Chapter 5
Conditional Induction of Oxidative Stress in RPE: A Mouse Model of Progressive Retinal Degeneration

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Abstract An appropriate animal model is essential to screening drugs or designing a treatment strategy for geographic atrophy. Since oxidative stress contributes to the pathological changes of the retinal pigment epithelium (RPE), we are reporting a new mouse AMD model of retinal degeneration by inducing mitochondrial oxidative stress in RPE. *Sod2* the gene for manganese superoxide dismutase (MnSOD) was deleted in RPE layer using conditional knockout strategy. Fundus microscopy, SD-OCT and electroretinography were used to monitor retinal structure and function in living animals and microscopy was used to assess pathology *post mortem*. Tissue specific deletion of *Sod2* caused elevated signs of oxidative stress, RPE dysfunction and showed some key features of AMD. Due to induction of oxidative stress, the conditional knockout mice show progressive reduction in ERG responses and thinning of outer nuclear layer (ONL) compared to non-induced littermates.

Keywords Retinal degeneration · Oxidative stress · Geographic atrophy · Retinal pigment epithelium · Superoxide dismutase · Age related macular degeneration · Knockout mice

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5.1 Introduction

Age related macular degeneration (AMD) is one of the major causes of vision loss among the elderly population in industrialized nations (de Jong 2006). Degeneration of the neural retina and of the retinal pigment epithelium (RPE) is associated with the advanced dry form of AMD, while vascular leakage and scarring characterize the neovascular form of the disease. Mitochondrial oxidative stress and RPE dysfunction may contribute the disease phenotype (Khandhadia and Lotery 2010) (Jarrett and Boulton 2012). The RPE is considered as one of the critical sites for oxidative injury to cause retinal degeneration in AMD (Cai et al. 2000; Hageman et al. 2001; Liang and Godley 2003). Geographic atrophy is the term used to describe the degeneration of the RPE and overlying photoreceptors in the advanced form of dry AMD (Holz et al. 2014). Anti-oxidant enzymes including manganese superoxide dismutase (MnSOD, coded for by the mouse *Sod2* gene) and catalase play an important role in regulating oxidative stress by reducing the levels of superoxide and hydrogen peroxide, respectively. Developing a mouse model of oxidative stress leading to geographic atrophy will enhance to understand the mechanisms of retinal degeneration and help to develop therapeutic strategy to prevent AMD. Previously, ribozyme mediated knockdown of MnSOD (*Sod2*) mice model was developed to study retinal degeneration (Justilien et al. 2007), but was subject to variability associated with subretinal injections. Using a cre/*lox* system, we developed a mouse model of RPE specific mitochondrial oxidative stress by deleting *Sod2* in RPE. This deletion results progressive retinal degeneration due to induction of oxidative stress in RPE.

5.2 Materials and Methods

5.2.1 Experimental Animals

All animal handling procedures and protocols were followed the guidelines of ARVO statement and approved by the IACUC of University of Florida. In order to generate transgenic mice, two different mice strains were used. One was inducible RPE-specific *cre* mice carrying RPE-specific *VMD2* promoter to drive tetracycline-inducible transactivator gene (rtTA), which, in turn, controlled the expression of *cre* (Le et al. 2008). These mice were crossed with $Sod2^{flox/flox}$ mice in which exon 3 of *Sod2* gene is flanked by *loxP* sites (Strassburger et al. 2005c). In order to maintain pure lines, both the strains were back-crossed to C57Bl/6J mice up to 10 generations. Mutations in rd1 and rd8 were regularly monitored, to maintain good breeding lines. To obtain mice homozygous for the floxed *Sod2* gene and hemizygous the *cre* transgene ($Sod2^{flox/flox}$-*VMD2-cre*), males heterozygous for *VMD2-cre* and for $Sod2^{flox}$ were bred with $Sod2^{flox/flox}$ females. Rodent chow containing doxycycline (dox) at 200 mg/kg was fed to nursing dams from P1 (postnatal day 1) to P14 to induce *cre* expression.

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5.2.2 Genotyping and PCR Analysis

To determine the genotype of mice, tail samples were processed to obtain genomic DNA using Sigma REDExtract-N-Amp™ Tissue PCR Kit. PCR analysis using genomic DNA was used to differentiate VMD2-*cre* mice from non-transgenic mice. To determine the *Sod2* genotype, the following primers were used: forward 5′-CTTGTGACATCTGGCTGACG-3′ and reverse 5′-CCCAGATCTGCAATTTCCAA-3′. Genetic deletion of exon 3 of SOD2 gene in *Sod2* $^{flox/flox}$*-VMD2-cre* mice (with or without doxycline food) was verified using genomic DNA isolated from RPE/choroid. The primers to verify *Sod2* deletions were designed from the available sequences located in intron 2 and intron 3 of the *Sod2* gene.

5.2.3 RPE Flat Mount and Staining For Oxidative Stress Marker

In order to process RPE for flat mount, the eyes were enucleated and fixed in 4 % paraformaldehyde for 15–30 min on ice. Cornea, lens, retina and extraocular tissue were removed, and only the RPE was collected in PBS by careful dissection. A rabbit polyclonal zona occludens (ZO-1) antibody (Invitrogen, 1:200) was used to analyze morphologic changes in both control and dox-induced mice. Using RPE flat mount, immunohistochemistry for MnSOD (Millipore, 1:300) was performed to detect changes in *Sod2* level in both no-dox control and dox-fed experimental mice. RPE flat mounts were stained with antibody to 8-hydroxydeoxyguanosine (8-OHdG, Abcam, 1:200 dilution), an oxidative stress marker to study induction of oxidative stress in experimental mice.

5.2.4 Monitoring Structural and Functional Changes

In order to measure functional and structural changes in dox-induced experimental mice in comparison with control, electronretinography (ERG), fundus imaging and spectral-domain optical coherence tomography (SD-OCT) were used. Using an LKC visual electrodiagnostic system, ERG was recorded on dark adapted mice following dilation with 2.5 % phenylephrine. Scotopic ERGs were recorded with 10-ms flashes of white light at following intensity of light 0 db (2.68cds/m^2), 10dB (0.18cds/m^2) and −20 dB (0.02cds/m^2). Structural abnormalities in retina of living mice were analyzed by Micron III fundus imaging system. In order to measure subretinal morphology and changes in outer nuclear thickness (ONL), an ultra-high resolution instrument (Bioptigen) was used. Linear B-scans (around 300) were obtained from an anesthetized mouse and 30 images were averaged to get better resolution. To determine the changes in ONL thickness, measurements were done at four different points around the optic nerve maintaining same distance.

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5.3 Results

5.3.1 Generation of Sod2 Knockout Transgenic Mice

In *Sod2*$^{flox/flox}$*VMD2-cre* mice cre was induced by feeding doxycylcine chow to the nursing dam and led to deletion of exon 3 of *Sod2* as evident in PCR analysis of genomic DNA isolated from 5 week old mice (Fig. 5.1a). Dox fed *Sod2*$^{flox/flox}$/*VMD2-cre* mouse produced only a 400 bp product characteristic of the deleted allele, whereas the no-dox control produced 1100 bp band signifying no

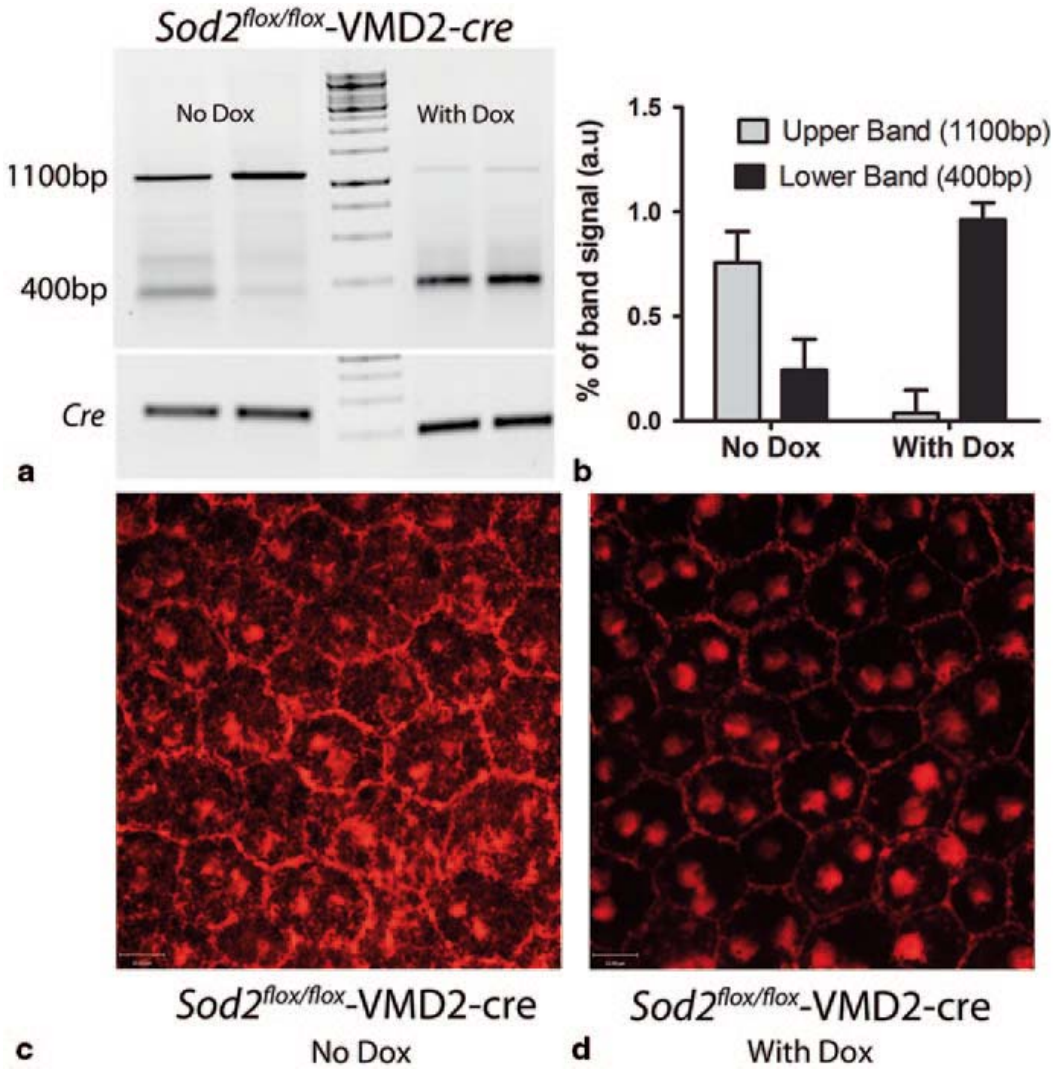

Fig. 5.1 RPE Specific *sod2* deletion in *Sod2*$^{flox/flox}$*VMD2-cre* mice. **a** Image of PCR analysis using genomic DNA from RPE/choroid from 5 week old *Sod2*$^{flox/flox}$*VMD2-cre* mice on doxycline (dox) chow (P0-P14) deleted allele (400 bp) and the full length *Sod2* product is 1100 bp **c** Representative image of *Sod2* immuno-staining of an RPE flat-mount from 2 month old no dox mouse; D: *Sod2* staining of a flat mount from a dox fed mouse

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deletion. Quantification of the signal strength of amplified bands in both groups indicated more than 90 % deletion of exon-3 in dox-induced mice occurred compared to the no-dox group (Fig. 5.1b). Extensive immunostaining of MnSOD on RPE flatmount was seen in *Sod2*$^{flox/flox}$/VMD2-*cre* mouse without dox food (Fig. 5.1c), whereas the immunostaining was significantly reduced in the dox-fed group (Fig. 5.1d).

5.3.2 Functional and Structural Abnormality

Deletion of *Sod2* in RPE caused elevated level of oxidative damage to the DNA. Flat mounts of 6-week old *Sod2*$^{flox/flox}$*/VMD2-cre* (no dox) mouse showed minimal immunostaining for 8-OHdG (Fig. 5.2a), while dox fed mice of that genotype revealed strong immunostaining, signifying the extent of oxidative injury in the RPE (Fig. 5.2b) due to deletion of *Sod2*. Increase in autofluorescence in aging retina is one of the characteristics of AMD (Lois N 2002). The frozen sections of four month old dox fed *Sod2* $^{flox/flox}$*/VMD2-cre* showed the increased level of fluorescence in choroid and RPE compared to control no dox group (data not shown). Fundus imaging of the experimental mice showed retinal atrophy that was apparent after 6 and 9 months (Fig. 5.2c and d). ERG responses (both a- and b-wave) from the dox treated group progressively declined, and differed significantly from the control (no-dox)

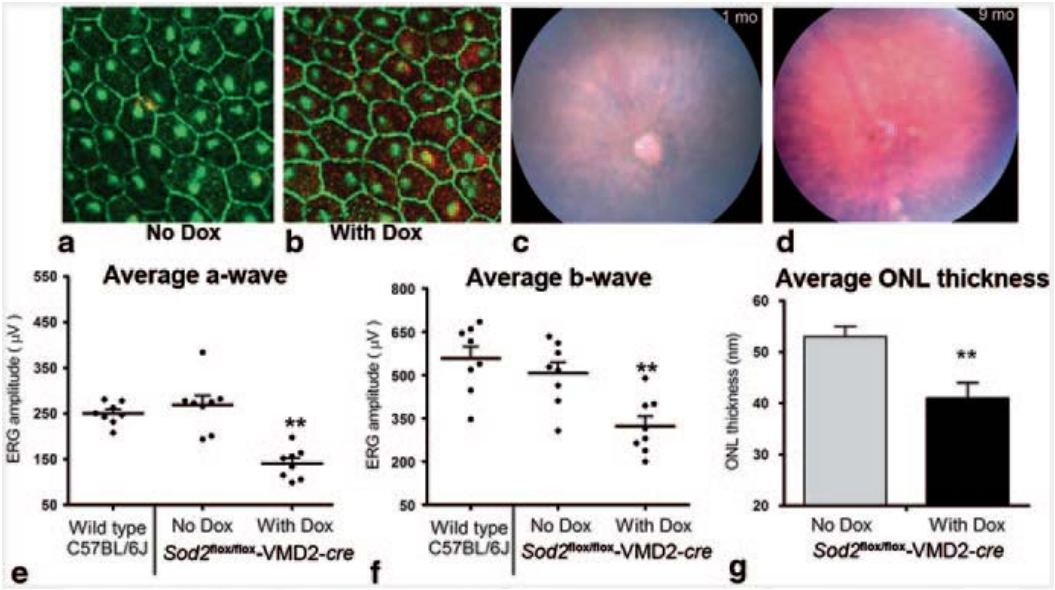

Fig. 5.2 Functional and structural abnormalities due to *sod2* deletion. **a, b** Representative images of RPE flat mount stained for ZO-1 (*green*) and 8-OHdG (*red*). **c, d** Representative fundus images from a dox induced transgenic mice shows extensive degenerated retina in 9 month old mice, **d** compared to a one month old, **c** Average a-wave amplitude, **e** and b-wave, **f** at 2.7 cds/m^2 (ERG responses are significantly reduced in 9 month old dox fed transgenic mice compared to no dox treated transgenic group and wild type C57BL/6J mice). OCT measurement shows the significant reduction, **g** in the thickness of outer nuclear layer in the dox treated group compared to no dox group

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group by the age of 6 months. By 9 months, a major loss of a-wave and b-wave was observed (Fig. 5.2e, f) revealing functional abnormalities in *Sod2* deleted mice. SD-OCT on those mice showed the thinning of ONL that was clearly significant by the age of 9 months (Fig. 5.2g).

5.4 Discussion

Reactive oxygen species generated in mitochondria are thought to contribute to the development of AMD (Jarrett and Boulton 2012), and oxidative stress stimulates inflammatory pathways that may become uncontrolled in this disease (Kauppinen et al. 2012; Suzuki et al. 2012). Oxidized lipids and proteins are deposited in the form of lipofuscin in the RPE and eventually as drusen beneath the RPE (Delori et al. 2000; Handa 2012). Several groups have generated mouse models lacking protective enzymes, such as *Sod1* (Imamura et al. 2006)*)*, or regulators of antioxidant pathways, such as *Nrf2* (Zhao et al. 2011). Developing a mouse model to test the role of mitochondrial oxidative stress in the RPE required cre*/lox* technology. Genetic deletion of exon 3 of *Sod2* led to significant reduction of MnSOD in the RPE. We observed increased oxidative stress in RPE as evident from 8-OHdG staining. Progressive reduction of the ERG a-wave and b-wave in *Sod2* deleted mice reflected retinal degeneration that was documented the thinning of outer nuclear layer as measured by SD-OCT.

In summary, inducible genetic defect only in RPE to promote oxidative stress allows this model to recapitulate RPE and retinal degeneration similar to that occurring in geographic atrophy. This model can be used to study drug-based or gene-based treatment approaches that may attenuate oxidative stress directly or the inflammatory processes arising from reactive oxygen species.

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