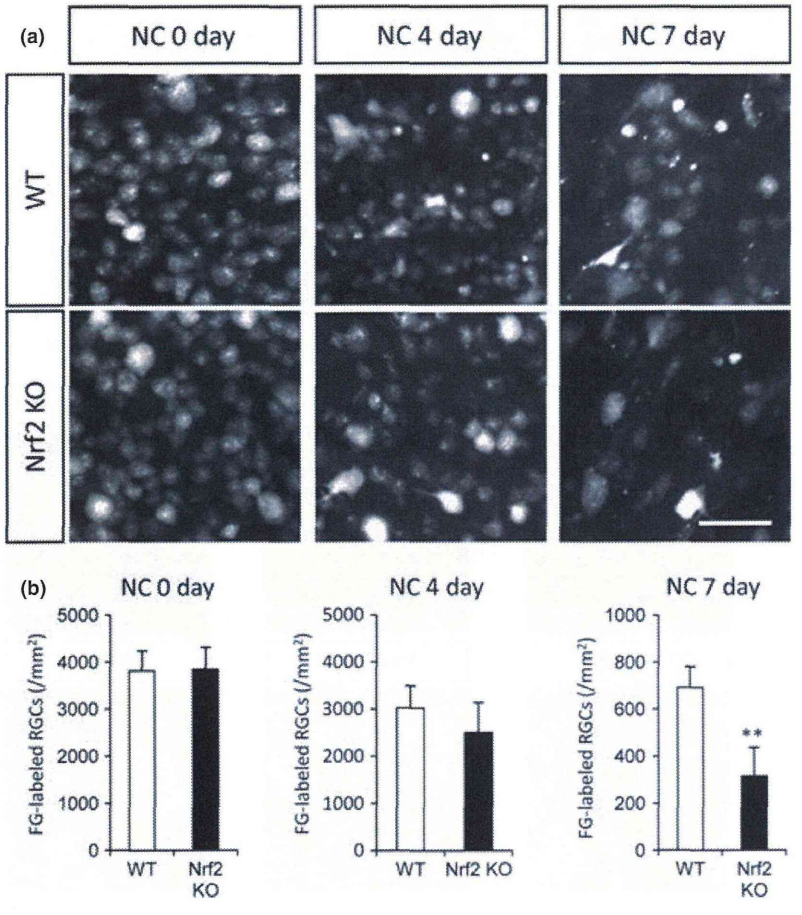

Fig. 3 Vulnerability of Nrf2 deficient mice to nerve crush (NC) injury. (a) Representative photographs of retinal ganglion cell (RGC)s in flat mounted retinas. Scale bar 50 μm. (b) Quantitative data on the density of RGCs after NC injury (n = 5–6 in each group). $^{**}p < 0.01$.

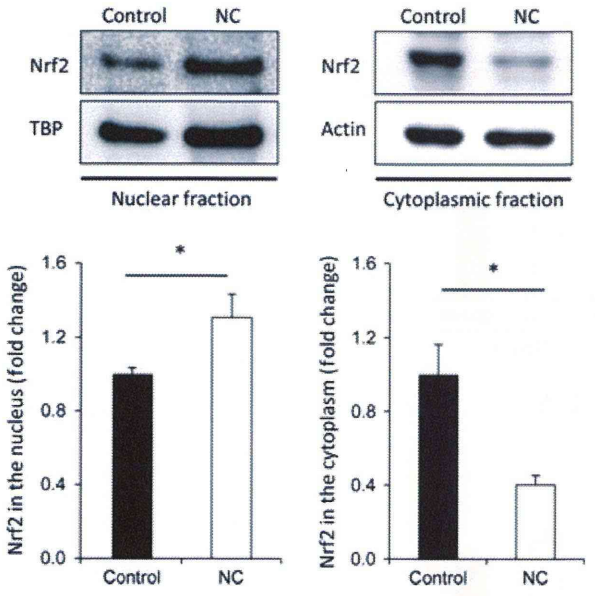

Fig. 4 We extracted nuclear and cytoplasmic fractions from the retinas. TATA binding protein (TBP) and β-actin (Actin) were used as internal controls for the nuclear and cytoplasmic fractions. Bar graphs show cumulative data for immunoblotting band densities (n = 3 in each group).

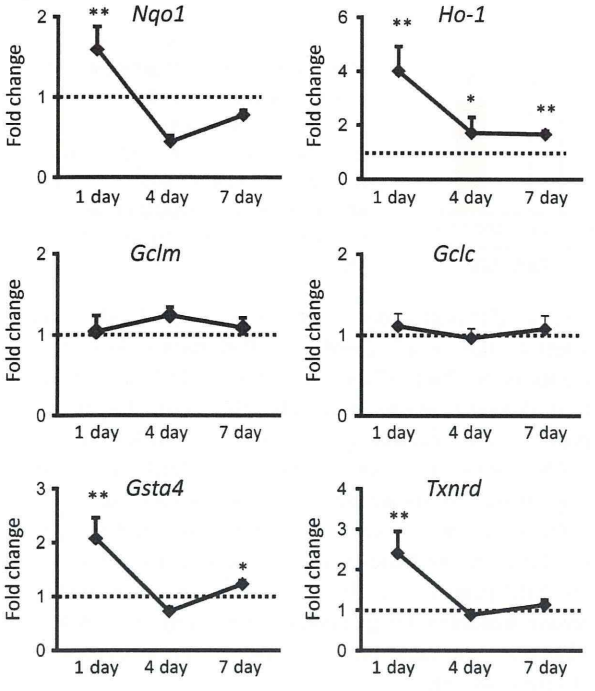

Fig. 5 Changes in retina gene expression. mRNA expression of antioxidant enzymes in the retina increased at 1, 4 and 7 days after nerve crush (n = 3–6 in each group). The graphs show the ratio of expression to sham-operated wild-type mice. *p < 0.05, **p < 0.01.

Nuclear accumulation of Nrf2 1 day after NC

We investigated the role of Nrf2 in axonal damage-induced RGC death, and were able to detect Nrf2 1 day after NC. Immunoblot analysis showed that NC promoted nuclear accumulation of Nrf2 ($p < 0.05$; Fig. 4). We confirmed the efficacy of cell fractionation by using anti-TATA binding protein and anti-β-actin antibodies as loading controls to detect nuclear and cytoplasmic proteins. Our results showed that Nrf2 immediately translocates into the nucleus from the cytoplasm after NC.

The gene expression pattern of Nrf2-related factors after NC

We examined changes in gene expression in the extracted retinas at 1, 4 and 7 days after NC (Fig. 5). The results were normalized to expression levels in retinas that had not undergone NC. One day after NC, we found that expression of *Nqo1*, *Ho-1*, *Gsta4*, and *Txnrd* had increased ($p < 0.01$). Four days after NC, *Ho-1* continued to have a high level of expression ($p < 0.05$). Seven days after NC, moreover, expression of *Ho-1* had increased still further, while expression of *Gsta4* had also risen ($p < 0.01$, $p < 0.05$). However, *Gclm* and *Gclc*, enzymes that directly regulate levels of glutathione, did not change their expression after NC.

CDDO-Im induces phase II and antioxidant genes

To test whether CDDO-Im, a potent activator of Nrf2, influences Nrf2 in the eye, WT and Nrf2 KO mice were treated with CDDO-Im daily for 3 days. Following this, phase II and antioxidant gene expression was measured in the retina. A statistically significant induction of these genes occurred in the retina following a dose of 30 μmol/kg (data not shown). CDDO-Im induced the expression of many genes in WT mice that were not induced in Nrf2 KO mice, such as *Nqo1*, *Ho-1*, *Gclm*, *Gclc*, *Gsta4*, and *Txnrd*, indicating that these responses are Nrf2 dependent (Fig. 6). Enzyme levels for the WT and Nrf2 KO mice were both set at 1 for the vehicle groups only to more easily show relative changes. The absolute mRNA levels of the vehicle-group Nrf2 KO mice were not the same as of the vehicle-group WT mice. In fact, mRNA levels of antioxidant enzymes were lower in the Nrf2 KO mice than in the WT mice.

Oxidative stress-induced RGC death inhibition with CDDO-Im

We investigated the activation of Nrf2 by CDDO-Im, and the possibility of a preventive effect against oxidative stress-induced RGC death. Seven days after NC, the density of FG-labeled RGCs in WT mice treated with CDDO-Im was significantly higher than in those treated with vehicle (1337 ± 219 cells/mm^2 and 819 ± 185 cells/mm^2, respectively, $p < 0.01$; Fig. 7c). In contrast, Nrf2 KO mice treated with CDDO-Im (490 ± 77 cells/mm^2) showed no difference from those treated with vehicle (450 ± 92 cells/mm^2) 7 days after NC.

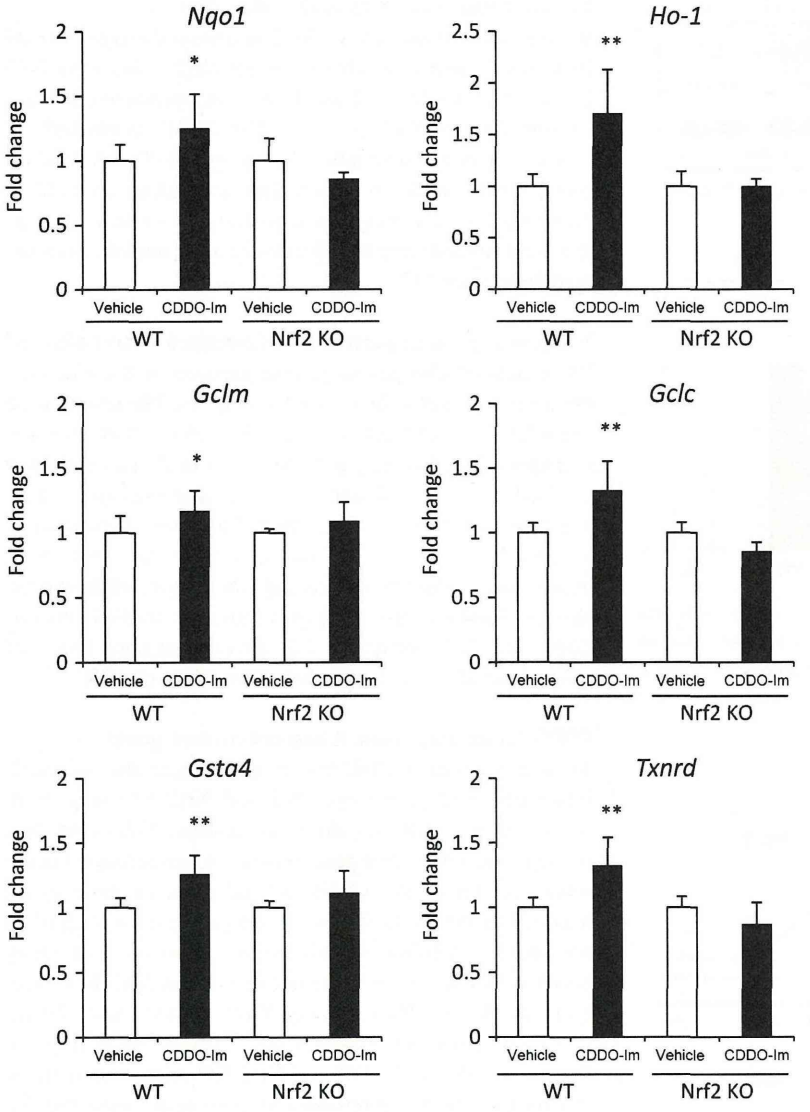

Fig. 6 Changes in mRNA levels measured by quantitative RT-PCR in mice retinas after treatment with 1-(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-pyl)imidazole (CDDO-Im). Wild-type (WT) and Nrf2 deficient (Nrf2 KO) mice were administered CDDO-Im or vehicle at 1 day intervals over the course of 3 days. Open columns: WT or Nrf2 KO mice treated with vehicle, and solid columns: WT or Nrf2 KO mice treated with CDDO-Im (30 μmol/kg; $n = 4$–6 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$.

Discussion

The results of this study suggest that the *Nrf2* gene is an important factor in protecting RGCs from axonal damage-induced RGCs death because of oxidative stress conditions. We compared the density of FG-labeled RGCs in WT and Nrf2 KO mice both with 7 days after NC or without NC. We observed that there was no difference of FG-labeled RGCs without NC, while significantly fewer FG-labeled RGCs in the Nrf2 KO mice than in the WT mice. This suggests that Nrf2 deficiency augments NC-induced RGC death. In contrast, CDDO-Im, an extremely potent activator of Nrf2, also demonstrated a neuroprotective effect against NC-induced RGC death *in vivo*. These data strongly suggest that Nrf2 has a neuroprotective effect on RGCs.

Oxidative stress is implicated in neuronal cell death in many neurodegenerative diseases, such as Alzheimer's disease (Ramamoorthy *et al.* 2012), amyotrophic lateral sclerosis (Lee *et al.* 2009), and Parkinson's disease (Clements *et al.* 2006; Choi *et al.* 2012). Nrf2 activation has been demonstrated to be a viable therapeutic target for the prevention of chronic neurodegeneration (Clements *et al.* 2006; Vargas *et al.* 2008). Our results clearly and strongly suggest that glaucoma should also be considered a chronic neurodegenerative disease associated with oxidative stress. Furthermore, we believe we have shown that activation of the Nrf2 pathway has the potential to be a new neuroprotective treatment for glaucoma, particularly normal tension glaucoma (the main type of glaucoma in Asian countries) (Iwase *et al.* 2004).

As RGCs represent only a small fraction of the total retinal cell population, many important responses in RGCs are easily masked. In an effort to identify changes more specific to RGCs, Fischer *et al.* studied gene expression in individual

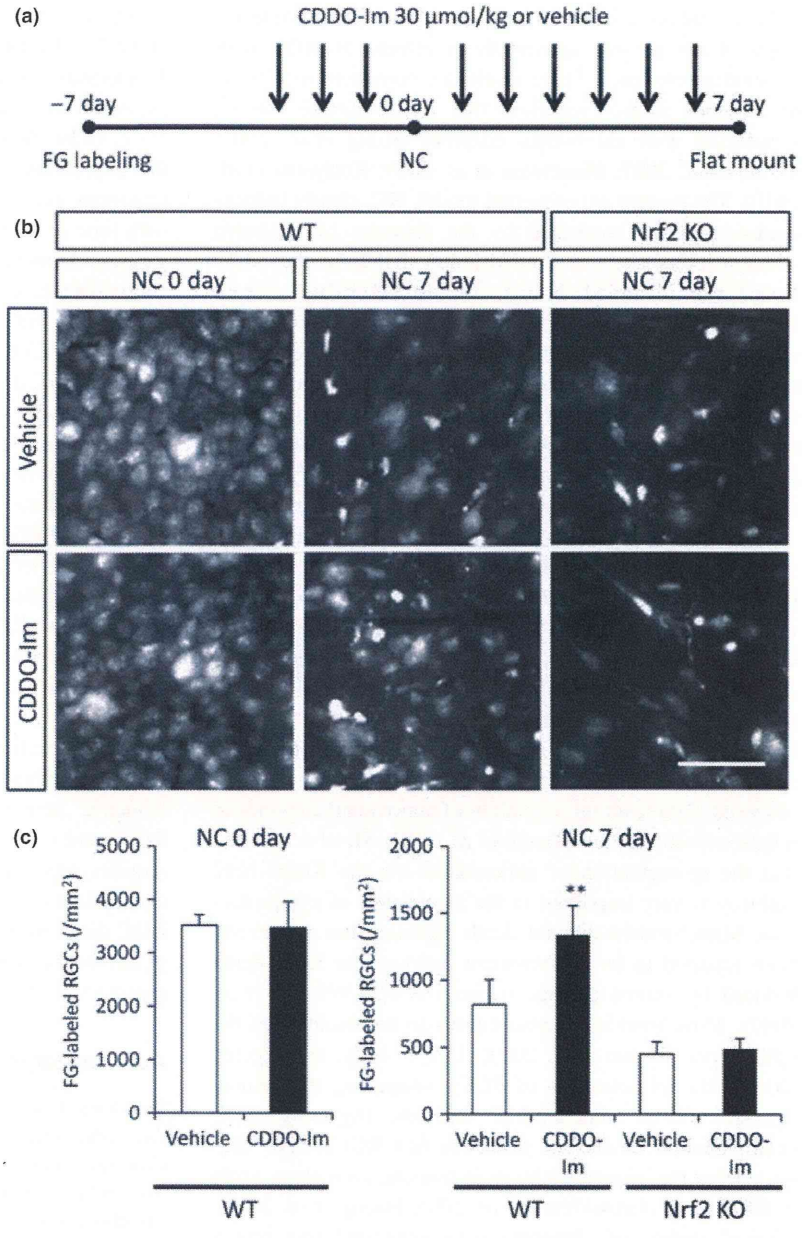

Fig. 7 Effects of activation of Nrf2 by 1-(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-pyl)imidazole (CDDO-Im) on retinal ganglion cell (RGC) death. (a) Protocol for evaluating CDDO-Im. The arrows indicate the days gavage of CDDO-Im was performed: CDDO-Im at 30 μmol/kg body weight every 24 h before nerve crush (NC) for 3 days, and after NC for six more days. (b) Representative photographs of RGCs in flat mounted retinas. Scale bar 50 μm. (c) Quantitative data on the density of RGCs after NC injury with and without CDDO-Im treatment (30 μmol/kg; $n = 4$–6 in each group). $^{**}p < 0.01$.

rat RGCs sorted with FACS (Fischer *et al.* 2004). In this study, we showed that adult primary neurons could also be isolated in mice. We modified a FACS-based cell purification technique and successfully used it to isolate RGCs from the retina. RGCs normally represent less than 0.5% of cells in the retina. The combination of sorting cells by retrograde fluorescent label and cell size allowed us to obtain a high purity of RGCs in a simple one-step procedure. By isolating RGCs, we were able to confirm that *Nrf2* and *Keap1* were expressed not only in the retinas but also in the RGCs of mice. These data may suggest that the RGC purification technique would be necessary and helpful for exploring the glaucoma pathogenesis because RGC was small population in the retinal cells.

Previous reports showed that oxidative stress could induce the apoptosis of RGCs (Maher and Hanneken 2005a,b; Inomata *et al.* 2006). Recently we also demonstrated that Nrf2 KO mice were susceptible for hyperglycemia-induced RGC death (Shanab *et al.* 2012), where the oxidative stress and subsequent intracellular calcium influx has pivotal roles in RGC death. ROS play a beneficial role in cell signaling, but their uncontrolled production can lead to cell damage through necrosis or apoptosis. ROS, which include free radicals such as superoxide anions, hydroxyl radicals, lipid

radicals, and nitric oxide, are a major contributor to oxidative stress. Cells protect against these effects of ROS with antioxidant enzymes. These results are consistent with those of previous studies reporting that RGC damage can be suppressed with antioxidant enzymes (Kong *et al.* 2007; Tanito *et al.* 2007; Munemasa *et al.* 2009; Koriyama *et al.* 2010). The present experimental model, NC, clearly induces oxidative stress, confirmed by the presence of oxidative stress markers such as 8-OHdG and 4HNE in the GCL. Keap1 was identified through a yeast two-hybrid screen. It suppresses Nrf2 activity by specifically binding to its evolutionarily conserved *N*-terminal Neh2 regulatory domain. When cysteine residues of Keap1 are modified by oxidative stress, the yeast two-hybrid screen changes. As a result, the suppression of Keap1 for Nrf2 is deactivated. Nrf2 subsequently translocates to the nucleus from the cytoplasm and activates the transcription of cytoprotective enzyme.

We found that RGCs in Nrf2 KO mice were more susceptible to NC-induced RGC death, and that the survival ratio of RGCs in the Nrf2 KO mice was lower than in WT mice both *in vivo* and *in vitro*. This is the first demonstration that Nrf2 plays a critical role in NC-induced RGC death. After NC, there is nuclear accumulation of Nrf2, and transcription of its downstream targets is activated, resulting in up-regulation of antioxidant and phase II detoxifying enzymes. After nuclear translocation of Nrf2, Tanito *et al.* observed significant up-regulation of antioxidant enzymes in a light exposure group (Tanito *et al.* 2007). He also reported that the up-regulation of antioxidants via the Keap1-Nrf2 pathway is very important in the mechanism of cytoprotection. Mitochondrial-derived death signaling has previously been reported to be an important pathway for RGC death induced by axonal damage (Chierzi *et al.* 1999; Qi *et al.* 2007). Mitochondria are also known to be abundant in the optic nerve (Barron *et al.* 2004). In this study, we detected ROS in the mitochondria of RGCs, suggesting that axonal damage affects mitochondrial function, triggering RGC death. Several studies of nerve-crushed RGCs have suggested that the caspase pathway in mitochondria plays a role in RGC death (Grosskreutz *et al.* 2005; Huang *et al.* 2005). Clinical studies of glaucoma have suggested that retinal microcirculation in the optic disc is disturbed in the early stages of the disease (Caprioli *et al.* 2010), and that as a result, axonal damage occurs in the lamina cribrosa. This axonal damage and mild chronic ischemia may induce ongoing oxidative stress in the axon and RGC body (Lieven *et al.* 2006). In this study, we have confirmed that oxidative stress is also involved in NC-induced RGC death. Oxidative stress induces ROS generation in mitochondria, causing the activation of Nrf2 (Calkins *et al.* 2005; Shih *et al.* 2005; Imhoff and Hansen 2009). Our results thus strongly suggest that the prevention of oxidative stress in mitochondria should be regarded as a candidate for the treatment of glaucoma.

In this study, we have also demonstrated that the activation of Nrf2 by CDDO-Im can protect against RGC death *in vivo*. To elucidate the effect of Nrf2 on RGC death after NC, we examined the expression of the Nrf2-responsive genes *Nqo1*, *Ho-1*, *Gclm*, *Gclc*, *Gsta4*, and *Txnrd*. We had assumed that the expression in the retina of *Nqo1*, one of the most important genes related to Nrf2 activation, would increase with time or the type of stimulation after injury. In addition, we have demonstrated that the expression of *Nqo1* and other antioxidant genes is up-regulated in WT mice treated with CDDO-Im, but not in Nrf2 KO mice. When Nrf2 was activated by CDDO-Im, a neuroprotective effect against NC-induced RGC death was also apparent *in vivo*. Antioxidant enzymes such as *Nqo1*, *Gclc*, and *Ho-1* protected the mitochondria from losing membrane potential and complex activity, and improved mitochondrial function (Feng *et al.* 2010). Our data thus suggest that Nrf2 plays a critical role in NC-induced RGC death. *Gclm* and *Gclc* did not change their expression after NC (as shown in Fig. 6), while CDDO-Im induced significant expression of *Gclm* and *Gclc* (as shown in Fig. 7). This CDDO-Im-induced expression did not occur in the Nrf2 KO mice. This would indicate that CDDO-Im has a stronger Nrf2-activating effect than NC, though further experiments are necessary to determine this.

Neuroprotective strategies have been proposed and are being investigated as new goals for glaucoma therapy. Effective neuroprotection, aimed at salvaging functional RGCs and their axons before they are irreversibly damaged, requires early intervention and the targeting of upstream events. Identification of the early clinical molecular events in RGC death would add to our understanding of the nature of glaucomatous injury and provide potential targets for neuroprotective strategies.

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Original Contribution

The role of the Nrf2-mediated defense system in corneal epithelial wound healing

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ABSTRACT

The corneal epithelium exists at the surface of cornea and is easily damaged by external stresses such as UV radiation or physical injury. The Nrf2-mediated defense system plays a central role in protecting cells by activating genes against these types of stress. In this study, we investigated the role of the Nrf2-mediated defense system in corneal epithelial wound healing by using Nrf2-knockout (KO) mice. Nrf2 was expressed in the corneal epithelium of wild-type (WT) mice, but not in KO mice. Observation of wounds after 24 h of healing revealed that healing of the corneal epithelium was significantly delayed in the Nrf2 KO mice, whereas Nrf2 was activated in the corneal epithelium of WT mice. Ki-67 staining revealed that the number of Ki-67-positive proliferating cells was significantly lower in the Nrf2 KO mice than in the WT mice at 24–36 h after injury; however, these numbers were approximately equivalent by 48 h. To clarify the role of Nrf2 during wound healing, we performed in vitro experiments with siRNA for Nrf2 and its suppressor Keap1. Nrf2 knockdown significantly delayed corneal epithelial cell migration, but did not affect cell proliferation. Conversely, Keap1 knockdown significantly accelerated cell migration. These results suggest that Nrf2 contributed to the corneal epithelial wound-healing process by accelerating cell migration, and Nrf2 would therefore be a good target for the treatment of corneal epithelial diseases such as dry eye or chronic corneal epithelial defect.

The cornea is a transparent tissue located in the anterior chamber of the eye. It consists of three layers—the epithelium, stroma, and endothelium. The corneal epithelium spans the entire surface of the cornea and plays a role in the defense of the ocular surface against external injury or infection. However, the corneal epithelium is easily damaged by external stresses such as UV radiation or physical injury. These external stresses have been known to involve oxidative stress [1–3], and the influence of oxidative stress on the corneal epithelium has been increasingly studied in recent years [4–7].

Additionally, whereas studies have discussed the effects of oxidative stress on corneal epithelium wound healing, they did not discuss the participation of the defense systems against oxidative stress. Thus, both the role and the mechanism of the defense system in corneal epithelial wound healing remain unclear.

The antioxidative stress factor Nrf2 is a transcriptional factor that systematically regulates the expression of various cytoprotective enzymes such as NAD(P)H dehydrogenase, quinone 1 (NQO1), heme oxygenase-1 (HO-1), and glutathione *S*-transferase [8–10]. Under normal conditions, Nrf2 is bound to Keap1 in the cytoplasm, and this complex is carried to proteasomes where it undergoes decomposition [11]. However, Nrf2 will dissociate from Keap1 when Keap1 is subjected to oxidative or electrophilic stress. After its translocation into nuclei, Nrf2 binds with the antioxidant response element and upregulates downstream genes [12,13]. Interestingly, recent studies have suggested that Nrf2 not only is involved in xenobiotic metabolism but also plays important roles in cancer generation and wound healing in some tissues [14–16]. However, neither the specific role nor the mechanism of action of the Nrf2-mediated defense system has been elucidated in wound-healing processes associated with the corneal epithelium or other tissues. Therefore, in this study, we attempted to clarify the role played by Nrf2 in corneal epithelial wound healing, migration, and proliferation by using Nrf2-knockout (KO) mice and Nrf2- or Keap1-specific small interfering RNA (siRNA).

Materials and methods

Animals

This study was performed in accordance with the ARVO *Statement for the Use of Animals in Ophthalmic and Vision Research* and was

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