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Methionine Sulfoxide Reductase B2 is Highly Expressed in the Retina and Protects Retinal Pigmented Epithelium Cells from Oxidative Damage

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Abstract

Methionine sulfoxide reductase B2 (MSRB2) is a mitochondrial enzyme that converts methionine sulfoxide (R) enantiomer back to methionine. This enzyme is suspected of functioning to protect mitochondrial proteins from oxidative damage. In this study we report that the retina is one of the human tissues with highest levels of MSRB2 mRNA expression. Other tissues with high expression were heart, kidney and skeletal muscle. Over-expression of a MSRB2-GFP fusion protein increased the MSR enzymatic activity three-fold in stably transfected cultured RPE cells. This overexpression augmented the resistance of these cells to the toxicity induced by 7-ketocholesterol, tert-butyl hydroperoxide and all-trans retinoic acid. By contrast, knockdown of MSRB2 by a miRNA in stably transfected cells did not convey increased sensitivity to the oxidative stress. In the monkey retina MSRB2 localized to the ganglion cell layer (GLC), the outer plexiform layer (OPL) and the retinal pigment epithelium (RPE). MSRB2 expression is most pronounced in the OPL of the macula and foveal regions suggesting an association with the cone synaptic mitochondria. Our data suggests that MSRB2 plays an important function in protecting cones from multiple type of oxidative stress and may be critical in preserving central vision.

Keywords

retina; macula; methionine sulfoxide reductase B2; mitochondria; antioxidant

1. Introduction

The methionine sulfoxide reductases (MSRs) are a family of enzymes capable of converting methionine sulfoxides back to methionine (Brot et al., 1983). This process plays a decisive role in recovering protein functionality and in protection against oxidative stress (Weissbach et al., 2002). Methionine sulfoxidation produces two diastereoisomers: Met(S)O and Met(R)O.

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MSRAs are responsible for reducing the S form (Brot et al., 1981) and MSRBs the R form (Grimaud et al., 2001) of the sulfoxides.

In mammals, MSRAs are coded by one gene regulated by two distinct promoters (Lee et al., 2006, Pascual et al., 2009). Their protecting role against oxidative stress has been well established in multiple cell lines (Moskovitz et al., 1998; Yermolaieva et al., 2004; Kantorow et al., 2004 and Picot et al., 2005). MSRAs may also play a role in senescence. Age-related decreases in MSRA expression and activity has been shown in rat tissues (Petropoulos et al., 2001) and the overexpression of MSRA in the fruit fly increases lifespan (Ruan et al., 2002). MSRA knockout mice exhibit abnormal behavior and neurodegeneration (Moskovitz et al., 2001, Pal et al., 2007, Oien et al., 2008. and Salmon et al., 2009). In addition, MSRAs have been implicated in the pathogenesis of aging diseases (Moskovitz, 2005) including Alzheimer's (Gabbita et al., 1999) and Parkinson's (Wassef et al., 2007 Liu et al., 2008). By contrast, the functions of the MSRBs in providing resistance to oxidative stress and in the aging process are not as well understood. The MSRBs are coded by three different genes: MSRB1, MSRB2 and MSRB3 (Kim and Gladyshev, 2004). MSRB2, also known as CBS-1 (Jung et al., 2002), is a mitochondrial protein of 182 amino acids long (Huang et al., 1999). Overexpression of MSRB2 has been reported to protect leukemia cells from H₂O₂ (Cabreiro et al., 2008) and from zinc induced oxidative stress (Cabreiro et al., 2009). Down regulation of MSRB2 with siRNAs has been reported to increase oxidative stress-induced cell death in lens cells (Marchetti et al., 2005).

In the retina more than 60% of the total MSR activity is due to the MSRBs (Lee et al., 2006) but little is known about their expression and function. In this study we determined the localization of MSRB2 in the monkey retina and examined its protective function in cultured RPE cells. Our data suggests that MSRB2 may play a decisive role in protecting the retina, (especially macula and fovea), from oxidative stress.

2. Materials and Methods

2.1 Materials

7-Ketocholesterol (7KCh) was purchased from Steraloids, Inc. (Newport, RI). All trans retinoic acid (ATRA), tert-butyl hydroperoxide (TBHP), Dabsyl Chloride, Methionine, Methionine sulfoxide, Tryptophan, hydroxypropyl- β -cyclodextrin (HPBCD) were purchased from Sigma-Aldrich Co. (St Louis, MO). DMEM and DMEM/F12 media were purchased from Atlanta Biologicals (Atlanta, GA). DNase I, TRIzol®, and SuperScript III reverse transcriptase were purchased from Invitrogen Corp. (Carlsbad, CA). Monkeys (*Macacca mulatta*) eyes were provided by the Pathology Department of the Division of Veterinary Resource after completion of approved protocols. All animal studies were performed in accordance to the guidelines for animal research at NIH and in adherence to the ARVO statement for the use of animals in ophthalmic and vision research.

2.2. Immunohistochemistry in monkey retina

A fresh monkey eye from a 7 year old female Rhesus was collected immediately after euthanasia and immersed overnight in ice-cold, freshly prepared 4% formaldehyde (Polysciences, Inc., Warrington, PA) in 1× PBS. After fixation, vibrotome sections of 100 μ m were prepared as previously described (Lee et al., 2006). Sections were incubated overnight at 4°C with the mouse anti-MSRB2 monoclonal antibody (1:100, Abnova Corp, Taipei, Taiwan). The sections were developed using a goat anti-mouse Alexa Fluor 633 secondary antibody (1:300, Invitrogen Corp, Carlsbad, CA). Nuclei were stained with DAPI and capillary endothelium cells were stained with Isolectin GS-IB4 Alexa Fluor 488 (Invitrogen Corp). The sections were imaged by confocal microscopy (model SP2, Leica mycosystems, Exton, PA).

2.3 Subcellular localization of MSRB2 in D407 cells

D407 cells were co-transfected (2 µg each) with MSRB2-GFP fusion construct and the pDsRed2-Mito plasmid (Clontech, Palo Alto, CA) as a mitochondrial marker. The cells were plated in two-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL). The nuclei were stained with DAPI. The cells were imaged by confocal microscopy.

2.4. RNA isolation and real time RT- PCR

Total RNA was isolated from monkey retinal tissues and D407 human RPE cell line using TRIzol® (Invitrogen). Total RNAs from neural retina and other human tissues were purchased from BD Biosciences (San Jose, CA). RNA (2 µg) was treated with DNase I and cDNA was synthesized with SuperScript III reverse transcriptase. Real-time PCR was performed using SYBR Green in an ABI 7500 instrument (Applied Biosystems, Foster City, CA). Values were calculated according to a standard curve. Primers for MSRB2 and the ribosomal 18S rRNA are represented in Table 1.

2.5. Construction of the pMSRB2 and pMSRB2-GFP expression plasmids

Two MSRB2 expression constructs were generated by cloning the MSRB2 open reading frame with and without the natural stop codon into the pcDNA 3.1 CT-GFP-TOPO vector (Invitrogen Corp). Thus, one construct (pMSRB2) generates the normal MSRB2 and the other (pMSRB2-GFP) generates the MSRB2 fused to green fluorescent protein (GFP). The MSRB2 open reading frame was amplified from human retina cDNA using specific primers (see Table 1 for sequences) and Platinum Taq polymerase (Invitrogen Corp).

2.6. DNA sequencing

All plasmid constructs were verified by direct DNA sequencing. Sequencing of the different plasmid constructs was performed using the BigDye terminator v3.1 cycle sequencing kit and an ABI 3130 Genetic Analyzer instrument (Applied Biosystems, Inc.) following the manufacturer's protocol.

2.7. Cell cultures

D407 cells were a kind gift from Richard Hunt (Department of Pharmacology and Microbiology, University of South Carolina, Columbia, SC). Cells were grown in DMEM medium supplemented with 4% fetal bovine serum (FBS). Penicillin 10 U/ml, streptomycin 100 µg/ul and 2mM of L-glutamine were added to both mediums.

2.8. Cell transfection

Transfections were performed on 2×10^6 cells by electroporation using the Cell Line nucleofactor V Kit and the Nucleofector™ II instrument (Amaxa Biosystems Inc., Gaithersburg, MD) according to the manufacturer's protocol.

2.9. Construction of stable D407 cell lines overexpressing MSRB2

Stably transfected D407 cells overexpressing MSRB2 were generated using the lepidopteran transposon piggyBac vector (Ding et al., 2005). The initial plasmid PB [Act-RFP] (Ding et al., 2005) was modified by removing the actin promoter (Act) and the red fluorescent protein (RFP) and inserting the CMV promoter with the MSRB2-GFP coding region followed by the Blasticidin resistance gene (controlled by the EM7 promoter) for selection. The transposase (PB) recognitions sites were preserved in the flanking regions. To generate the stable cell lines the PB-MSRB2-GFP was co-transfected with the transposase expression plasmid Act-PBase (Ding et al., 2005). The transposase cleavages the DNA between the PB sites and incorporates

it multiple times into the genome. The selection of stable cells was carried out during one month in a restrictive medium with 4 µg/ml of Blasticidin (Invitrogen Corp).

2.10. Construction of stable D407 cell lines expressing MSRB2 micro RNA (miRNA)

A Pol II promoter-driven miRNA expression vector system (Invitrogen) was used to make pcDNA-MSRB2-miRNA. The miRNA oligos (Table 1.) were annealed and cloned into the pcDNA 6.2 GW vector (Invitrogen) according to the manufacturer's instructions. The CMV-MSRB2-miRNA fragment was subcloned into the lepidopteran transposon piggyBac vector (Ding et al., 2005). D407 cells were transfected with PB-MSRB2-microRNA expression vectors containing either the MSRB2 miRNA insert or a control LacZ miRNA insert. Stably transfected D407 cells overexpressing MSRB2 miRNA were selected as described above.

2.11. Treatment with TBHP, ATRA and 7KCh

Stable D407 cells overexpressing MSRB2 were seeded in 24 well plates (7×10^4 /per well) and treated with different doses of TBHP, ATRA, and 7KCh in serum free media. ATRA was dissolved in DMSO and TBHP in water. A 1 mM working solution of 7KCh was prepared containing 4.5% HPBCD in 1× PBS.

2.12. Cell viability assay

Cell viability was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). This assay determines cell viability by measuring cellular dehydrogenase activity. The plates were read in a Wallac 1420 Victor2 instrument (Perkin Elmer Inc, Waltham, MA) according to the manufacturer's instructions.

2.13. Immunoblot Analysis

Monkey neural retina and RPE/choroid regions were separated in sterile 1× PBS. Proteins were extracted by homogenization in PBS 1× containing the Complete® protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at $600 \times g$ for 5 min. In D407 stably transfected cell lines, the mitochondria were purified using the Mitochondria Isolation Kit for Cultured Cells (Pierce Biotechnology). Cell pellets containing 20×10^6 cells were used according to the manufacturer's instructions. Proteins (20-40 µg) were separated by SDS-PAGE using 10% SDS-polyacrylamide gels (Nupage, Invitrogen Corp) at 200 volts, 50 minutes. The proteins were transferred to a nitrocellulose membrane using iBlot dry blotting system (Invitrogen Corp). Blots were blocked with 5% non fat milk (Bio Rad Laboratories, Inc) and incubated overnight at 4°C with the primary antibodies. The primary antibodies were as follows: mouse anti-MSRB2 (1:500, Abnova Corp) monoclonal antibody, rabbit anti-GFP (1:5000, Invitrogen Corp) polyclonal antibody, mouse anti-β-actin monoclonal antibody (1:5000, Sigma-Aldrich Co), mouse anti-MSRB1 monoclonal antibody (1:200, Abcam Inc, Cambridge, MA), rabbit anti-Prohibitin mitochondrial marker (1:100, Abcam Inc), and mouse anti- Caspase 3 antibody (1:250 Cell SignalingTechnology, Inc Danvers, MA) The blots were incubated with a goat anti-mouse-HRP (1:10000, Jackson ImmunoResearch Labs Inc, West Grove, PA) or with a goat anti-rabbit-HRP (1:2000 Cell SignalingTechnology, Inc) secondary antibodies, respectively. The blots were developed using Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford).

2.14. Preparation of dabsyl methionine sulfoxide substrate

The L-MetO (R/S)-DABS racemic mixture was prepared as previously described (Chang et al., 1983). In brief, L-methionine sulfoxide (10 mg) was dissolved in 2 ml of 200 mM sodium bicarbonate buffer pH 9.0 and added 2 ml of 50 mM Dabsyl chloride dissolved in acetone. The mixture was heated to 50°C in a refluxing apparatus with a constant rotation and nitrogen flow to prevent sulfoxone formation. After drying the L-MetO-DABS was dissolved in water and

bound to a SepPak cartridge (Waters Corp. Milford, MA). The bound L-MetO-DABS was washed with water and eluted with 100% methanol. The L-MetO-DABS was separated from contaminating DABS-OH by preparative HPLC using a C-18 column. Similar reactions were performed to synthesize L-Met-DABS and Trp-DABS to use as standards. L-MetO(R/S)-DABS were purified by preparative HPLC to greater than 99% purity.

2.15. Measurement of MSR activity by HPLC

Cellular extracts were prepared by homogenizing the cellular pellets in M-PER protein extraction buffer (Pierce Biotechnology) containing Complete protease inhibitor cocktail (Roche diagnostics). Nuclear debris was removed by centrifugation at $3000 \times g$. Mitochondrial and cytosolic fractions were isolated as described above. The reaction mixture was composed of 19 μ l protein extract, 1 μ l 0.5 M DTT, 4 μ l of 4 mM L-MetO-DABS and 1 μ l 1 mM Trp-DABS (used as an internal standard) to a final volume of 25 μ l. The reaction was incubated at 37°C for 30 min and then stopped by the addition of 25 μ l of methanol. After centrifugation, an aliquot of the reaction mixture was directly injected into the HPLC for quantification of Met-DABS. Protein was measured using the Coomassie Plus assay kit (Pierce Biotechnology).

2.16. HPLC chromatography

HPLC was performed in a Waters 2790 instrument (Waters Corp, Milford, MA) equipped with a 996 photodiode array detector. Analytical chromatography was performed using a 4.6×250 mm Varian XRs C-18 column running at 1 ml/min. The initial mobile phase consisted of 50 mM ammonium acetate pH 4.2 (80%) and acetonitrile (20%). The samples were eluted in a linear gradient until the acetonitrile concentration reached 40% in 20 min. The column was then eluted with 100% acetonitrile and re-equilibrated. The extinction coefficients for Met-DABS and Trp-DABS were determined at 463 nm (λ_{max} for Met-DABS under these conditions) using standards of known concentrations. Quantification was performed by peak area integration of the Met-DABS peak at 463 nm. Values were normalized to the internal standard value (Trp-DABS) then calculated as pmol Met-DABS/ μ g of protein then normalized again to control (value of 1.0).

2.17. Statistical analysis

All values are expressed as mean $\pm$ SEM. Statistical significance was analyzed by two-way analysis of variance (Two way ANOVA) followed by t-test subjected to the Bonferroni correction, $P < 0.05$ were considered significant. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. Statistical analysis was performed using Graph-Pad Prism software (San Diego, CA, USA).

3. Results

3.1. MSRB2 expression in human tissues, monkey retina and D407 cells

MSRB2 mRNA was measured by qRT-PCR in total RNA from various human tissues (Fig 1A). The values were normalized to the 18S ribosomal RNA and are shown from highest to lowest. Although neural retina RNA used in this experiment is a mixture of more than ten different cell types, it expresses significant levels of MSRB2. The retina is one of the tissues with highest expression of MSRB2 mRNA similarly to brain (cortex). Other human tissues with high levels of MSRB2 mRNA were kidney, skeletal muscle and heart. The levels in heart muscle are 10 fold greater than those of most other tissues. This may be partially attributed to the high mitochondrial content in this tissue. These results are consistent with those previously reported which have shown high expression in heart muscle (Jung et al., 2002 and Hansel et al., 2003). In retina tissues, the level of protein expression was higher in MPEC than in MNR (Fig. 1C). The D407 cells also contain of MSRB2 protein as detected by immunoblot analysis (Fig. 1C). The molecular weight for MSRB2 in monkey seems to be slightly lower than the

human in the D407 cell line (Fig 1C). The difference in the apparent molecular weight may be explained by slightly different sequence and/or processing of the monkey MSRB2.

3.2. Effect of GFP on the MSRB2 activity and expression

To determine if GFP may interfere with MSRB2 enzymatic activity, D407 cells were transiently transfected with equal amounts of pMSRB2 and pMSRB2-GFP expression plasmids and the total MSR activity was measured (Fig. 2A). The MSR activity was measured on MetO(R/S)-DABS substrate and the Met-DABS formation quantified by HPLC (see section 2.14-2.16). The results indicate that there is essentially no difference between the increase in MSR activity generated by the pMSRB2 and pMSRB2-GFP in the transiently transfected cells extracts. The experiment was repeated four times to ensure reproducibility and accuracy. The MSRB2 protein expression (representative experiment in Fig. 2B) was quantified by immunoblots with a relative values for control (701 ± 66.5), MSRB2-GFP (5231 ± 1665) and MSRB2 (6166.5 ± 731.5) showing an equivalent increase in the MSRB2 over-expression by both plasmids. These data suggest that GFP does not interfere with MSRB2 expression or activity.

3.3. Increase of mitochondrial MSRB2-GFP expression and activity in stably transfected D407 cells

Although the transiently transfected D407 cells demonstrated high MSRB2 expression and activity (Fig 2), the stress of the transfection seemed to cause an increase in sensitivity to the oxidative stress agents and a corresponding increase in variability between experiments. To avoid these problems a stably transfected D407 cell line was constructed over-expressing the MSRB2-GFP fusion protein. For this purpose we used the piggyBac vector (Ding et al., 2005) as described above (section 2.9). The advantage of this vector is that all the cells that are cotransfected with the transposon piggyBac construct and the transposase enzyme plasmid are converted into stably transfected cell by inserting the MSRB2-GFP into the genome multiple times (Ding et al., 2005).

To determine if the MSRB2-GFP fusion protein generated in this stably transfected cell line properly expressed in the mitochondria, mitochondrial and cytosolic fractions of these cells were prepared and analyzed for expression (immunoblot, section 2.13) and MSR activity (HPLC, sections 2.15-16). The experiment was done three times. The increase in MSR activity (over controls) was significantly different and almost three times higher in the mitochondrial fraction than the cytosolic fraction of MSRB2 over expressing cells (Fig. 3A). Moreover, the MSRB2-GFP protein was detected only in the mitochondrial fraction (Fig. 3B). The data indicates that MSRB2-GFP is correctly targeted to the mitochondria in this stably transfected cell line and that it is enzymatically active.

To further verify the mitochondrial localization, D407 cells were transiently co-transfected with pMSRB2-GFP (gree) and the mitochondrial marker pDsRed2-Mito plasmid (red) (Fig. 4). The conditions for the co-localization were performed as previously described (Lee et al. 2006, section 2.3). Both proteins mostly co-localized although there seemed to be some small amounts of cytosolic expression (Fig. 4C). This seems to be due to some cleavage of the GFP. Immunoblots of the transfected cells detected small amount of free GFP that was apparently cleaved from the fusion product (data not shown).

3.4. Stably transfected D407 cells overexpressing MSRB2-GFP are resistant to various forms of oxidative stress

In order to determine whether increased MSRB2 expression conveyed resistance to oxidative damage in RPE derived cells, controls and stably transfected D407 cells were treated with different doses of t-butyl-hydroperoxide (TBHP), all-trans retinoic acid (ATRA) and the highly

toxic oxysterol, 7-KCh (Fig. 5). The rationale for using these three different compounds is that their modes of oxidative toxicity are mechanistically different from each other. TBHP is a small freely diffusible general oxidant that generates reactive oxygen species throughout the cell. All-trans retinoic acid generates ROS by causing mitochondrial damage (Rigobello et al., 1999 and Schmidt-Mende et al., 2006) and it is known to be present in the retina in relatively high amounts (McCaffery et al., 1996). 7-KCh is a highly toxic oxysterol also suspected to cause mitochondrial and endoplasmic reticulum damage although the precise mechanism for generating ROS and/or causing oxidative stress is not fully understood (Miguet-Alfonsi et al., 2002 and Han et al., 2007). This oxysterol has been recently shown to be present in significant levels in the retina and RPE (Moreira et al., 2009) and to cause a variety of inflammatory responses (Moreira et al., 2009 and Joffre et al., 2007). Control cells are shown in white bars and MSRB2-GFP overexpressing D407 cells are shown in grey bars (Fig. 5). Cell viability was measured 24 h after treatment for TBHP and 48h after treatment for ATRA and 7KCh (Fig. 5A, B and C, respectively). MSRB2 over-expression conveyed a significant increase in resistance to cytotoxicity in all three oxidative treatments. No particular differences were observed between them. This suggests that the MSRB2-mediated methionine sulfoxide reduction is providing a broad range of protection to oxidative stress.

3.5. Mode of cell death in D407 cells treated with TBHP, ATRA and 7KCh

To determine the mode of cell death (apoptosis or necrosis) caused by the different treatments two independent methods were used, procaspase-3 degradation, measured by immunoblot (Fig. 6A) and nuclei staining with Hoechst 33342 dye (Fig. 6B). Staurosporine was used as a control for apoptosis. Staurosporine and ATRA completely degraded the 32 KDa procaspase-3 band (Fig. 6A). This degradation also occurs in TBHP treatment but less markedly. 7-KCh did not cause any degradation of the procaspase-3 band. MSRB2 overexpression clearly decreases the procaspase-3 degradation induced by ATRA but the protection against TBHP induced apoptosis is not as clear (Fig 6A). The Hoechst 33342 nuclei staining support the procaspase-3 measurements (Fig. 6B). Staurosporine and ATRA caused the formation of the classical pyknotic or condensed nuclei. TBHP caused less piknotic nuclei while 7KCh did not cause any apparent nuclear changes (Fig. 6B). These results indicate that MSRB2 overexpression is able to protect against apoptosis induced by ATRA and necrosis induced by 7KCh and probably TBHP. This dual protection by MSRB2 overexpression has been previously reported by Cabreiro et al., 2008.

3.6. Silencing of MSRB2 does not increase susceptibility to oxidative stress

Since MSRB2 over-expression demonstrated clear protection from oxidative damage we wanted to determine if under-expression or knockdown increased susceptibility. For this purpose a stably transfected cell D407 cell line was generated that over-expressed an artificial MSRB2 miRNA (see section 2.10 above). Cytosolic and mitochondrial preparations were made and the expression of MSRB2, MSRB1, prohibitin (mitochondrial marker) and β -actin (cytosolic marker) were measured by immunoblot (Fig. 7A). The results demonstrate that the cell line with the MSRB2 miRNA does not express MSRB2 but clearly expresses MSRB1. However, when these cells were treated with TBHP, there was no significant increase in susceptibility (Fig. 7B). These results suggest that the MSR system is redundant and other MSRs can compensate for the loss of MSRB2. These data is supported by a previously published study which also showed no difference in oxidative stress susceptibility with a siRNA knockdown (Cabreiro et al., 2008).

3.7. Localization of MSRB2 in monkey retina

The localization of MSRB2 was performed in monkey retina by immunofluorescence and imaged by confocal microscopy (see section 2.2) (Fig. 8). The MSRB2 immunoreactivity was

developed using an Alexa 633 secondary antidody (red). The capillaries endothelial cells were stained with isolectin-IB4 labeled with Alexa 488 (green) and the nuclei were stained with DAPI (blue). In the peripheral retina, MSRB2 localized mainly to the ganglion cell layer (GCL), RPE and the outerplexiform layer (OPL) with weaker staining in other regions (Fig. 8A). In the macular region, MSRB2 immunoreactivity occurs in the same areas as in peripheral retina but more intensely in the OPL and RPE regions (Fig. 8B). In the fovea MSRB2 immunoreactivity is most intense in the OPL synapses (Fig. 8C). Interestingly, no staining was observed in the photoreceptor inner segments which are known be rich in mitochondria. The no primary control is shown in Fig. 8D. Some background staining is observed in the RPE.

4. Discussion

The methionine sulfoxide reductases (MSRs) are a group of enzymes that reverse the oxidation of methionine sulfoxide back to methionine preventing permanent damage to proteins and cellular processes (Weissbach et al., 2002). In the retina, due to its high metabolic rate and photo-oxidative environment, the MSR system may be of critical importance. Although much is known about MSR function (Cabreiro et al., 2006) and their involvement in protecting cells from oxidative damage (Moskovitz et al., 1998) and in senescence (Petropoulos et al., 2001 and Ruan et al., 2002), little is known about their specific roles in the retina. MSRB2 was initially detected in the ciliary body (Escribano et al., 1995) and it was later found to be expressed in several other ocular tissues including the retina (Huang et al., 1999). MSRB2 is a mitochondrial protein (Kim and Gladyshev, 2004) and the highest expression of MSRB2 occurs in muscle tissues, principally the heart (Jung et al., 2002) where mRNA expression is 8 to 10-fold greater than in other tissues (Fig. 1).

One of the key findings in our study is that by increasing the MSRB2 activity (Fig. 2), we increased the ability of cells to survive multiple forms of oxidative damage (Fig. 5). While previous studies have used H₂O₂ (Cabreiro et al., 2008 and Sreekumar et al., 2005), TBHP (Marchetti et al., 2005) or Zinc (Cabreiro et al., 2009), we used two other toxic agents, ATRA and 7KCh which are known to be present in the neural retina and RPE (McCaffery et al., 1996, Moreira et al. 2009). These two agents are likely to better simulate some of the oxidative stress conditions that RPE cells would encounter *in vivo*. All-trans retinoic acid is generated by the photoreceptors and RPE as a by-product of the visual process (McCaffery et al., 1996). Retinoic acids induce reactive oxygen species (Hail et al., 2006) and apoptosis in ARPE19 cells (Samuel et al., 2006). At high concentration ATRA is toxic to the mitochondria (Rigobello et al., 1999 and Schmidt-Mende et al., 2006). 7-KCh is a highly toxic oxysterol present in high levels in atherosclerotic plaques (Lyons et al., 1999). This oxysterol is suspected of causing foam cell formation and vascular endothelial cell toxicity (Hakamata et al., 1998 and Jessup et al., 2002). Recently, 7KCh has been localized in the monkey retina associated with lipoprotein deposits (Moreira et al., 2009). At sublethal doses, 7KCh has potent inflammatory properties causing the induction of cytokines like VEGF and IL-8 (Moreira et al., 2009 and Joffre et al., 2007). Dose response curves using TBHP, ATRA and 7KCh demonstrated a statistically significant cell survival in MSRB2 overexpressing cells (Fig. 5). The results also indicate that MSRB2 overexpression can protect cell from both necrosis and apoptosis modes of cell death (Fig. 6). These results suggest that increasing MSRB2 activity in the RPE may provide resistance to multiple types of oxidative damage and thus help sustain RPE function.

The knockdown of MSRB2 expression by a miRNA failed to increase the susceptibility of D407 cells to oxidative stress (Fig. 7). This is consistent with the previously published work by Cabreiro et al. (2008) but contradicts another study by Marchetti et al. (2005). When we used the identical siRNAs described in the Marchetti et al. (2005), we also failed to observe any increased susceptibility (data not shown). Our results suggest that loss of MSRB2 is not

compensated by the cytosolic MSRB1 since we did not notice an increase in protein expression (Fig. 7). The loss of MSRB2 may be compensated by MSRB3. MSRB3 has two isoforms one located in the mitochondrial and the other in the endoplasmic reticulum (Kim and Gladyshev, 2004). We were unable to measure the MSRB3 protein levels but mRNA levels of both MSRB1 and MSRB3 do not change in the stably transfected cells carrying the MSRB2 miRNA (data not shown). By contrast, the loss of MSRA does not seem to be compensated and previous studies have shown that MSRA loss does convey increased susceptibility to oxidative stress (Kantorow et al., 2004; Lee et al., 2006 and Marchetti et al., 2006).

In the monkey retina we localized MSRB2 to the GCL, OPL and RPE (Figs. 8). The high expression in the macular region and especially in the foveal OPL (Fig. 8C) suggests high expression in cone synaptic pedicles. The more interesting aspect of the MSRB2 localization are its similarity to MSRA (Lee et al., 2006). Both MSRA and MSRB2 seem to be highly expressed in cone synaptic mitochondria with significantly less expression in the rod inner segment mitochondria (Figs. 8). The differences between rod and cone mitochondria have been well documented (Perkins et al., 2004 and Johnson et al., 2007). Cones seem to have greater ATP demands than rods and thus generate more ATP which is reflected in their mitochondrial composition (Johnson et al., 2007). Thus, we speculate that the MSRs, especially MSRB2 and mitochondrial MSRA (Lee et al., 2006) are highly expressed in cones to protect these highly active mitochondria.

Loss of MSR activity has been associated with senescence (Picot et al., 2004; Petropoulos et al., 2001 and Koc et al., 2007) and with age-related diseases such as Alzheimer's disease (Gabbita et al., 1999) and Parkinson's (Wassef et al., 2007 and Liu et al., 2008). In the aging retina a group of diseases collectively known as age-related macular degeneration (AMD) is characterized by loss of RPE function (Bonnell et al., 2003). This loss of RPE function is particularly severe in the macular region leading to significant loss of cone photoreceptors. The pathogenesis of AMD is not well understood but oxidative damage and mitochondrial dysfunction have been linked to AMD (Beatty et al., 2000; Glotin et al., 2008; Hollyfield et al., 2008 and Jarret et al., 2008). The high expression and localization of MSRB2 and mitochondrial MSRA (Lee et al., 2006) in the primate macula suggests that their potent anti-oxidative functions may be of importance to AMD.

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Abbreviations

RPE	retinal pigment epithelium
CH	choroid or choriocapillaris
ROS	rod outer segments
RIS	rod inner segments
ONL	outer nuclear layer
OPL	outer plexiform layer
INL	inner nuclear layer
IPL	inner plexiform layer
GCL	ganglion cell layer
MSRB2	methionine sulfoxide reductase B2
MNR	monkey neural retina
MPEC	monkey RPE-choroid
TBHP	tertiary-butyl hydroperoxide ATRA, all-trans retinoic acid
7KCh	7-ketocholesterol
L-Met O-DABS	dabsyl L-methionine sulfoxide
Met-DABS	dabsyl methionine
Trp-DABS	dabsyl tryptophan

Fig. 1. Expression of MSRB2 in human tissues, monkey retina and RPE cell lines

A. MSRB2 mRNA expression in various human tissues measured by qRT-PCR. B, Brain; C, Cerebellum; H, Heart; K, Kidney; Li, liver; Lu, Lung; Pl, placenta; Pr, prostate; R, Neural retina; SI, Small Intestine; SM, Skeletal Muscle; Sp, Spleen; St, Stomach; T, Testis. A relative value of 1 was given to the retina. Data were normalized to the 18S ribosomal RNA and presented as the mean $\pm$ S.E.M. **B.** Immunoblot demonstrating MSRB2 protein expression in monkey retina and in D407 cells. MNR, monkey neural retina; MPEC, monkey RPE and choriocapillaris.

Fig. 2. Effect of the GFP tag on the activity and expression of MSR2 in D407 cells

D407 cells were transiently transfected with pMSRB2-GFP or pMSRB2 (with stop codon after open reading frame). **A.** MSR2 activity in D407 cells was measured by HPLC using L-MetO (R/S)-DABS as substrate. The Met-DABS values were normalized to the internal standard Trp-DABS and to μg of protein then again to control (value of 1.0). The data is shown as mean $\pm$ S.E.M. **B.** Immunoblot analysis using anti-MSRB2 antibody of D407 cells transiently transfected with the same plasmids described above. β -Actin was used as protein loading control.

Fig. 3. MSRB2-GFP expression and activity in stably transfected D407 cells after mitochondrial fractionation

A. Total MSR activity in cytosolic (Cyto.) and mitochondrial (Mito.) fractions of D407 cells stably transfected with a control vector (C) or with pMSRB2-GFP. Data, normalized by μg of protein and related to the control, are expressed as mean $\pm$ S.E.M. * $p < 0.05$. **B.** Immunoblot analysis using anti-GFP antibody of the same cells described above after mitochondrial fractionation. Anti-Prohibitin and anti- β -actin antibodies were used as mitochondrial and cytosolic controls respectively.

Fig. 4. Cellular sub localization of MSRB2 in cultured RPE cells

D407 cells were co-transfected with the pMSRB2-GFP fusion construct and pDsRed2-Mito plasmid mitochondrial marker (Clontech, PaloAlto, CA). The nuclei were stained with DAPI (blue) and imaged by confocal microscopy. **A** pDsRed2-mitochondrial marker (Red). **B**. MSRB2-GFP (Green). **C**. Merged.

Fig. 5. Cell viability of MSRB2-GFP stably transfected D407 cells after treatment with TBHP, ATRA and 7KCh

Cell viability of control D407 cells (white bars) and MSRB2-GFP stably transfected D407 cells (grey bars) 24 h after treatment for TBHP and 48h for 7KCh and ATRA. **A.** Treatment with 0-100 μ M TBHP. **B.** Treatment with 0-25 μ M ATRA. **C.** Treatment with 0-20 μ M 7KCh. Cell viability was measured using the Cell Counting Kit-8 which determines cell viability by measuring cellular dehydrogenase activity. Data are expressed as mean $\pm$ S.E.M, n=4. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 6. Mode of cell death induced by 7KCh, TBHP and ATRA in D407 cells

A. Immunoblot detecting procaspase-3 cleavage (activation) in D407 cells stably transfected with a control vector or MSRB2-GFP after treatment with 7KCh (20 μM), TBHP (100 μM) and ATRA (25 μM). **B.** Image of the same cells stained with Hoechst 33342 after various treatments. Staurosporine was used as a positive control for apoptosis.

Fig. 7. Effect of TBHP on stably transfected D407 cells expressing MSRB2 miRNA (MSRB2 knockdown)

Two stably transfected cell lines one containing a lacZ miRNA control (C) and the other containing a MSRB2 miRNA were tested for expression of various proteins and susceptibility to TBHP. **A.** Immunoblot analysis for MSRB2, MSRB1, prohibitin (mitochondrial marker) and β -actin (cytosolic marker) in cytosolic (Cyto.) or mitochondrial (Mito.) preparation of the stably transfected cells. **B.** Cell viability of the same cell lines in response to TBHP treatment. Cell viability was measured using the Cell Counting Kit-8.

Fig. 8. Immunofluorescent localization of MSRB2 in monkey retina vibrotome sections

The MSRB2 immunoreactivity was detected using an rabbit anti-mouse AlexaFluor-633 secondary antibody (red). Capillaries were localized using Isolectin-IB4 labeled with AlexaFluor-488 (green). Nuclei were stained with DAPI (blue). **A.** Peripheral retina. **B.** Macula. **C.** Fovea. **D.** No primary antibody control. CH, choriocapillaris; RPE, retinal pigment epithelium; POS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Size bar is 75 μ m.

Table 1

Primers used for the preparations of expression constructs and qRT-PCR.

Primers for Real time		
MSRB2	F	CACAAGAGAAAAGGGAACGGAACC
	R	GTCGCAGCACACGCAATGATAC
18S	F	ATGCTCTTAGCTGAGTGTCCCG
	R	ATTCCTAGCTGCGGTATCCAGG
Primers to clone MSRB2 ORF		
	F	ATGGCGCGGCTCCTCTGGTT
GFP construct	R	CGTGTTTCCTTGTTTGAAC TTCAAAG
With stop codon	R	TCAGTGTTTCCTTGTTTGAAC TTCAA
Primers to clone MSRB2-GFP into PiggyBac vector		
	F	AGAATTCGCGATGTACGGGCCAGATATA
	R	TCGTACGGGTTTAATTCATTATTTGTAGAGC
miRNA primers cloned into Piggybac vector		
	F	TGCTGATACATTCCTGCTTCCTTGTTGTTTGGCC ACTGACTGACAACAAGGACAGGAATGTAT
	R	CCTGATACATTCCTGCTTCCTTGTTGTCAGTCAGTGG CCAAAACAACAAGGAAGCAGGAATGTATC