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NRF2 and Age-Dependent RPE Degeneration

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1. Introduction

Retinal pigment epithelium (RPE) is a single layer of epithelial cells lined between the neurosensory retina and choriocapillaris. It is part of the blood-retinal barrier and is a central component of the visual phototransduction pathway. RPE cells regenerate 11-cis-retinal by RPE65 isomerase and its related enzymes and chaperones (Moiseyev et al., 2006; Xue et al., 2004). They are professional phagocytes and are responsible for the clearance of daily shed photoreceptor outer segments (POS) (Young, 1967; Young & Bok, 1969). The multi-step process of phagocytosis includes receptor-mediated binding of POS to the RPE (Finnemann et al., 1997), internalization (Feng et al., 2002; Finnemann & Silverstein, 2001), transport to lysosome and degradation. The importance of RPE phagocytosis has been clearly illustrated by the Royal College of Surgeons (RCS) rats, which carry a mutation in *Mertk* gene (D'Cruz et al., 2000). MERTK is a membrane-associated receptor tyrosine kinase and is activated upon binding of POS to the RPE (Feng et al. 2002). In RCS rats, loss-of-function mutation of *Mertk* causes defects in phagocytosis and consequently these animals develop inherited retinal dystrophy and photoreceptor apoptosis (Tso et al., 1994). In addition to their roles in the visual cycle, RPE cells provide vital support for the structure and function of the outer retina. They transport ions, water and nutrients between choroidal blood supply and the retina, and synthesize melanin which absorbs light and shields the retina. RPE-produced growth factors, such as vascular endothelial growth factor (VEGF), are indispensable for the choroidal vasculature (Saint-Geniez et al., 2009).

Degeneration of the RPE with aging is an initiating event in age-related macular degeneration (AMD), a major cause of blindness in elderly people. Approximately 11% of persons between ages 65 and 74 have AMD, with prevalence rates rising to 30% in individuals at age 75 or older (Lee et al., 2003). Vision loss in AMD occurs through photoreceptor loss in the macula, the central area of the retina, and results either from a gradual "geographic atrophy" of the RPE (dry or atrophic disease) or from leakage and/or bleeding from choroidal neovascularization (CNV) (wet or neovascular disease). During CNV, blood vessels break through Bruch's membrane, leading to rapid loss of central vision in many cases. In recent years anti-VEGF agents have achieved unprecedented success in preserving visual acuity in patients with CNV (Brown et al., 2006; Rosenfeld et al., 2006; Galbinur et al., 2009). Detailed clinical aspects of wet AMD and anti-VEGF therapy are covered by other chapters of this book.

The genetic and biochemical mechanisms of RPE degeneration in dry AMD, however, remain largely unknown. Several hypothetical models have been proposed, including accumulation of lipofuscin and its bisretinoid fluorophore (Sparrow et al., 2003; Zhou et al., 2006), iron overload (Dunaief, 2006; Hahn et al., 2004), autoimmune response (Hollyfield et al., 2008) and exposure to double strand RNA (Ambati, 2011; Kaneko et al., 2011). All of them have suggested clinical associations with AMD and their causal relationships to the disease have been demonstrated by respective animal models (Ramkumar et al., 2010). Oxidative stress is a common mechanism underlying these diversified pathological processes. Photooxidation of the bisretinoids can produce singlet oxygen and release methylglyoxal to form advanced glycation end product (Wu et al., 2010). Iron overload increased isoprostane, a marker of lipid peroxidation, in the RPE/choroid (Hadziahmetovic et al., 2008). Mice immunized with serum albumin conjugated with carboxyethylpyrrole, an oxidation product of docosahexaenoic acid, developed signs of RPE degeneration and deposition of complement proteins in the Bruch's membrane (Hollyfield et al., 2008). Oxidative stress can downregulate DICER1, a RNA processing enzyme whose deficiency was shown to cause Alu RNA-induced cytotoxicity and RPE apoptosis (Kaneko et al., 2011).

Results from earlier clinical and laboratory studies also support the contributing roles of oxidative stress to AMD. Smoking is the strongest environmental risk factor of AMD (Cano et al., 2010; Smith et al., 2001) and has been clearly associated with oxidative stress (DeBlack, 2003; Mitchell et al., 2002; Pryor et al., 1983; Smith et al., 2001). A number of interventional studies showed that antioxidant supplementation had protective effects against development of AMD or limiting its progression. Experimental animals fed with diets supplemented with antioxidants demonstrated an increased resistance to retinal degeneration (Ham et al., 1984; Organisciak et al., 1985; Tso et al., 1984). Results from the Age-Related Eye Disease Study (AREDS) showed that supplemental antioxidants (vitamin C, vitamin E and beta carotene) and zinc can decrease the risk of progression from intermediate AMD to advanced AMD by 25% (AREDS 2000 & 2001). Taken together, the findings from the research of the past two decades suggest that AMD is a multifactorial disease, with oxidative stress viewed as a common mechanism involved in the gene-environmental interaction of its etiololgy.

Oxidative stress is due to an imbalance between the generation of reactive oxygen species and their clearance by antioxidant systems. The RPE has powerful endogenous antioxidant capacity to overcome the high level of oxidative stress, which is caused by both focal light exposure and high metabolic rate of the retina. In addition to utilizing direct radical scavengers such as β-carotene, ascorbic acid and α-tocopherol, RPE cells have an elaborate enzymatic antioxidant system that can prevent and repair oxidative injury. Nuclear factor erythroid 2-related factor 2 (NRF2) is a master regulator of cellular antioxidant and detoxification responses (Kensler et al., 2007). We and others have shown previously that elevating the transcriptional activity of NRF2 can protect against oxidative injury to the RPE; while mice that are deficient of NRF2 developed pathological features similar to human AMD (Zhao et al., 2011; Cano et al., 2010). Oral zinc supplementation, which was used in the AREDS to slow AMD progression, can activate NRF2-dependent antioxidant system in the RPE (Ha et al., 2006). More recently, a newer class of NRF2 inducers, which are based on synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its

derivatives, have achieved potent protection in various models of retinal damage (Pitha-Rowe et al. 2009). In this chapter we will review past and recent literature reports, based on cell culture, animal models and human clinical studies, to address how NRF2 regulates RPE function both *in vitro* and *in vivo*.

2. NRF2-dependent antioxidant defense

NRF2 is a transcription factor that controls the expression of phase 2 detoxification genes. It heterodimerizes with members of the Maf family of transcription factors and binds to the *cis*-acting antioxidant response element in the promoter regions of various phase 2 genes (Katsuoka et al., 2005; Motohashi et al., 2004). The latter encode a group of enzymes, such as glutamate-cysteine ligase, glutathione S-transferase, glutathione peroxidase, heme oxygenase, NAD(P)H:quinone reductase and glutamate-cysteine exchanger, which are essential for detoxification of xenobiotics and endogenous reactive intermediates (Kensler et al., 2007; Wakabayashi et al. 2010). NRF2-deficient mice showed increased sensitivity to a variety of pharmacological and environmental toxicants (Kensler et al., 2007; Rangasamy et al., 2004). The protective effects of NRF2 inducers have been tested in a number of models of human diseases, including cancer, neurodegeneration, cardiovascular disease, and liver and lung injury (Kensler et al., 2007; Wakabayashi et al., 2010).

Activation of NRF2 is subjected to multiple levels of regulation. Under basal conditions, NRF2 is sequestered by its inhibitor protein, Keap1, and is targeted for Cullin 3/Rbx1-mediated ubiquitination and degradation (Cullinan et al., 2004; Furukawa & Xiong, 2005; Kobayashi et al., 2004). Upon conditions of oxidative stress or exposure to electrophilic compounds, NRF2 protein can be liberated from Keap1 and will translocate into nucleus to mediate gene trasncription. As illustrated in Fig. 1, there are six Neh (NRF2 ECH homology) domains that are responsible for most of the functions of NRF2. The Neh domains show amino acid sequence homology conserved between different species including human, rodents and chicken (McMahon et al., 2004; Zhang et al., 2007).

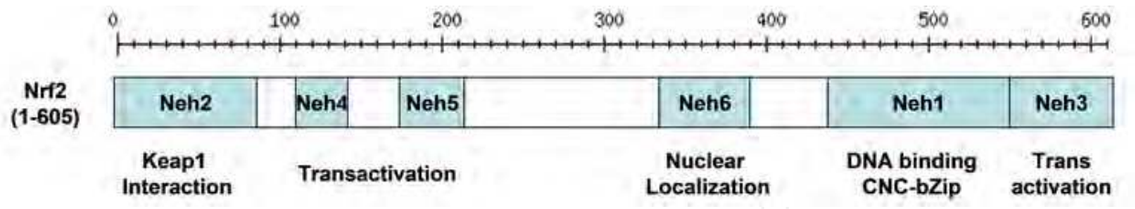

Fig. 1. Illustration of the Neh domains of the NRF2 protein. Human NRF2 is a polypeptide of 605 amino acids and contains 6 Neh domains. The relative positions of each domain and their putative functions are listed. Neh1 contains the signature cap-n-collar motif which is a highly conserved basic leucine zipper domain for DNA binding. The nuclear localization and export signals are present in both Neh6 and Neh1.

3. NRF2-mediated protection in cultured RPE cells

Compounds that promote the nuclear translocation of Nrf2 and elevate its transcriptional activity can protect against oxidative injury in cultured RPE cells. In 2001, Talalay and

colleagues first reported that sulforaphane could prevent RPE cell death caused by treatments with menadione, t-butyl hydroperoxide, 4-hydroxynonenal and peroxynitrite (Gao et al., 2001). Since then numerous other studies reported the protective effects of a wide range of structurally-different NRF2 inducers including isothiocyanates (sulforaphane) (Gao & Talalay, 2004), polyphenols (curcumin, resveratrol and flavonoids) (Alex et al., 2010; Johnson et al., 2009; Mandal et al., 2009), 1,2,dithiole-3-thiones (oltipraz) (Nelson et al., 2002), zinc (Ha et al., 2006) or triterpenoids (Pitha-Rowe et al., 2009). Many of them are naturally occurring compounds present in fruits and vegetables, making them ideal for dietary supplementation. Some of the compounds have either gone through human clinical trials or are currently used for other applications. For instances, zinc was used in the AREDS supplementation either alone or with antioxidant vitamins. Oltipraz, a dithiole derivate, is used in treating schistosomiasis and cancer chemoprevention (Jacobson et al., 1997). A common mechanism underlying the antioxidant and detoxification functions of NRF2 is to increase cellular glutathione (GSH) synthesis.

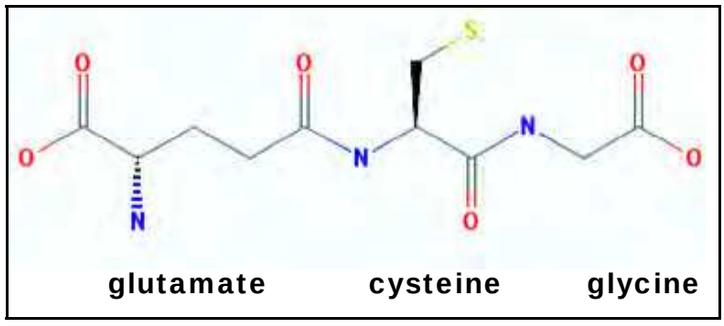

Fig. 2. Structure of glutathione. The γ-glutamylcysteine is formed by a peptide bond between the carboxylate group of glutamate and animo group of cysteine. The sulfhydryl group of cysteine is responsible for the antioxidant function of the tripeptide.

GSH is a tripeptide consisted of glutamate, cysteine and glycine. It contains a unique peptide bond between the amine group of cysteine and the carboxyl group of the glutamate side chain so that it is much more resistant to degradation by peptidase (Fig. 2). The sulfhydryl group of cysteine of GSH can be used by glutathione S-transferase to conjugate electrophilic centers on a wide variety of substrates (Pool-Zobel et al., 2005). GSH is also used by glutathione peroxidase to reduce lipid hydroperoxides and hydrogen peroxide to alcohols and water, respectively. The glutamate cysteine ligase (GCL) is the rate-limiting enzyme of GSH synthesis. It generates γ-glutamylcysteine from glutamate and cysteine. NRF2 inducers can elevate the mRNA levels of the catalytic and modulatory subunits of GCL. Cystine uptake by the RPE is mediated by a sodium independent, cystine/glutamate exchanger (Bridges et al., 2001; Ishii et al., 1992). The transporter is consisted of two subunits, xCT as the light chain and 4F2hc as the heavy chain (Wagner et al., 2001). NRF2 controls the expression of *xCT* gene (Sato et al., 1999). In *xCT* knock out mice, the plasma cystine concentration almost doubled, resulted from decreased tissue uptake (Sasaki et al., 2002). The $xCT^{-/-}$ mice showed more several renal injury caused by ischemia-reperfusion (Shibasaki et al., 2009). Thus, NRF2 inducers can increase both the rate of GSH synthesis and cellular concentration of its amino acid precursor.

Monitoring the RPE glutathione content is a reliable assay for initial screening of model compounds designed to activate NRF2. For instance, RPE cells pretreated with oltipraz showed increased total and mitochondrial GSH. At 50 μM, oltipraz increased total cellular GSH by 18% and mitochondrial GSH by 50%, and achieved significant protection against tert-butylhydroperoxide-induced RPE cell death (Nelson et al., 2002). Similar results were obtained from cells pretreated with dimethylfumarate (DMF) for 24 hours (Nelson et al., 1999). However, when the time course of the DMF was evaluated, a transient decrease in GSH levels was found that preceded the increase noted at later time points. Compared to vehicle-treated control cells, cells pretreated with DMF for 3 hours showed a significant reduction in viability when further challenged by peroxide (Nelson et al, 1999). Thus, the initial decrease of GSH after DMF treatment rendered the RPE cells more sensitive to oxidative injury, although it can subsequently lead to a feedback increase of GSH synthesis and a more robust antioxidant response (Nelson et al., 1999). Many of the NRF2 inducers are thiol-reacting compounds and may cause a similar initial depletion of cellular GSH. Therefore, although the *in vitro* culture system does not present the complexity of the retinal microenvironment and cell-cell interaction *in vivo*, it is a valuable tool for assessing both the pharmacological properties of new NRF2 inducers and their potential toxicities. For treatment of a chronic disease like AMD, the RPE cells are already stressed by oxidative burden and may not tolerate transient GSH depletion after repeated administration of agents that react with cellular thiols with low selectivity.

4. Ocular pathology of *Nrf2* knockout mice

Nrf2 knockout mice have normal embryonic development (Chan et al., 1996) and their basal level of antioxidant status in many tissues is not different from age-matched wild type mice. However, the *Nrf2* null mice show increased sensitivity to a variety of pharmacological and environmental toxicants (Cano et al., 2010; Kensler et al., 2007; Osburn & Kensler, 2008). Depending upon the stimuli, injuries occur in different organs and tissues. The phenotypes vary, but commonly involve oxidative and inflammatory stress. For ocular pathology, neonatal *Nrf2* knockout mice develop more severe retinal vaso-obliteration at early phase after hyperoxia exposure (Uno et al., 2010). NRF2 also modulates the innate immune response in the retina and iris-ciliary body in a mouse model of uveitis induced by intraperitoneal injection of lipopolysaccaride (Nagai et al., 2009).

Aging and smoking are the major demographic and environmental risk factor of AMD, respectively. Cano and colleagues (2010) reported that NRF2-deficient mice were more susceptible to smoking-induced retinal injury. At 8 months, *Nrf2* null mice showed a mild degree of ultrastructural change in the RPE. Comparing to age-matched wild type mice, RPE of the knockout mice exposed to cigarette smoking for 6 months (starting at 2 months) displayed markedly increased staining of 8-hydroxydeoxyguanosine, an indicator of accumulated oxidative DNA damage (Cano et al., 2010). On electron microscopy, *Nrf2*$^{-/-}$ smoking mice displayed abnormal RPE basal infoldings and vacuoles, without apparent changes of the choroidal endothelium or sub-RPE deposit formation (Cano et al., 2010). Thickening and deposits in the outer collagenous layer of Bruch's membrane were often observed in smoking mice. The data suggest that NRF2-mediated protection to the RPE is important against chronic environmental toxicities associated with AMD.

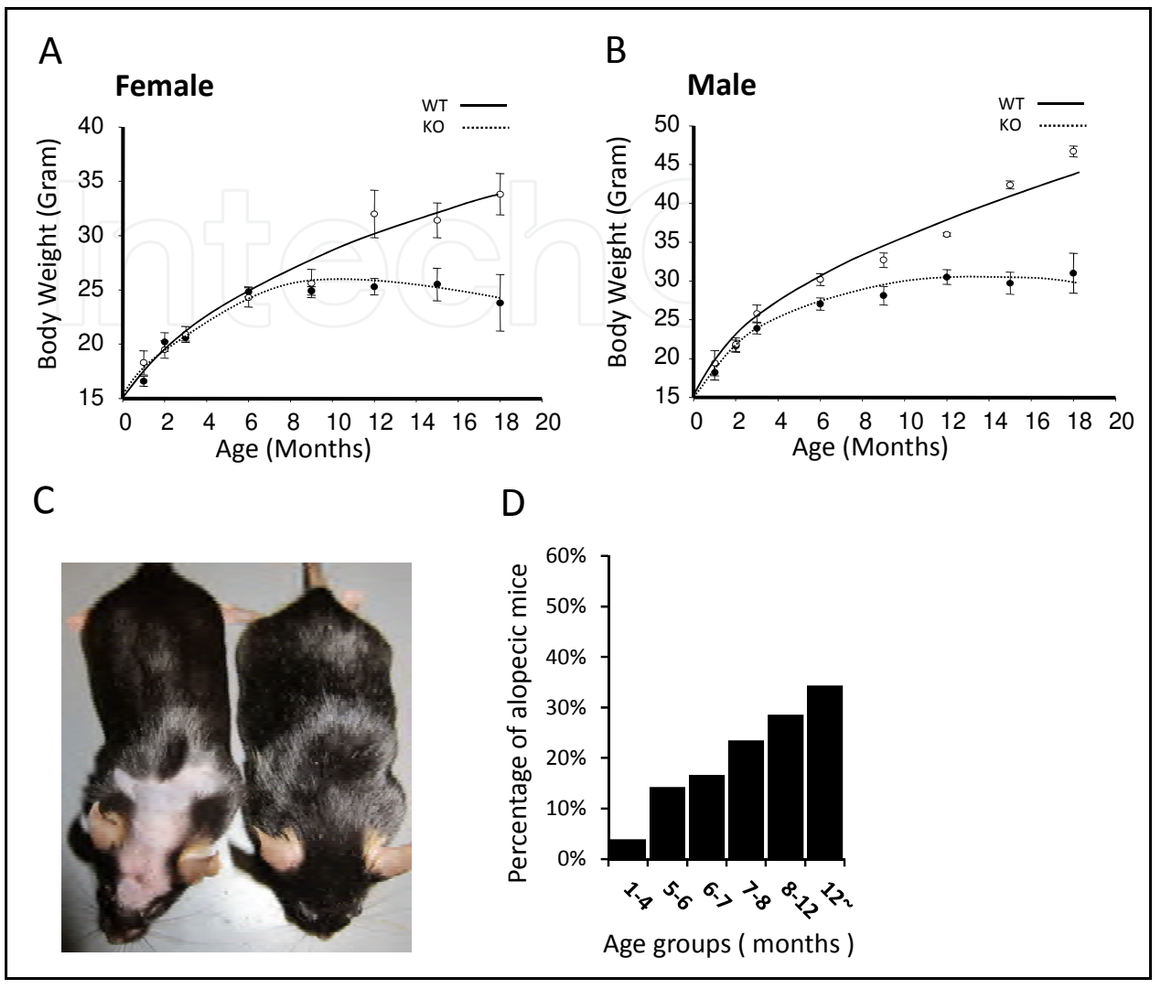

Fig. 3. Accelerated aging in *Nrf2*$^{-/-}$ mice. (A) and (B) Growth curves of male and female *Nrf2*$^{-/-}$ mice and their age-matched littermates. Knockout animals showed a lower body weight after the first year. (C) and (D) Hair loss in *Nrf2*$^{-/-}$ mice. A representative picture of a 12-month-old alopecic *Nrf2*$^{-/-}$ mice is shown in (C). Hair loss was often first observed between 5 to 6 months of age (D).

We recently reported that *Nrf2*$^{-/-}$ mice developed age-related RPE and choroidal degeneration resembling cardinal features of human AMD (Zhao et al., 2011). The *Nrf2*$^{-/-}$ mice have accelerated aging. Some of the animals exhibited extensive hair loss (alopecia), which began as early as 4 months and peaked at 8 months (Fig. 3). Interestingly, more female *Nrf2*$^{-/-}$ mice suffered from hair loss than male ones; this could possibly be attributed to the higher susceptibility of female mice to autoimmune diseases as reported by Takahashi and colleagues (Yoh et al., 2001). After 12 months, the *Nrf2*$^{-/-}$ mice started to show lower body weight than the age-matched wild type littermates (Fig. 3). The life expectancy of *Nrf2*$^{-/-}$ mice is about 20 months which is only 60% of wild type mice with the same genetic background (Pearson et al., 2008).

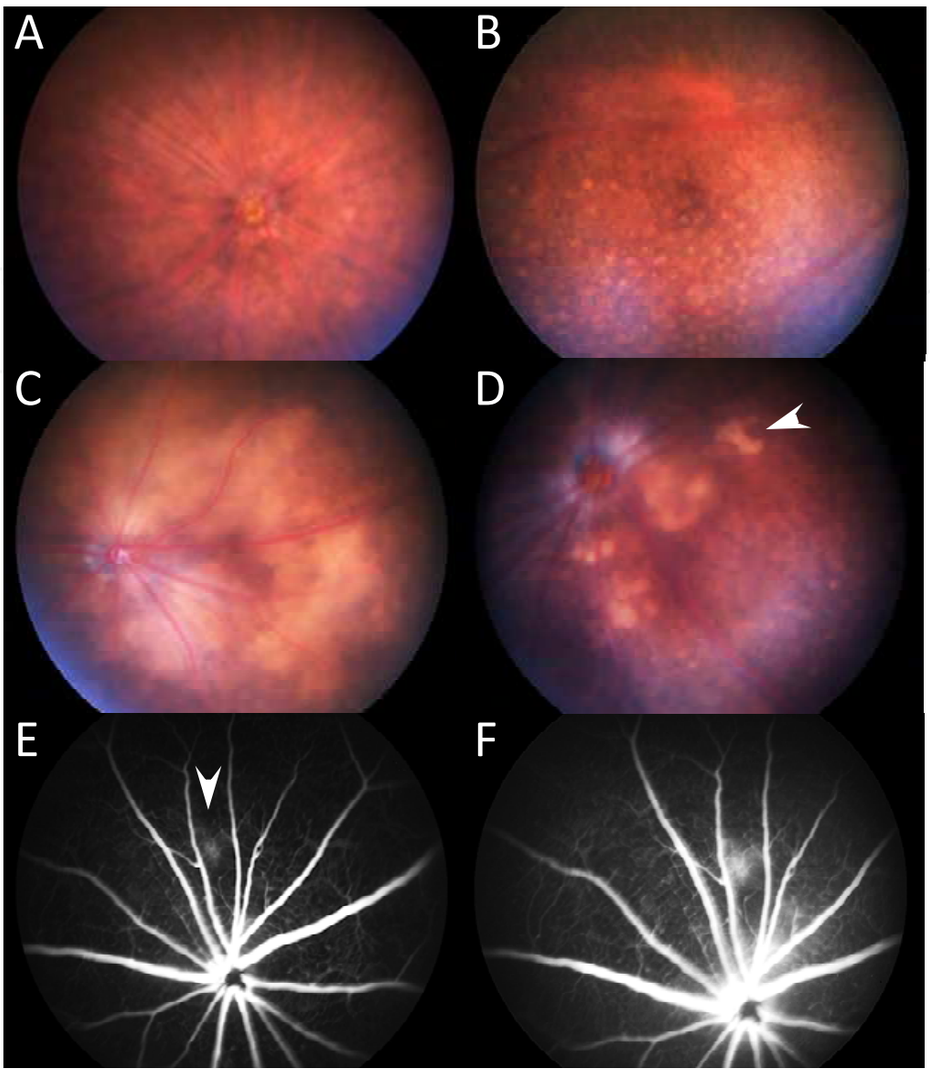

Fig. 4. Fundus examinations of *Nrf2*$^{-/-}$ mice. (A) Normal fundus image taken from a 12-month-old wild type mouse. (B) Drusen like deposits developed in the peripheral retina of an 8-month-old *Nrf2*$^{-/-}$ mouse. (C) Yellowish patchy lesions found in a 14-month-old *Nrf2*$^{-/-}$ mouse. (D-F) A 16-month-old knockout mouse developed extensive RPE lesions (D), one of which showed hyperfluorescence in both early (E) and late (F) phase of fluorescein angiography. Arrowheads in D and E indicate the same lesion.

Drusen-like deposits were noted in around 70% of eyes from *Nrf2*$^{-/-}$ mice, as examined by funduscopy between 8 to 11 months (Fig. 4B). With aging, these small, dome-shaped whitish spots in the fundus tended to become confluent yellowish lesions, gradually increasing in area (Fig. 4C). Atrophic RPE lesions were frequently seen in *Nrf2*$^{-/-}$ mice after the first year (Fig 4C and 4D). Some of these lesions would eventually develop into sites of CNV, which were identified by both fundus fluorescein angiography (Fig 4E and 4F) and histopathology (Zhao et al., 2011). Moderate but statistically significant decreases in both a- and b-wave amplitudes on ERG were observed between the *Nrf2*$^{-/-}$ and wild-type mice at 12 months of age (Zhao et al., 2011), indicating compromised visual function in knockout mice.

The fundus phenotype in aged *Nrf2*$^{-/-}$ mice was further confirmed by histology (Fig. 5 and Zhao et al., 2011), which showed drusen formation, extensive RPE atrophy with numerous vacuoles, increased autofluorescence inside the RPE layer and CNV. Thickening of the Bruch's membrane with age and basal laminar and basal linear deposit were found exclusively in *Nrf2*$^{-/-}$ mice by electron microscopy (Zhao et al., 2011). Immunostaining of eye sections revealed increased deposition of proteins that are related to innate immunity (i.e., C3d, vitronectin and serum amyloid P) and marker of oxidative injury (nitrotyrosine) between the RPE and Bruch's membrane in *Nrf2*$^{-/-}$ mice (Zhao et al., 2011). The same proteins have been found in drusen and Bruch's membrane of human AMD eyes (Crabb et al., 2002; Mullins et al., 2000).

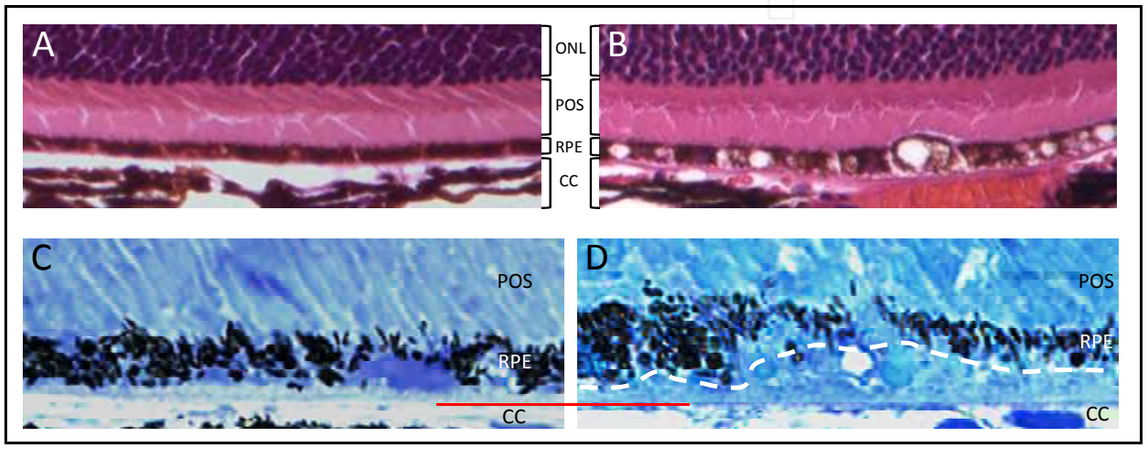

Fig. 5. Histology examination of retina of *Nrf2*$^{-/-}$ mice. (A) A 14-month-old wild type mouse showed normal structure of the outer retina. (B) Representative image of RPE degeneration with big vacuoles, taken from a 14-month-old *Nrf2*$^{-/-}$ mouse. (C-D) Semi-thin sections from a 12-month-old wild-type mouse (C) and an age-matched *Nrf2*$^{-/-}$ mouse (D). Bruch's membranes of the two were aligned at the same level (red line). Note that the RPE layer was elevated due to heterogeneous deposits (under the dotted line) in the sub-RPE space. (A and B: Paraffin section with hematoxylin and eosin staining. C and D: Plastic section with toluidine blue staining. ONL: outer nuclear layer; POS: photoreceptor outer segment; CC: choriocapillaris)

The accelerated degeneration after middle age and the typical pathology of the RPE/choroid indicate that the *Nrf2*$^{-/-}$ model shares many features of human AMD. At advanced age, the retinal pathology progressed from atrophic form to neovascularization and about 15% of the *Nrf2*$^{-/-}$mice developed spontaneous CNV (Zhao et al., 2011). Photoreceptor degeneration was moderate and was probably secondary to RPE dysfunction. Rodents do not have macula and, therefore, cannot be used to generate ideal models of AMD. On the other hand, mechanistic studies exploring the molecular and biochemical mechanisms of age-related RPE degeneration and CNV can greatly benefit from animal models that at least partially reproduce representative lesions commonly seen in human AMD eyes. Animal models, such as the *Nrf2*$^{-/-}$ mice, will display the dynamic process of the disease and offer windows of intervention that can either slow down or accelerate the progression. Similar experiments will be difficult if not impossible to perform with human eyes mainly at late stages of AMD.

5. Pharmacological interventions that activate NRF2 *in vivo*

A number of *in vivo* studies have investigated the protective roles of NRF2 inducers in models of retinal injury and inflammation. A study by Yodoi and colleagues (Tanito et al., 2005) showed that sulforaphane, a prototypic NRF2 inducer, could upregulate thioredoxin in both the RPE and neural retina, and was effective in protecting photoreceptors from photo-oxidative damage. Compared to vehicle-treated controls, mice received sulforaphane showed fewer apoptotic cells in the outer nuclear layer and RPE, and had moderate but statistically significant improvement of both a- and b-wave amplitudes. At four days after light exposure, the ONL was significantly thicker in sulforaphane-treated mice (Tanito et al., 2005). Sulforaphane also delayed photoreceptor cell death in *tubby* mouse, a model of Usher syndrome (Kong et al., 2007). Homozygous *tubby* mice develop progressive photoreceptor degeneration shortly after birth. Sulforaphane-treated *tub/tub* mice showed significantly increased ONL thickness and b-wave amplitude at P28 and P34, as compared to vehicle-treated animals (Kong et al., 2007).

For human clinical studies, AREDS reported (2001) that supplementation with zinc alone, or antioxidants plus zinc, decreased the risk of progression towards advanced AMD by 20%. We showed that zinc could activate NRF2 both in cultured RPE cells and in RPE of NRF2 reporter mice (Chen et al., data not shown). In an ancillary study of AREDS, we analyzed the effects of long-term zinc supplementation on plasma thiol metabolites and their redox status (Moriarty-Craige et al., 2007). There was a significant decrease in plasma cystine concentration in the zinc-supplemented group. The systemic effects may be due to increased tissue uptake of cystine, as NRF2 regulates the transporter protein xCT (Sasaki et al., 2002). These results prove the concept that long term dietary supplementation of an NRF2 inducer is a feasible approach for treating early stage AMD patients.

A new class of synthetic triterpenoids derivatives of oleanolic acid have been tested both in cultured RPE cells and *in vivo*. These agents exerted highly potent activity at concentration as low as 10 nM. They reacted with a broad range of accessible protein thiols and activate NRF2 about 10 times more potently (by the ARE reporter assay) than previously used compounds (Pitha-Rowe et al., 2009). The *in vivo* protection against light-induced retinal toxicity has been demonstrated. Mice receiving 200 mg/kg CDDO-trifluoroethylamide (-TFEA) showed significantly increased ONL thickness after light-induced retinal degeneration (Pitha-Rowe et al., 2009). CDDO-imidazolide decreased mouse leukocyte adherence to retinal vasculature after lipopolysaccaride treatment, and reduced expression of inflammatory mediators including ICAM-1, IL-6, COX-2, TNF-α and MCP-1 (Nagai et al., 2009; Cano et al., 2010). CDDO-methyl ester inhibited neutrophil infiltration in vitreous and internal limiting membrane after retinal ischemia-reperfusion induced by high intraocular pressure, and inhibited degeneration of retinal capillary (Wei et al., 2011). The CDDO compounds are currently under clinical trials for chronic kidney disease and type 2 diabetes. Their potential applications in treating dry AMD can be explored in human studies in the near future.

6. Signaling pathways that regulate NRF2 activation

The interaction between Keap1 and NRF2 is considered as a major determinant of the stability and function of NRF2 (Dinkova-Kostova et al., 2002; Hong et al., 2005). Electrophilic compounds, such as sulforaphane, can directly react with various cysteine residues of Keap1 and consequently cause dissociation and activation of NRF2 (Eggler et al., 2005). Keap1-

deficient hepatocytes had increased NRF2 activity and were more resistant to acetaminophen (Okawa et al., 2006). In addition to thiol modification and redox regulation, it is well established that there are cross-talk between the protein kinase pathways and NRF2-dependent antioxidant system (Sherratt et al., 2004).

Several phosphorylation sites of NRF2 protein have been mapped out and associated to its activity (Fig. 6). Phosphorylation of NRF2 at Serine 40 by protein kinase C promotes its dissociation from Keap1 and translocation into the nucleus (Bloom and Jaiswal, 2003; Huang et al., 2002). Phosphorylation at Tyrosine 568 by a Src subfamily kinase Fyn controls the export and inactivation of NRF2 at the late phase of induction (Jain and Jaiswal, 2006; Salazar et al., 2006). Other Src subfamily kinases, Src, Yes and Fgr, can also function as negative regulators of NRF2 by phosphorylating the protein at Tyr568 (Niture et al., 2011). A recent study by Rada et al (2011) demonstrated that a serine cluster in the Neh6 domain (Ser335, 338, 342, 347, 351, and 355) (Fig. 1) of NRF2 can be phosphorylated by glycogen synthase kinase-3β (GSK-3β). The phosphorylation enhanced the association between Nrf2 and SCF/β-TrCP, which is an adaptor protein for ubiquitin ligase and targets NRF2 for cullin-1/Rbx1-mediated degradation (Rada et al., 2011). Thus, phosphorylation of NRF2 by GSK-3β will facilitate it proteosomal degradation and inhibit its transactivation function. GSK-3β may also act upstream of Src family kinases (Jain and Jaiswal, 2006; Kaspar and Jaiswal, 2011). It remains elusive whether those two mechanisms work independently or additively. Mitogen-activated protein kinases (MAPKs) have been shown to phosphorylate NRF2 at Ser215, 408, 558, 577 and Tyr559; however, impacts on NRF2 location and activity were marginal after phosphorylation at those residues (Sun et al., 2009).

Results from the functional studies consistently showed that inhibition of the PI3K/Akt pathway decreased NRF2 activation induced by a variety of stimuli in different cell lines, while expression of a constitutive active mutant of Akt increased NRF2 activity, indicating that PI3K/Akt signalling is a positive regulator of NRF2 (Chen et al., 2009; Jain and Jaiswal, 2006; Kang et al., 2000; Lee et al., 2001; Wang et al., 2008). PI3K/Akt controls NRF2 via multiple indirect mechanisms. They can facilitate translocation of NRF2 into the nucleus via rearrangement of cytoskeletal actin (Kang et al., 2002). They are upstream kinases of GSK-3β. Akt phosphorylates GSK-3β at Ser9 and inhibits its kinase activity, which in turn will potentiate NRF2 activation because GSK-3β is its negative regulator (Jain and Jaiswal, 2006; Niture et al., 2011; Rada et al., 2011; Salazar et al., 2006).

There are other kinases that can be positive regulators of NRF2. PKR-like endoplasmic reticulum kinase phosphorylates and activates NRF2 under conditions of ER stress (Cullinan and Diehl, 2004; Cullinan et al., 2003). Casein kinase 2 phosphorylates endogenous NRF2 and regulates its activity and degradation (Pi et al., 2007). MAPK family proteins, extracellular signal-regulated protein kinases (ERKs) and the c-Jun N-terminal kinases (JNK), also play positive roles in NRF2-signaling pathway (Shen et al., 2004; Yu et al., 2000a; Zipper and Mulcahy, 2003). However, the positive regulation by ERKs and JNK may not through direct phosphorylation of NRF2 (Shen et al., 2004; Zipper and Mulcahy, 2003). Instead, they may upregulate NRF2 activity by phosphorylating and activating Nrf2 binding partner, such as the nuclear transcriptional coactivator CBP (Shen et al., 2004; Yu et al., 2000a). The p38 kinase may either stimulate or inhibit NRF2 activity, depending on the different type of cells and the pharmacological agents used for the studies (Yu et al., 2000b; Zipper and Mulcahy, 2000).

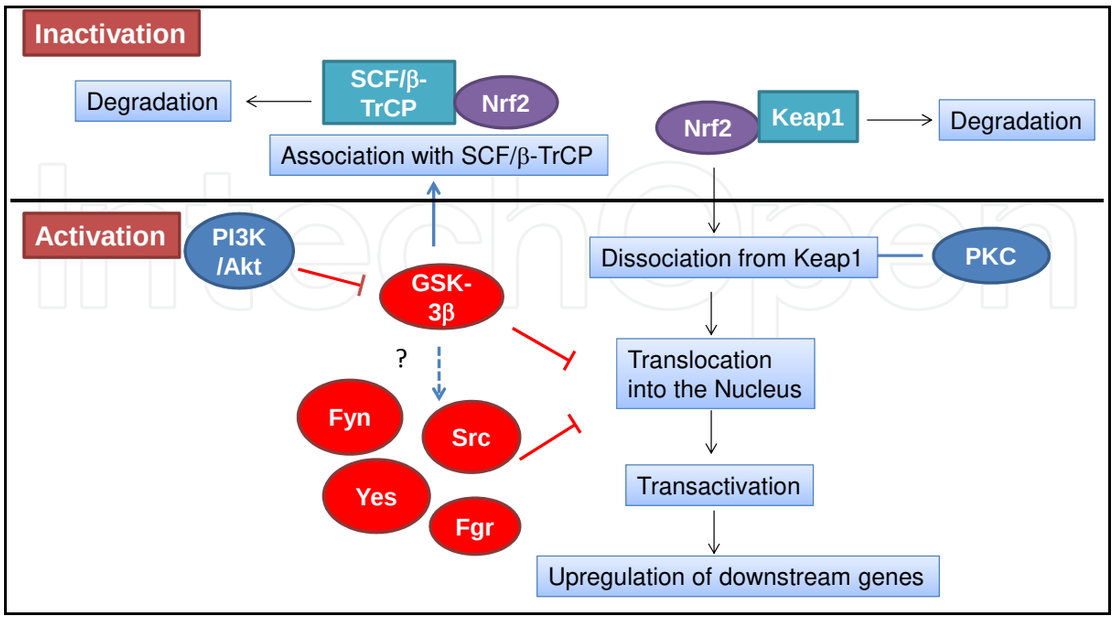

Fig. 6. Regulation of NRF2 activity by protein phosphorylation. There are positive and negative regulators of upstream kinases that function at various steps of the signalling pathway. Phosphorylation can leads to either its activation or degradation/inactivation.

Because of the multiple putative phosphorylation sites of NRF2, and its dual regulation by cellular redox status and protein phosphorylation, it is difficult to clearly define its upstream signalling network both at basal level and in response to oxidative stress. Identification of authentic phosphorylation sites and development of antibodies specific for phosphorylated NRF2 can greatly advance our knowledge in this area. More importantly, most of the works on signal transduction of NRF2 have been performed in transformed cancer cells, which harbour genetic and biochemical variations and function quite differently from the RPE. Future mechanistic studies of NRF2 will be needed to address cell type-specific signalling mechanisms involved in RPE aging and degeneration.

7. Potential mechanisms linking NRF2 to AMD

A unique pathology of AMD is that RPE degeneration occurs before severe loss of photoreceptors, a retinal phenotype also seen in NRF2-deficient mice. In contrast, in model systems of retinal toxicities, animals exposed to excessive levels of oxidative stress often showed much more severe retinal degeneration than the RPE damage. Compared to epithelial cells, neurons are less efficiently protected by the endogenous antioxidant system. The outer segments of rods and cones have very low GSH (Winkler 2008). As shown in both the SOD1- and SOD2-deficient mice, severe loss of neurons occurred before or at the same time of RPE degeneration (Imamura et al. 2006; Justilien et al., 2007). In *Vldlr*$^{-/-}$ mice, antioxidant supplementation protected retinal degeneration and improved the retinal electrophysiology (Dorrell et al., 2009). The fact that *Nrf2*$^{-/-}$ mice showed preferential loss of RPE suggests that NRF2 can have functions other than antioxidant protection.

It is noteworthy that the retinal ultrastructure of aged *Nrf2*$^{-/-}$ mice showed signs of dysregulated autophagy (Zhao et al., 2011). Autophagy is a major self-renewal process which is essential for organelle turnover and removal of aggregated proteins that cannot be processed by proteasome (Klionsky 2007). During autophagy, unwanted proteins and organelles are sorted to double-membrane autophagosomes, which are further delivered to and fused with lysosomes to degrade the sequestered cargos. The accumulation of various intermediate forms of autophagic vacuoles and multivesicular bodies in the RPE and Bruch's membrane was evident on EM images of aged *Nrf2*$^{-/-}$ mice (Zhao et al. 2011). This can be caused by either increased autophagic flux or decreased final degradation by lysosome. Similar findings of dysregulated autophagy were reported in another study using human AMD eyes (Wang et al., 2009).

Autophagy is of particular importance in non-dividing cells like neurons and RPE which, unlike proliferating cells, are incapable of diluting the waste products by mitosis. Dysregulated autophagy is considered as pathogenic in various neurodegenerative diseases; and the underlying mechanisms are disease-specific. In Alzheimer's disease, mutations in presenilin-1 impairs lysosomal targeting of v-ATPase V0a1, which is essential for lysosome acidification and protease activation (Lee et al., 2010). In Parkinson's disease, mutated α-synuclein cannot be efficiently degraded by autophagy (Cuervo et al., 2004). In Huntington's disease, mutant huntingtin may impair the initial cargo assembly of autophagic vesicles (Martinez-Vicente et al., 2010). It has been hypothesized that dysregulated autophagy is also involved in AMD (Wang et al., 2009; Kaarniranta 2010).

NRF2 can be an important regulator of RPE autophagy via multiple mechanisms. In normal RPE cells, autophagy is responsible for the removal of ubiquitinated and/or aggregated proteins. Cargos inside autophagosomes will be delivered to lysosome for degradation and recycled for catabolism. NRF2 is likely involved in autophagosome formation. Several previous studies reported that p62, which is a receptor protein of ubiquitinated proteins and essential for the initial assembly of autophagosome, is transcriptionally regulated by NRF2 (Komatsu et al., 2010). Whether NRF2 controls other specific molecular components of the autophagy pathway remains to be characterized by future studies. Accelerated accumulation of lipofuscin was observed in *Nrf2*$^{-/-}$ RPE (Zhao et al., 2011). Reactive metabolites from bisretinoids inhibit lysosome-mediated autophagic degradation. NRF2-dependent detoxification can be protective in both formation and elimination of lipofuscin-related metabolic waste products. Thus, compromised NRF2 signalling can impact both the early and late stages of RPE autophagy.

NRF2 may also be involved in the innate immune response that amplifies the initial RPE lesions in AMD. As shown in the uveitis model, NRF2-deficient retina had higher number of infiltrated leukocytes and increased production of pro-inflammatory cytokines (Cano et al., 2010). Thioredoxin 1, a downstream protein of NRF2, can interact with complement factor H and regulate its activation (Inomata et al., 2008). Autophagy can be a possible mechanistic link between oxidative stress and inflammation (Levine et al., 2011). Elevated cellular stress will cause increased damage to proteins and organelles and overwhelm the degradation capacity of RPE autophagy. Consequently, the undigested wastes could be exported into Bruch's membrane via exocytosis and deposited in the sub-RPE space (Wang et al., 2009). The exported proteins, possibly in oxidatively modified forms, may further promote drusen formation and cause local inflammation mediated by complement proteins and

macrophages. Loss of endothelial fenestration was observed in choriocapillaris of aged *Nrf2*$^{-/-}$ mice (Zhao et al., 2011). In human AMD eyes, choroidal vascular degeneration occurs in areas of geographic atrophy (McLeod et al., 2009; Mullins et al. 2011). Decreased transport function of choroidal vessels can facilitate the accumulation of damaged proteins in the sub-RPE space and Bruch's membrane.

Single nucleotide polymorphisms (SNPs) in the coding region of *NRF2* gene have been detected in human cancerous tissues (Shibata et al. 2008). Functional polymorphisms in the promoter region of *NRF2* have been reported (Marzec et al., 2007). However, according to the GWAS data (Chen et al., 2010), NRF2 is not a major risk allele of AMD and SNPs of *NRF2* are unlikely to be a major genetic factor. A recent study showed that age-dependent decline of NRF2 function could be caused by upstream regulatory mechanisms, such as GSK-3β, that control its localization and activity (Tomobe et al., 2011). Defining these mechanisms will open up new revenues of intervention to prevent oxidative injury and RPE loss during dry AMD. Unlike the inherited genetic variations, the biochemical changes associated with RPE aging are likely treatable.

8. Conclusion

NRF2 is a protein that has been extensively studied in cancer and other chronic human diseases. Accumulating evidence suggests that NRF2-mediated signalling pathways have central roles in protecting the RPE cells from aging and age-related degeneration. The *Nrf2*$^{-/-}$ mice represent a new model for translational and mechanistic studies of AMD. Agents that activate Nrf2 are potential candidates for treating AMD and other retinal diseases involving oxidative and inflammatory stress.

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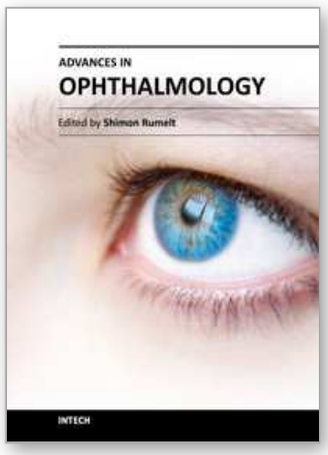

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This book focuses on the different aspects of ophthalmology - the medical science of diagnosis and treatment of eye disorders. Ophthalmology is divided into various clinical subspecialties, such as cornea, cataract, glaucoma, uveitis, retina, neuro-ophthalmology, pediatric ophthalmology, oncology, pathology, and oculoplastics. This book incorporates new developments as well as future perspectives in ophthalmology and is a balanced product between covering a wide range of diseases and expedited publication. It is intended to be the appetizer for other books to follow. Ophthalmologists, researchers, specialists, trainees, and general practitioners with an interest in ophthalmology will find this book interesting and useful.

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