APPROACHES FOR RAISING THE LEVEL OF FOXO3A IN ANIMAL CELLS

by

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A Thesis Submitted to the Faculty of

The Charles E. Schmidt College of Medicine

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Florida Atlantic University

Boca Raton, Florida

May 2012

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Howard M. Prentice, Department of Biomedical Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ACKNOWLEDGEMENTS

I would first like to acknowledge my advisor, Dr. Howard Prentice for his constant optimism and understanding during my thesis. I would like to thank Dr. Weissbach for his support, guidance, and all the valuable lessons that have helped me grow as both a researcher and a person. His tenacity and love for research are an inspiration. I am very grateful to Dr. Diane Baronas-Lowell, as well, for her encouragement and advice during my thesis. Her exuberance and motivation drove me to finish my thesis. I would also like to thank my committee members, Dr. Milton and Dr. Huang for taking the time out of their busy schedule to guide me and advise me throughout my thesis work.

I want to thank all of the people who I've had the pleasure of working with both in the lab and within the graduate community during my years at FAU. Dr. Sagher, Dr. Brunell, Dr. Kesaraju, Dr. Brot, Dr. Chamely-Wiik, Dr. Louda, Dr. Haky, and Manas, thank you for your unconditional support and kindness.

I want to thank my family and my parents and brother, whose love and support enabled me to succeed. I would also like to thank my boyfriend for always cheering my successes and giving me strength during hard times. I would finally like to thank my friends who stuck with me through the good and the bad. Thank you.

This material is based upon work supported by the National Science Foundation under Grant No. DGE: 0638662. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

ABSTRACT

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Title: Approaches for Raising the Levels of FOXO3a in Animal Cells

Institution: Florida Atlantic University

Thesis Advisor: Dr. Howard Prentice

Degree: Master of Science

Year: 2012

The turtle is a unique model of anoxic survival. The turtle's brain can tolerate total oxygen deprivation for hours to days as well as prevent high levels of mitochondrial-derived free radicals upon re-oxygenation. Because of its ability to prevent elevated free radical generation, the turtle has also become recognized as a model of exceptional longevity. We are employing the turtle model for an investigation into the regulation of a key antioxidant enzyme system - methionine sulfoxide reductases (Msrs), primarily MsrA and MsrB. The Msr system is capable of reversing oxidation of methionines in proteins and Msr subtypes have been implicated in protecting tissues against oxidative stress, as well as, enhancing the longevity of organisms from yeast to mammals. Preliminary data, unpublished results, indicate that MsrA protein and transcripts are elevated by anoxia. A recent study on *Caenorhabditis elegans*

demonstrated that FOXO is involved in activation of the MsrA promoter. Using the turtle MsrA promoter sequence we worked to determine which regions in the promoter are necessary for activation by anoxia. The results of the present study were 1) to prepare a TAT-FOXO3a fusion protein which could penetrate animal cells and 2) to construct a FOXO3a expression vector for transcription studies on MsrA expression.

APPROACHES FOR RAISING THE LEVEL OF FOXO3A IN ANIMAL CELLS

LIST OF FIGURES

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Trachemys scripta

The fresh water turtle *Trachemys scripta* is extremely efficient at enduring brain anoxia (complete depletion of oxygen) and hypoxia. *T. scripta* can survive from weeks to months, depending on the environmental temperature, in anoxic conditions without any deleterious effects (Lutz et al., 2003). These animals are capable of surviving massive anoxic insults without increases of reactive oxygen species, ROS, after reoxygenation and are well known for their longevity with little or no senescence (Milton et al., 2007; Pamenter et al., 2007). Upon reoxygenation in most cells, voltage–gated cationic channels (Na^+, K^+, Ca^{2+}) are susceptible to oxidative modification from the excessive ROS present causing depolarization, ATP is depleted, and excitatory amino acids (EAA), such as glutamate and dopamine, are elevated in the extracellular space becoming neurotoxic (Berger et al., 2002). Furthermore, it has been hypothesized that the functional changes in ion channels due to ROS and the increased susceptibility to EAA may be involved in the process of aging and in age relative neurodegenerative diseases (Lutz et al., 2003). A turtle bypasses these challenges by decreasing its metabolic rate such that energy utilization is matched to anaerobic energy production (Lutz et al., 2003). The turtle makes this possible through mechanisms for conserving ionic gradients and avoiding EAA toxicity by maintaining a balance between glutamate and extracellular

GABA (Milton and Lutz, 1998; Milton et al., 2002). The survival of anoxia and reoxygenation is achieved in the turtle by decreasing brain energy-demanding activities, maintaining adequate ionic gradients and neuronal ATP levels, and decreasing energy consumption (up to an 80% decrease) so that the metabolic demand can be met through anaerobic glycolysis (Lutz et al., 2003). During anoxia, the turtle down-regulates ion function in the brain by arresting ion channels, possibly mediated by a decrease in N-methyl-D-aspartate receptor activity (Pamenter et al., 2008), thus resulting in a decrease in excitability and reducing costs of transmembrane ion pumping (Chih et al., 1989). The turtle avoids the EAA challenge by maintaining a balance between dopamine (Milton and Lutz, 1998) and glutamate release (Milton et al., 2002) while actively maintaining its reuptake mechanisms. GABA is a major inhibitory neurotransmitter and in the turtle during anoxia there is a sustained increase of extracellular GABA and an increase in GABA (A) receptor density (Nilsson and Lutz, 1991; Nilsson and Lutz 1992). The increase in GABA during anoxia further inhibits excitatory neurotransmission (Nilsson and Lutz, 1991). Furthermore, GABA may play an important role in healthy aging due to its protection of neurons in some models of age related neurodegenerative disorders (Brewer, 1998).

The turtle has become recognized as an important model of longevity. Turtles cannot only survive oxygen deprivation, but in light of its ability to produce lower levels of ROS concentrations on reoxygenation, it may also be intrinsically resistant to the aging process. Brain aging has been associated with an increase in neuronal dysfunction. Since ROS are implicated in aging and age related neurodegenerative disorders, it is likely that the processes that protect the turtle brain from ROS during anoxia and reoxygenation are

less robust in mammals, leading to mammalian senescence. Upon reoxygenation, turtles, unlike mammals, exhibit ROS levels not significantly different from that of basal normoxic conditions. These low ROS levels during reoxygenation can be contributed either to reduced ROS production or efficient scavenging or both (Milton and Prentice, 2007). Turtles are able to survive anoxia and reoxygenantion due to the low amounts of ROS produced during reoxygenation and through protective mechanisms against ROS production and ROS damage (Lutz et al., 2003). Turtles maintain high levels of antioxidants (catalase, superoxide dismutase, and alkyl hydroperoxide reductase) and have enhanced defenses against oxidative damage (Willmore and Storey, 1997 a, b). Brain hypoxia tolerance and survival during reoxygenation is achieved through the expression of adaptive molecules. C-fos, c-jun, and HSP-70, which also play a role in protecting the brain against age related and degenerative processes, are known to protect mammalian brains in hypoxia (Lutz and Prentice, 2002). Furthermore, these molecules are increased in the turtle during anoxia (Lutz et al., 2003). There is accumulating evidence that oxidative stress is implicated in the aging process and it has been suggested that part of the aging process might be due to harmful changes in ion channel activity induced by ROS and other free radicals. There is evidence correlating the rate of endogenous oxidative damage and the rate of aging in animals (Barja, 2002). A low rate of free radical production in the proximity of DNA and a higher rate of DNA repair might account for the slow rate of DNA damage accumulation resulting in slower aging rate of long-lived animals (Barja, 2002).

Methionine Sulfoxide Reductases

The methionine sulfoxide reductases (Msrs), MsrA and MsrB, are repair enzymes that function to reduce, in a stereospecific manner, methionine sulfoxide (Met(o)) residues in oxidated proteins back to methionine (Met), restoring the protein's function (Weissbach et al., 2005) . Oxidation of proteins and other biomolecules occurs due to the production of ROS and other superoxide radicals during many normal cellular processes or following oxygen deprivation. ROS is formed as a result of oxygen being incompletely reduced during respiration occurring in the mitochondria (Murphey, 2009; Chen et al., 2003). These ROS have the ability to oxidize many cellular components including proteins, nucleic acids, and lipids. Oxidative damage to proteins and other biomolecules by ROS has been implicated in a number of diseases, as well as, in the process of aging (Stadtman, 2006). The Msrs function to protect cells from oxidative damage and are implicated in the process of aging and neurodegenerative disorders (Weissbach et al., 2005).

Methionine sulfoxide reductase A (MsrA) was first discovered in *Escherichia coli* over 30 years ago during studies on the ribosomal protein L12 (Caldwell et al., 1978) after the observation that the ribosomal protein L12 was readily inactivated in the presence of hydrogen peroxide despite the fact that it lacked several amino acids known to be susceptible to oxidation. The inactivation of ribosomal protein L12 was due to the oxidation of its methionine residues (Brot et al., 1981). These studies led to the first evidence of two enzymes with the ability to reduce Met(o) back to Met (Brot et al., 1981; Ejiri et al., 1979). When Met undergoes oxidation, two Met(o) epimers result due to the asymmetric sulfur center, referred to as Met-R-(o) and Met-S-(o). It is now known

that MsrA reduces free and bound Met-S-(o) while MsrB reduces primarily Met-R-(o) in proteins (Weissbach et al., 2005).

Methionine sulfoxide reductase B (MsrB) was first identified in *Nisseria gonorrhoae*'s PilB protein. PilB has a homologous region to MsrA and a C-terminal region, which had no known function at the time (Weissbach et al. 2005). Confirmation of MsrB came from studies done in *E. coli* where the YeaA gene, which is homologous to the C-terminal region of PilB protein, was cloned and expressed revealing that it specifically reduced Met-R-(o) (Grimaud et al. 2001). While a single MsrA gene exists in humans and mice, three MsrB genes have been identified in mammals; MsrB1, MsrB2, and MsrB3 and each is distinct in its location whether cytoplasmic, mitochondrial, or both ER and mitochondrial (Kim and Gladyshev, 2004; Hansel et al., 2005). MsrA has been the most intensely studied enzyme of the Msr system. Rat studies revealed the MsrA protein to be present in both the cytosol and mitochondria even though there is only one MsrA gene in most organisms (Vougier, 2003).

MsrA and Resistance to Oxidative Stress

Oxidative stress occurs when there is an imbalance between the production of ROS and the ability to detoxify these reactive intermediates, causing the accumulation of oxidative damage to cellular macromolecules. The Msrs have been shown to render protection against oxidative stress by enzymatic repair through the reduction of Met(o) in oxidatively damaged proteins back to Met. Furthermore, Met residues in proteins have been proposed to act as efficient ROS scavengers due to their ability to react with ROS to form Met(o), which destroys the ROS. The Msr system regenerates Met allowing the Met

residues to function as catalytic antioxidants (Levine et al., 1996). The importance of MsrA in the protection against oxidative stress was first observed in studies on *E. coli* MsrA mutants using a hydrogen peroxide disk inhibition assay. These *E. coli* mutants showed greater sensitivity to oxidative stress than the wild type (Moskovitz et al., 1995). Furthermore, restoration of the wild type phenotype was successful by transfection with plasmids containing MsrA from *E. coli* and *Mycobacterium tuberculosis*, but not by transfection of enzymatically inactive MsrA (St. John et al., 2001). In contrast, *Saccaromyces cervisiae* overexpressing MsrA was shown to grow better, had a lower rate of Met(o), and had a better survival rate when exposed to hydrogen peroxide or paraquat oxidative stress, than the wild type or mutant MsrA (Moskovitz et al., 1998). Over-expression of MsrA rendered protection in PC-12 cells from hypoxia/reoxygenation injury. These cells had significantly lower levels of ROS and displayed enhanced cell survival (Yermolaieva et al., 2004).

The importance of protein oxidation in aging is supported by various observations of increased levels of oxidized proteins with animal age. MsrA gene expression, protein level, and activity were observed to decrease with age in rat liver, kidney, and brain suggesting that down regulation of MsrA can contribute to the accumulation of oxidized proteins associated with the aging process (Petropoulos et al., 2001). Oxidative damage proved to be an important determinant to lifespan in studies conducted in *Drosophila melanogaster* since overexpressing the MsrA gene in the nervous system showed an extension of lifespan and greater resistance to paraquat-induced oxidative stress (Ruan et al., 2002). In contrast, MsrA knockout mice exhibited enhanced sensitivity to oxidative

stress after exposure to 100% oxygen, and had higher levels of accumulated oxidized proteins under oxidative stress (Moskovitz et al., 2001).

Moreover, age-related accumulation of oxidation may reflect age-related increases in rates of ROS generation, decreases in antioxidant activities, or losses in the capacity to degrade oxidized proteins (Stadtman, 2006).

Cell Penetrating Peptides

In order to treat the animal cell of choice with FOXO3a, we needed to find a method in which the FOXO3a protein could cross the cell's plasma membrane without any deleterious effects. Translocation through the plasma membrane has been a major limiting step for the cellular delivery of macromolecules. An efficient strategy to overcome this problem is the chemical conjugation or fusion to cell penetrating peptides (CPP) derived from proteins able to cross the plasma membrane (Silhol wt al., 2002). CPPs are short sequences raging from 11-35 amino acids. One of these translocating peptides, transactivating transcription factor (TAT), was derived from the HIV-1 TAT protein (Silhol wt al., 2002). TAT peptides have been extensively used for the intracellular delivery of DNA, liposomes, and macromolecules (Albarran et al., 2005). Although translocation mechanisms are yet to be elucidated, studies have suggested that translocation is energy-independent and non-receptor mediated, in which most cells can be targeted. The introduction of CPPs to mice showed successful delivery of active enzyme to all tissues including the brain (Schwarze and Dowdy, 2000).

FOX Transcription Factors

Fox transcription factors are a winged-helix family of transcription factors that share a highly conserved 100-amino acid DNA binding domain, called the Forkhead box (FOX). There are 19 subclasses in mammals, FOXA – FOXS , and all differ in their location and levels of expression. Members of the FOXO subclass have been identified in *Caenorhabditis elegans* (Daf-16), *Drerio* (FOXO5), *D. melanogaster* (dFOXO), mouse (FOXO1, FOXO3, and FOXO4), and humans (FOXO1, FOXO3a, FOXO4, and FOXO6) (Arden, 2008). FOXO proteins are the most divergent members of the Forkhead family containing a unique insert of 5-amino acids (GDSNS), within the region of the DNA binding domain, that is directly involved in sequence-specific interaction with DNA binding sites (Barthel et al., 2005; Arden 2008). FOXO is involved in a variety of physiological processes including cellular proliferation, tumor suppression, metabolism, cell cycle arrest, apoptosis, and stress protection (Arden, 2008).

C. elegans was the first organism in which a FOXO ortholog, Daf-16, was shown to be important in dauer larva formation and lifespan (Riddle et al., 1981; Keyon et al., 1993). Lifespan of *C. elegans* was found to be regulated by the insulin/insulin-like growth factor (IGF)-1 receptor homolog, Daf-2, which is known to signal through the conserved PI-3 kinase/Akt pathway. This lifespan extension requires Daf-16 and it is thought to be the main target of the Daf-2 pathway in worms (Kenyon et al., 1993; Lin et al., 2001). Studies on the *D. melanogaster* homolog of Daf-16, dFOXO, showed that mutation of FOXO increased sensitivity to oxidative stress and an increase in lifespan when overexpressed (Giannakou et al., 2004; Sedding, 2008). Daf-16 was shown to induce expression of MsrA, which is responsible for 2/3 of the increase in life-span that

was observed (Minniti et al., 2009). Studies on mice revealed other roles of FOXO. All studies in mice had to be done using the FOXO1 $^{+/-}$ mouse because the FOXO1$^{-/-}$ mouse resulted in an embryonic lethal phenotype, with an average lifespan of 10.5 days (Hosaka et al., 2004). As in *C. elegans* and *D. melanogaster*, FOXO1 in mice plays a role in the insulin/IGF-1 receptor pathway (Nakae et al., 2002). However, other experiments showed FOXO to have functionally diverse roles in mammals (Arden, 2008; Hosaka et al., 2004).

Regulation and Functions of the FOXO subfamily

FOXO proteins are ancient, evolutionary conserved targets of insulin-like signaling pathway and are important in regulating metabolism in response to changes in nutrient availability and environment conditions (Barthel et al., 2005). Evidence of FOXO being negatively regulated by a PI-3 kinase-Akt (PKB) signal transduction pathway came from early studies done on *C. elegans*, after it was observed that activation of the insulin-like pathway resulted in the inactivation of Daf-16 (Arden, 2006, 2008; Lin et al., 2001). This mechanism of regulation through the Akt pathway is conserved in mammals. FOXO phosphorylation by Akt occurs after stimulation by growth factors, such as insulin and insulin-like growth factor. FOXO proteins contain three highly conserved predicted PKB phosphorylation sites that are not found in other Forkhead proteins. Phosphorylation of these sites by Akt/PKB results in the interaction with 14-3-3 proteins in the nucleus, which serve as chaperone molecules for FOXO's nuclear export (Barthelet al., 2005; Greer and Brunet, 2005). Phosphorylation and cytoplasmic localization also promote FOXO's ubiquitinylation and degradation (Barthel et al., 2005).

FOXO genes are subjected to multiple and complex regulatory mechanisms depending on the cell type, tissue type, differentiation status, and environment (Arden, 2006). In response to stress, the MAPK family member, JNK, is responsible for FOXO regulation in several organisms. JNK appears to phosphorylate 14-3-3 proteins, releasing FOXO, thus allowing FOXO's nuclei import. Furthermore, regulation of FOXO by JNK is suspected to modulate longevity in vertebrates (Greer and Brunet, 2005). Depending on the environmental cues or the signal from different pathways, phosphorylation of FOXO by the AKT pathway or the JNK pathway appear to work in opposition of each other to regulate FOXO (Arden, 2006).

FOXO's intricate regulation mechanisms are not limited to the AKT or JNK pathways. In mammals, FOXO interacts with the NAD-dependent deacetylase sirtuin-1, SIRT1, in response to oxidative stress by directly deacetylating FOXO. SIRT1 deacetylation of FOXO results in FOXO's ability to induce a subset of its target genes, including stress-resistant genes (Greer and Brunet, 2005). Furthermore, Furukawa-Hibi demonstrated an important role of FOXO in the regulation of the growth arrest and DNA damage, GADD45, gene and G_2-M checkpoint as a response to oxidative stress (Fukurawa-Hibi et al., 2002). In vitro studies showed that activation of FOXO3a led to increased expression levels of GADD45 and was inhibited in $SIRT^{-/-}$ cells, while over-expression of SIRT1 was observed to inhibit stress triggered apoptosis induced by FOXO3a (Vogt et al., 2005). Studies on mice revealed SIRT2 expression to be elevated in response to caloric restriction and oxidative stress. SIRT2 also binds to FOXO3a, deacetylating the transcription factor, resulting in the increased expression of $p27^{kip1}$, manganese superoxide dismutase (MnSOD), and Bim and reduction of cellular ROS,

while promoting apoptosis in cells under severe stress (Wang et al., 2007). SIRT1 and SIRT2 have been demonstrated to be up-regulated in response to dietary restriction, resulting in the deacetylation of FOXO3. These results suggested the possibility of a mechanism by which dietary restriction increased life-span that involved the insulin/IGF-1 receptor pathway and upregulation FOXO signaling (Partridge and Bruning, 2008).

A key discovery that is central to our study came from studies on *C. elegans* performed by Minniti et al., 2009. The worm MsrA promoter regions were found to contain two consensus DAF-16 (FOXO) binding elements. In addition, Daf-16 was also shown to regulate Msra-1 expression in *C. elegans* submitted to paraquat-induced oxidative stress. This Msra-1 elevation in response to oxidative stress was abrogated in worms containing a Daf-16 mutation (Minniti et al., 2009). Therefore, we wanted to investigate whether FOXO is involved in the regulation of MsrA expression.

SPECIFIC AIMS

Our long-term goal is to test whether FOXO3a is responsible for activating MsrA in turtle neurons under hypoxia. In this thesis, our goal was to try to elevate the levels of FOXO3a in cells in order to determine whether FOXO3a regulates Msr expression in animal cells. I investigated this hypothesis by addressing the following three specific aims:

1. To prepare a TAT-FOXO3a fusion protein.

2. To prepare a FOXO3a transfection vector for animal cells.

3. To test whether FOXO3a activates the MsrA promoter in an animal system.

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CHAPTER 2

MATERIALS AND METHODS

Transformation of *E. coli*

Chemically competent DH5α (Invitrogen), T7 express (Invitrogen), and Lemo21 (New England Biolabs) *E. coli* were allowed to thaw in ice. Once thawed, 1-3 µl of the ligation reaction were added to each thawed vial of cells. Cells were incubated in ice for 30 min and then heat shocked for 10 seconds in a 42°C water bath. Cells were incubated in ice for 5 minutes and then added 950 µl of super optimal broth (SOC) media. Cells were incubated at 37°C, 225 rpm for 1 hour. Ampicillin-LB plates or kanamycin/chloramphenicol plates were pre-warmed before plating. Cells were diluted 10-fold and 100-fold before plating 150 µl of transformed cells.

Retinal Pigmented Epithelial Cell Maintenance

The ARPE-19 cells were purchased from ATCC. ARPE-19 cells are a human retinal pigmented epithelial cell line. ARPE-19 cells were maintained in T-75 flasks in a 1:1 mixture of Dulbecco's modified Eagles medium (DMEM) and Ham's F12 medium, containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. Cells were split 1:4 upon reaching a confluency of 90-95%. Before trypsinization, cells were washed twice with phosphate buffer saline (PBS) and trypsinized with 3 ml of 0.05%

tripsin, 0.53mM EDTA. Cells were incubated at 37°C for 5 minutes and then added 6 ml of complete medium, to deactivate trypsin. Cells were then spun at 125 x g (1000 rpm) for 5 minutes. The supernatant was discarded and the cells were resuspended in 4 ml of complete medium for splitting. Cells were kept to a third passage before experimental use.

Thawing and Freezing of ARPE-19

Cells were taken out from liquid nitrogen storage and quickly thawed in a water bath at 37°C without letting the cap of the tube touch the water bath. Cells were aseptically transferred to a 15-ml tube and 5 ml of supplemented DMEM:Ham's F12 media was added. Cells were spun at 125 x g (1000 rpm) for 5 minutes. The supernantant was discarded and cells were resuspended in supplemented DMEM:Ham's F12 medium. Cells were plated in a T75 flask. Twenty-four hours after thawing the medium was changed and after 48 hours the cells were split.

Cells were frozen in a single 1 ml vial from a confluent T75 flask. Cells were washed twice with PBS and then treated with 3 ml of 0.05% trypsin, 0.53mM EDTA. Cells were incubated at 37°C for 5 minutes and then 6 ml of complete medium were to deactivate the trypsin. Cells were then centrifuged at 125 x g (1000 rpm) for 5 minutes. The supernatant was discarded and cells were resuspended in 1 ml of complete media containing 5% DMSO. Cells were incubated at 4°C for 5 minutes, at -20°C for 15 minutes, and at -80°C overnight, and then properly stored in liquid nitrogen.

Transfection of ARPE-19 cells with pcDNA3.1-FOXO3a

ARPE-19 cells were plated in 60mm culture dishes (4×10^5 cells) 48 hours before transfection and maintained in DMEM:Ham's F12 medium, containing 10% FBS, 1%

penicillin/streptomycin, and 1% glutamine. 24 hours before transfection, cells were changed to a DMEM:Ham's F12 medium containing no additives, no serum, and no antibiotics. Cells were transfected at a 90% confluency using Invitrogen's Lipofectamine-2000, employed as per manufacturer's protocol.

ARPE-19 Protein Extraction

48 hours after transfection, cells were washed twice with phosphate saline buffer and then treated with protein extraction buffer (Invitrogen). Culture dishes were scrapped using cell scrapers and then collected. Lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C and supernatants were collected. Protein concentrations were determined by BCA assays.

Western Blotting

Equal amounts of protein were electrophoretically separated by gradient SDS-PAGE (4-12%) (NOVEX by Life Technologies) at 150 V for 1 hour. The separated proteins were transferred to polyvinylidene fluoride transfer (PVDF) membranes (PALL Life Sciences). Membranes were blocked in 5% dried nonfat milk in TBST (25 mmol/L Tris-Cl, pH 7.5, at 24ºC, 150 mmol/L NaCl, 0.1% Tween 20) for 1 hour for nonspecific binding. Primary antibodies were prepared in 5% BSA in TBST and incubated overnight at 4°C. As a control for protein loading variation, actin was employed (1:1000) (Sigma). The employed primary antibodies include: methionine sulfoxide reductase A (prepared previously in this laboratory) (1:1000), superoxide dismutase 2 (Abcam, Cambridge, MA; 1:5000), FOXO3a (Cell Signaling Technology, Beverly, MA; 1:1000). The

secondary antibody (goat anti-rabbit) was obtained from Assay Designs (Ann Harbor, MI). Membranes were washed 3X with TBST before being incubated for 2 hours at room temperature with the HRP-conjugated secondary antibody, prepared in 5% milk. Proteins were detected by ECL chemiluminescence (Life Technologies).

MsrA activity assays

MsrA activity was performed as described previously (Sagher et al., 2006). To summarize protein samples (~280 μg) were incubated for 1 hour at 37°C with 0.1M Tris, pH 7.4, 15 mM diothiothreitol, (DTT), and 100 nM 4-N,N-dimethylaminoazobenzene-4-sulfonyl-Met(O), (DABS-Met(O)). DTT was used as an in vitro reductant in place of thioredoxin (Trx), the biological reductant for MsrA,. DABS-Met(O) was used as substrate and the product DABS-Met was assayed by HPLC analysis.

Cell Viability Assays

CellTiter 96 aqueous cell proliferation assay (Promega) was employed for ARPE-19 viability assays. CellTiter 96 is a colorimetric method for determining cell viability. This assay uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and an electron coupling reagent, phenazine methosulfate (MTS). MTS is bioreduced by viable cells into a formazan product that can be measured at 490 nm. Cell viability assays were done as per manufacturer's protocol.

Treatment of ARPE-19 Cells with Hydrogen Peroxide

ARPE-19 cells were plated in 96-well plates (0.4×10^5 cells/well) 48 hours before transfection. Cells were maintained for 24 hours in DMEM:Ham's F12 medium,

containing 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. Medium in the cells was changed to DMEM:Ham's F12 without supplements 24 hours before transfection with pcDNA3.1-FOXO3a and pcDNA3.1. The cells were treated with 0, 0.25, 0.5, 1, 1.5, 2, 3, and 4mM hydrogen peroxide (H_2O_2) in DMEM:Ham's F12 containing 2% FBS, 24 hours after transfection.

CHAPTER 3

RESULTS

TAT-FOXO3a Plasmid Construction

The human FOXO3a cDNA was obtained from OriGene. The FOXO3a open reading frame and Kozak sequence were PCR amplified to include the 5' Hind III and 3' Xho I restriction enzyme sites. The bacterial vector pET28b, containing a polyhistidine tag, and the FOXO3a amplicon were restriction digested at the Hind III and Xho I enzyme restriction sites. The FOXO3a amplicon was subsequently subcloned into pET28b using NEB quick DNA ligase. The TAT cell penetrating peptide oligo sequence (~ 39 bp) was custom made by IDT DNA to contain the 5' Nde I and 3' HindIII restriction enzyme sites. The TAT coding sequence was subcloned into the pET28b-FOXO3a clone using restriction sites Nde I and Hind III as shown in the plasmid map, figure 1. After verification by restriction digestion, as shown in figure 2, the pET28b-TAT-FOXO3a construct was sent out to The University of Florida Sequencing Services to verify the sequence of the FOXO gene and the TAT peptide.

Induction of TAT-FOXO3a protein in *E. coli*

Initially, BL21 cells were employed for transfection with pET28b-TAT-FOXO3a for high expression of TAT-FOXO3a fusion protein. A stationary culture was set up 16 hours

before protein induction experiments. The stationary culture was diluted to reach an OD 600nm of 0.1. Cultures were allowed to grow at 37°C, 225 rpm until an OD 600nm of 0.4 was reached. For protein induction, the transformed BL21 cells were then treated with either 0.1mM IPTG or 0.5mM IPTG and incubated at 25°C, 225 rpm for 4 hours. Cells were then pelleted and resuspended in lysis buffer +T/G buffer (50mM HEPES, pH 7.4, 10% glycerol, 0.1% Triton X-100, 0.5M KCl, 40ug/ml DNase, 1mM $MgCl_2$, 15mM methionine, 0.5mM Imidazole, and EDTA-free protease inhibitor). Cells were lysed by sonication and centrifuged at 35,000 x g for 30 minutes at 4°C. The supernatant was saved and the pellet was resuspended in +T/G buffer. The pellet was kept due to the possibility of TAT-FOXO3a being present in inclusion bodies. Protein concentration of the supernatants and resuspended pellets were established by Bradford assays and an SDS-PAGE gel followed by Comassie blue staining to detect production of the TAT-FOXO3a fusion protein. The TAT-FOXO3a fusion protein was expected to be 72 kDa. After various trials, it was established that TAT-FOXO3a was toxic to *E. coli* BL21. There was inhibition of BL21 cells growth after IPTG addition, and in some cases death of BL21 cells after IPTG addition.

The NEB *E. coli* cell line Lemo21 (DE3), a derivative of BL21 cells, was employed in order to control the T7 expression after IPTG induction. These cells were employed with the idea to produce less TAT-FOXO3a to alleviate the formation of inclusion bodies and toxicity of the protein. Levels of expression were achieved in Lemo21 cells by varying the level of lysozyme (lysY), which is an inhibitor of T7 RNA polymerase. The levels of lysY were controlled by addition of different concentrations of L-rhamnose (0, 0.1, 0.25, 0.5, 1, and 2mM) to the expression culture. Once the

expression cultures reached an OD 600nm of 0.4 - 0.6, 400 μM IPTG was added to begin TAT-FOXO3a induction. Cultures were incubated for 8 hours at 30°C, 225 rpm. Cells were pelleted and resuspended in lysis buffer containing 8M urea, 200mM HEPES, pH 7.9, 100mM NaCl. Cells were lysed by sonication and centrifuged at 35,000 x g for 30 minutes at 4°C. Pellets were resuspended in lysis buffer and an SDS-PAGE gel followed by Comassie blue staining was performed. The pellets showed no evidence of TAT-FOXO3a production. A suspected reason for *E. coli*'s inability to produce quantifiable concentrations of TAT-FOXO3a is perhaps the lack of codons that are native to *E. coli*, or also the size of the construct.

It is apparent that the two bacterial strains used were not able to remain viable when TAT-FOXO3a plasmid was induced. One important future strategy would be to employ additional bacterial strains. In our first TAT-FOXO3a strategy we employed BL21 bacteria and our bacteria were killed upon IPTG induction. This finding is in contrasts to the results of Dr. Essafi et al. (2011) where BL21 bacteria were successfully employed for small scale TAT-FOXO3a production. However, no large-scale production has been carried out of TAT-FOXO3a from BL21 cells to date. It is likely that this line of bacteria is not optimal for certain such TAT fusion protein strategies.

For preparation of TAT-FOXO3a fusion protein in the future, we will need to find strains of *E. coli* in which high level T7 promoter activity can be used to drive TAT-FOXO3a expression without eliciting bacterial toxicity.

FOXO3a Plasmid Construction

The mammalian vector pcDNA3.1, containing a CMV promoter, was employed to construct the FOXO3a plasmid. FOXO3a cDNA and the pcDNA3.1 vector were digested with restriction enzymes HindIII and XhoI. Digested bands were gel purified and quantified. FOXO3a and pcDNA3.1 were then ligated using NEB quick ligase, as shown in the plasmid map, figure 3. Invitrogen's DH5α cells were employed for transfection with the pcDNA3.1-FOXO3a construct. 24mM β-mercaptoethanol was added to DH5α cells and cells were incubated in ice for 10 minutes before transformation with pcDNA3.1-FOXO3a in order to facilitate the uptake of the 7385 bp construct. Verification by restriction digestion (figure 4) was performed followed by sequencing.

Turtle MsrA Promoter Plasmids Construction

In order to begin our studies for our long-term goal of elucidating whether FOXO3a is responsible for activating MsrA in turtle neurons during hypoxia and reoxygenation, we began by constructing two MsrA promoter plasmids. The construction of these plasmids will allow for future experimentation into the role of FOXO3a during hypoxia in the turtle brain.

The turtle MsrA promoter sequence was obtained from Paul Kirchman at Florida Atlantic University. The sequence was analyzed by Dr. Lina Shehadeh, from The University of Miami, and revealed three putative sites for FOXO3a transcription factor binding, shown in figure 5. A region of the MsrA promoter (-1020 bp to -1 bp) was selected and sent to IDT DNA for making custom plasmids (pIDTDNA-TM1020), containing 5' Mlu I, 5' Bgl II, and 3' Hind III restriction enzyme sites. The promoter-less

pGL3-basic vector driving luciferase reporter gene was selected to incorporate the promoter cassette. The pGL3 vector (Promega) was digested with restriction enzymes Mlu I and Hind III, gel purified, and quantified. Similarly, the pIDTDNA-TM1020 plasmid was digested with restriction enzymes Mlu I and Hind III to release the 1020bp insert turtle MsrA promoter. The insert was then gel purified and quantified for sub-cloning. To obtain the smaller promoter region (561bp), the pIDTDNA-TM1020 plasmid was digested with Bgl II and Hind III restriction enzymes, gel purified, and quantified. The pGL3 vector was also digested with Bgl II and Hind III restriction enzymes, gel purified, and quantified. Both vectors and inserts were then ligated using Fermentas quick ligation kit. Plasmid maps of both turtle MsrA promoters -1020 and -560 are shown in figure 6 depicting their orientation and the luciferase reporter gene. The colonies were selected and plasmids were isolated using a mini plasmid isolation kit (Promega). The new plasmids were verified by restriction enzyme digestion for releasing the appropriate insert, 1020 bp band or 560 bp band as shown in figure 7, and subsequently sent for sequence verification (DNA Sequencing Center, University of Chicago).

For analysis of MsrA promoter activity in response to FOXO3a overexpression, co-transfection of pcDNA-FOXO3a and pMsrA promoter-luciferase should give a quantitative luciferase reading dependent on the activation properties of FOXO3a. All luciferase values are normalized to constitutively active internal control renilla luciferase plasmid.

MsrA and MnSOD Levels in ARPE-19 Cells Transfected with pcDNA3.1-FOXO3a

Protein extracts from ARPE-19 cells transfected with pcDNA3.1-FOXO3a and pcDNA3.1 were lysed and used for western analysis. Equal amounts of protein were loaded on an SDS-PAGE gel for protein separation and transferred to a PVDF membrane. Membranes were probed for FOXO3a (72 kDa), MsrA (26 kDa), MnSOD (24 kDa), and actin (42 kDa), as a control, shown in figure 8. FOXO3a was clearly visible in ARPE-19 cell extracts that were transfected with pcDNA3.1-FOXO3a compared to cells transfected with empty vector. However, levels of MsrA and MnSOD were not elevated in cells transfected with FOXO3a compared to control cells.

MsrA Activity Assay of ARPE-19 Cells Transfected with pcDNA-3.1-FOXO3a

ARPE-19 cells transfected with pcDNA3.1-FOXO3a and pcDNA3.1 were lysed 48 hours after transfection and protein extractions were prepared. MsrA assays were conducted with the protein extracted from transfected ARPE-19 cells (see methods). In this HPLC assay, the product, DABS(met), appears at 1.8 minutes, as seen in figures 9 A and B. There was no significant difference in MsrA activity observed between cells transfected with pcDNA3.1-FOXO3a or pcDNA3.1. The control, using purified bovine MsrA, shows a clear peak of DABS (met) formation.

Effect of FOXO3a Transfection in ARPE-19 Cells Treated with Hydrogen Peroxide

FOXO3a is known to protect cells against oxidative stress, thus we employed ARPE-19 cells for FOXO3a transfection and hydrogen peroxide treatment, as an oxidative stressor. ARPE-19 cells were plated in 96-well plates (0.4×10^5 cells/well 48

hours before transfection as described in methods. The cells were treated with 0, 0.25, 0.5, 1, 1.5, 2, 3, and 4mM hydrogen peroxide (H_2O_2) in DMEM:Ham's F12 containing 2% FBS for 14 hours. The MTS cell viability assays were performed. It was expected that FOXO3a would provide protection for the cells. However, unexpectedly both the control and FOXO3a transfected cells showed significant enhanced killing only in the presence of H_2O_2 (figure 10). Future studies are needed to clarify why this is happening.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Our ultimate goal is to test whether FOXO3a activates the MsrA promoter under oxidative stress in turtle neuronal cultures and to study the effect of activation and inhibition of FOXO3a on MsrA expression during oxidative stress. In order to accomplish this, we selected two ways by which FOXO3a levels in cells could be elevated. Our first approach was to sub-clone the DNA sequence encoding FOXO3a along with the cell penetrating peptide TAT in order to express the TAT-FOXO3a fusion protein in bacteria. This TAT-FOXO3a fusion protein would be introduced into mammalian cells and subsequently we would measure MsrA protein and transcript levels in animal cells subjected to oxidative stress. Unfortunately, induction of TAT-FOXO3a induction in bacteria proved to be toxic to *E. coli*. We believe that this toxicity could be due to high production of inclusion bodies or the lack of codons that are native to *E. coli*. Future studies are needed in order to obtain production of TAT-FOXO3a in *E. coli*. If the induction of TAT-FOXO3a had been feasible, the protein would have been subsequently purified using a nickel column and then used for the treatment of ARPE-19 cells and primary culture turtle neurons. Addition of TAT-FOXO3a to the cell culture would allow FOXO3a to successfully enter the plasma membrane, mediated by the TAT sequence, and deliver FOXO3a to the cell's cytoplasm. In this approach it would be interesting to investigate if the import of the TAT-FOXO3a into the nucleus requires either SIRT1

or SIRT2 activation or activation of the JNK pathway. Following transduction of TAT-FOXO3a protein into cells, it would be possible to examine levels of down-stream targets of FOXO, such as MsrA under a range of physiological and pathological conditions by western blot analysis.

Our second approach involved the construction of the FOXO3a clone into a mammalian vector. The potential advantage of this approach was that the foreign FOXO3a would be synthesized by the mammalian cells and may be more likely to be appropriately regulated by post-translational modifications. We successfully obtained elevated protein levels of FOXO3a in ARPE-19 cells transfected with FOXO3a. However, we did not observe elevated protein levels of MsrA, MnSOD, or enhanced MsrA activity in ARPE-19 cells transfected with FOXO3a. There is evidence of FOXO3a activation by post-translational modification occurring after oxidative stress and resulting in cell protection (Greer and Brunet, 2005). To investigate this possibility in our system, we treated ARPE-19 cells with H_2O_2 after FOXO3a transfection. Cell viability assays did not, however, show any protection rendered by FOXO3a. More studies are needed in order to understand why FOXO3a is not protecting the ARPE-19 cells treated with H_2O_2.

We also constructed two MsrA promoter clones containing a luciferase reporter gene: one containing the truncated sequence and another containing the full sequence of the turtle MsrA promoter. Co-transfection of these MsrA promoter clones along with the FOXO3a clone in turtle cells would confirm if FOXO3a truly activates the MsrA promoter through binding to this regulatory domain in a system where MsrA is regulated by FOXO3a.

There is still much research to be done to find how MsrA is regulated and the role of FOXO3a as pro-survival or pro-apoptotic in the turtle brain upon oxygen deprivation and reoxygenation. It would also be important investigating additional genes that FOXO3a up-regulates or down-regulates in order to protect the turtle neurons during anoxia and reoxygenantion. If MsrA is up-regulated by FOXO3a in the turtle, this would give us further insight into the role of MsrA in relation to anoxia tolerance and longevity.

APPENDIX

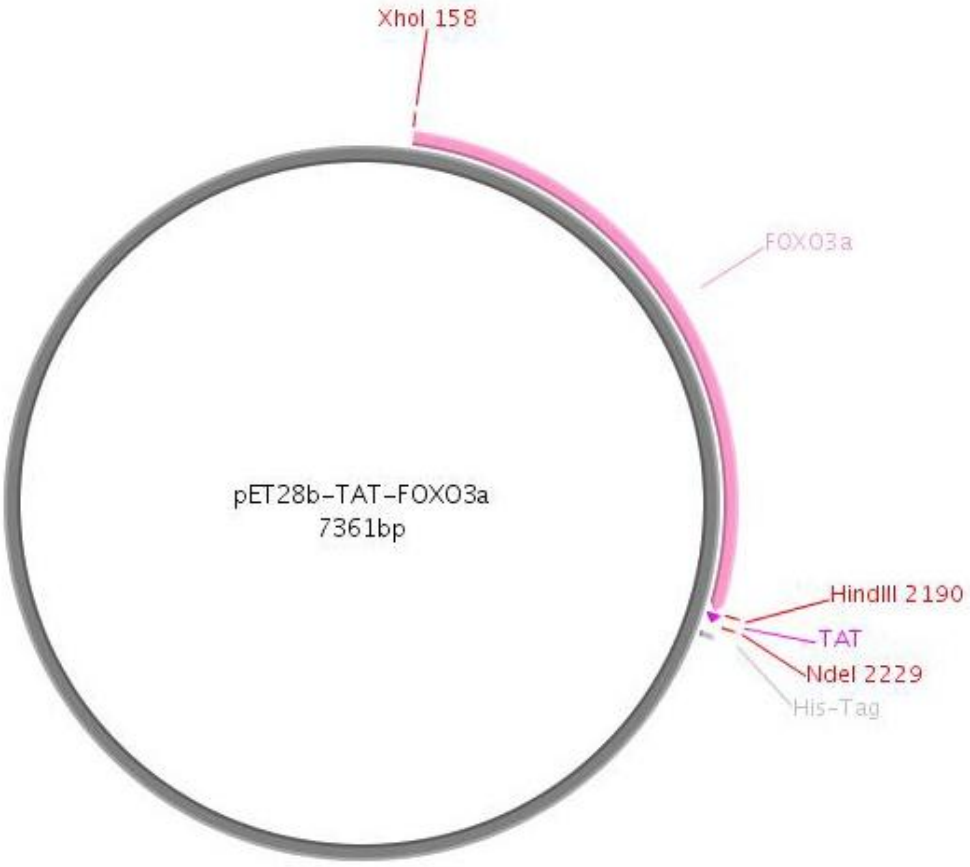


Figure 1. Plasmid map of TAT-FOXO3a construct.

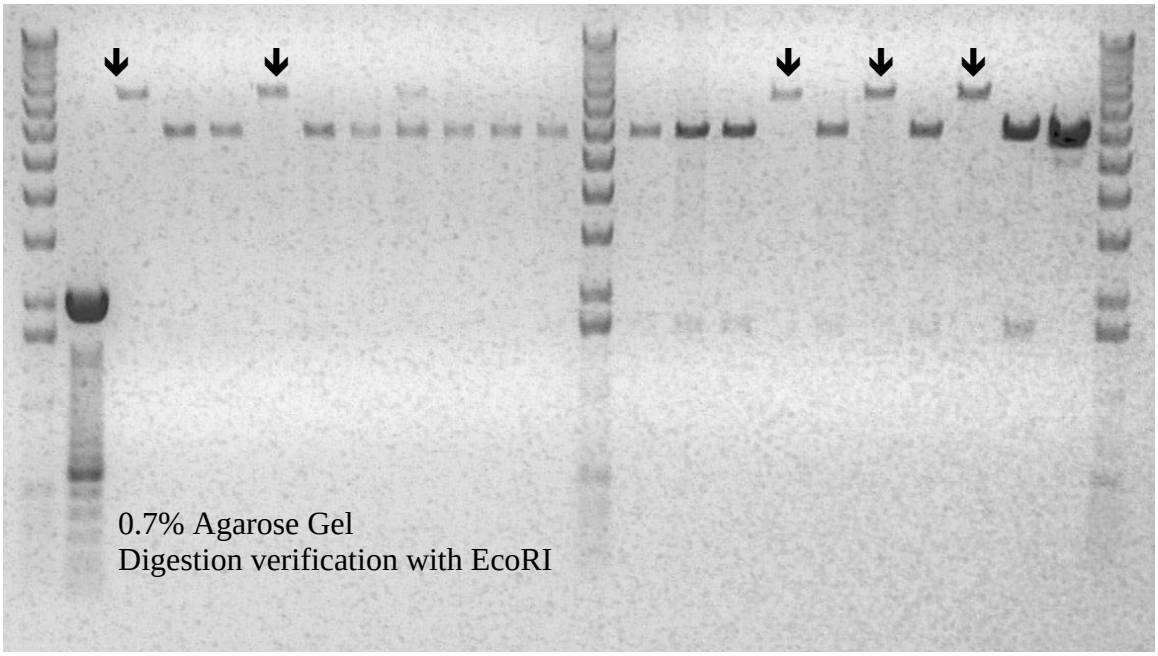


Figure 2. Restriction enzyme digestion verification of TAT-FOXO3a construct. TAT-FOXO3a constructs were digested with EcoRI and correct containing clones FOXO3a and TAT yielded fragments at 7215 bp and 144 bp, shown in black arrows.

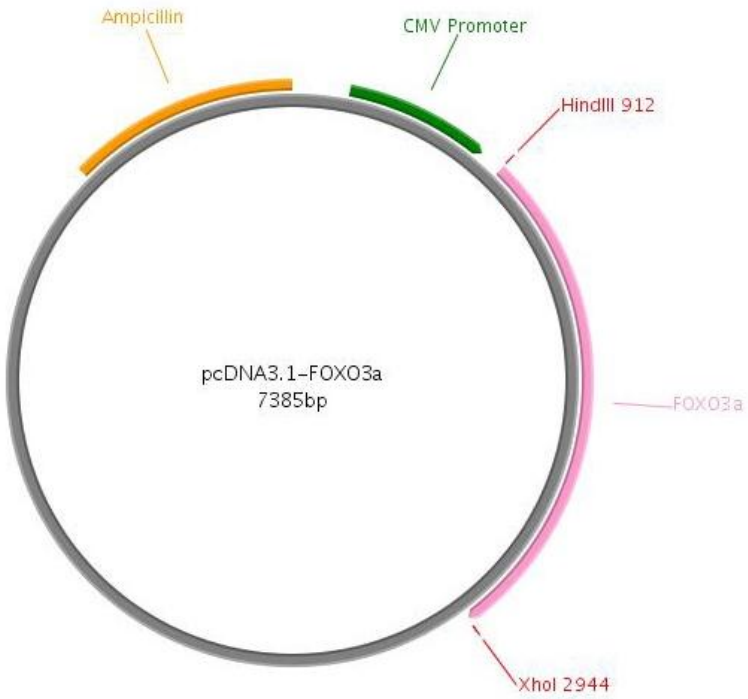


Figure 3. Plasmid map of pcDNA3.1-FOXO3a construct.

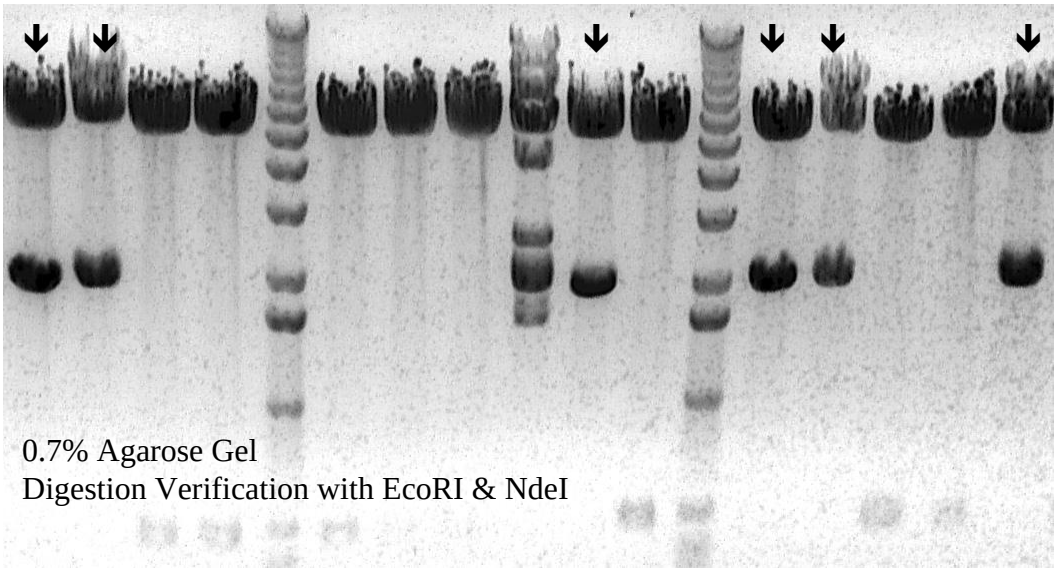
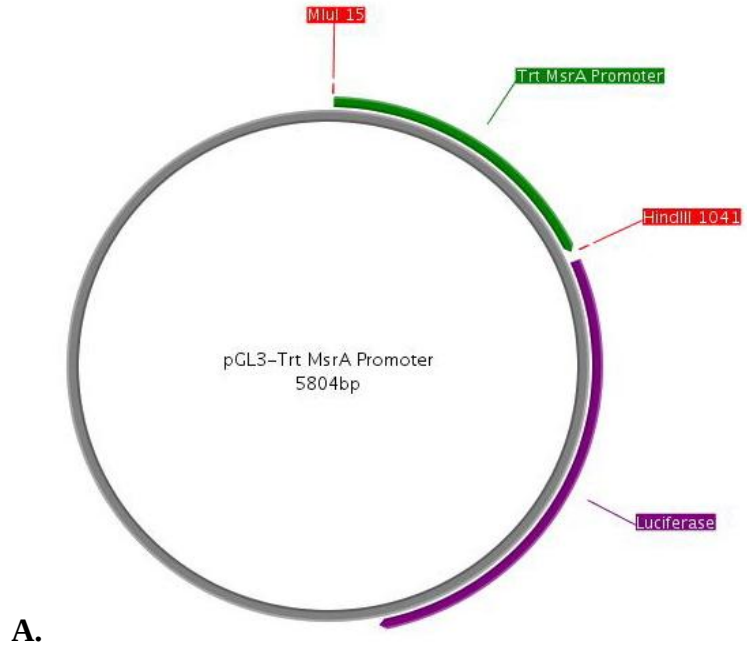
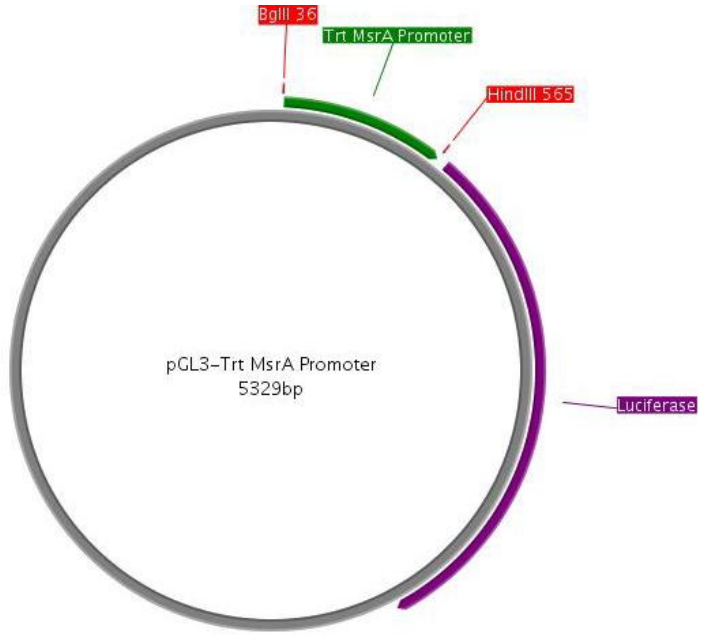


Figure 4. Restriction enzyme digestion verification of pc-DNA3.1-FOXO3a construct. pcDNA3.1-FOXO3a constructs were digested with EcoRI and NdeI releasing a 2492 bp fragment and a 4957 bp fragment, shown in black arrows.

Figure 5. Turtle MsrA promoter sequence including three putative sites for transcription factor FOXO3a binding, shown in red.



A.



B.

Figure 6. Turtle promoter MsrA plasmid maps. A. Full MsrA promoter plasmid map. **B.** Truncated MsrA promoter plasmid map.

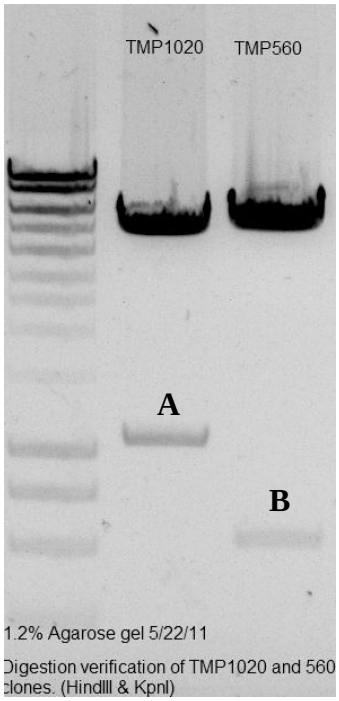


Figure 7. Restriction enzyme digestion of turtle MsrA promoter-1020 clone and turtle MsrA promoter-560 clone. A. Turtle MsrA promoter -1020 bp band **B.** Turtle MsrA promoter -560 bp band.

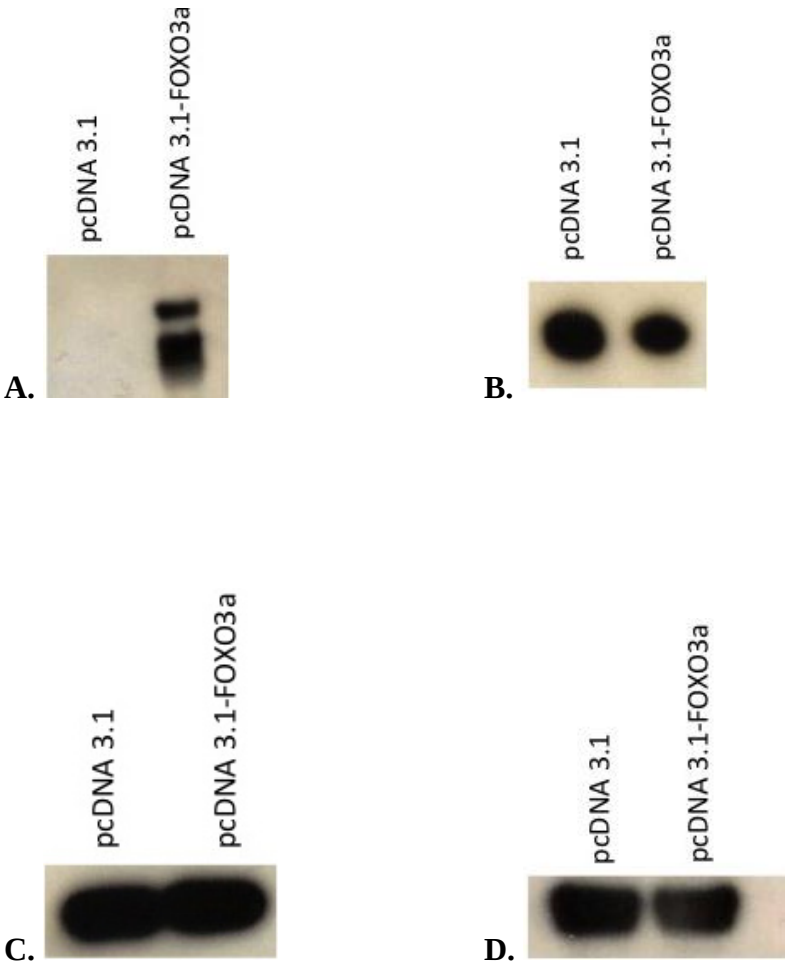
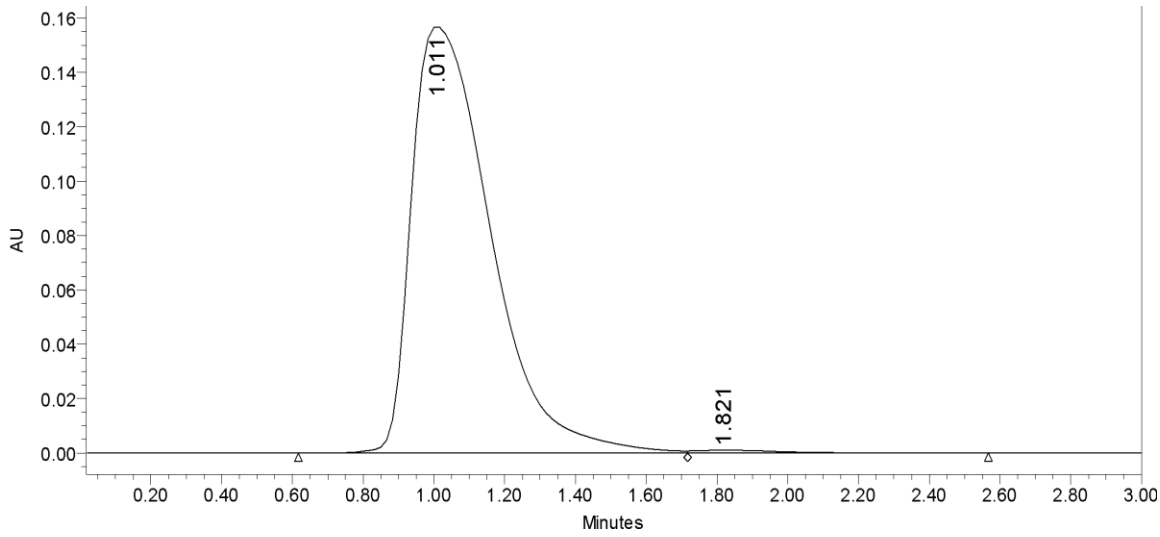


Figure 8. ***In vitro*** **levels of FOXO3a and downstream targets.** Analysis of **A.** FOXO3a, **B.** MsrA, **C.** MnSOD, and **D.** actin protein levels of protein extracts from ARPE-19 cells transfected with pcDNA3.1-FOXO3a or pcDNA3.1.

A.



B.

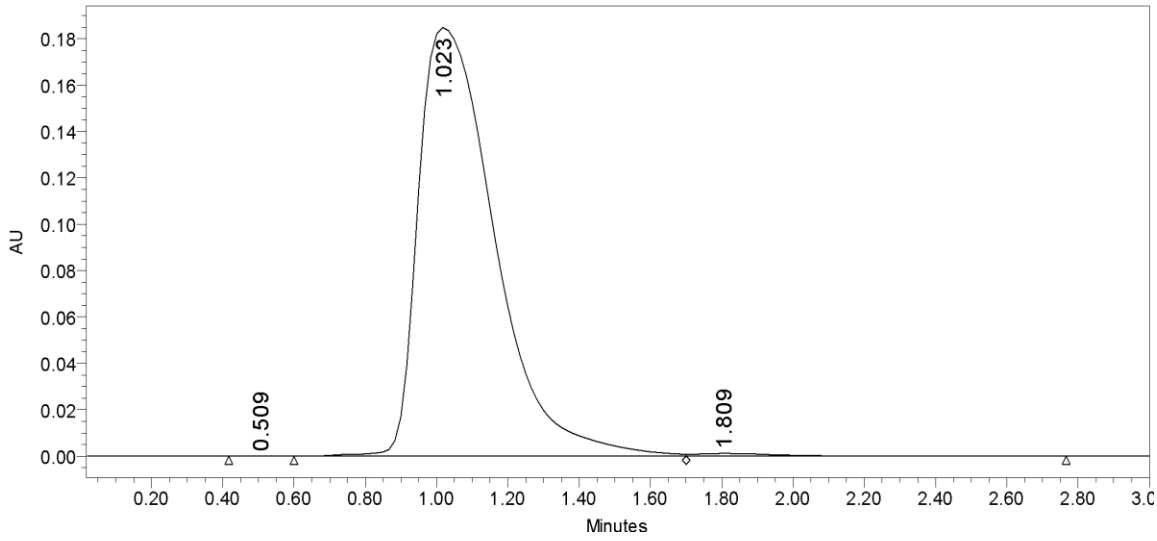


Figure 9 continued on next page

C.

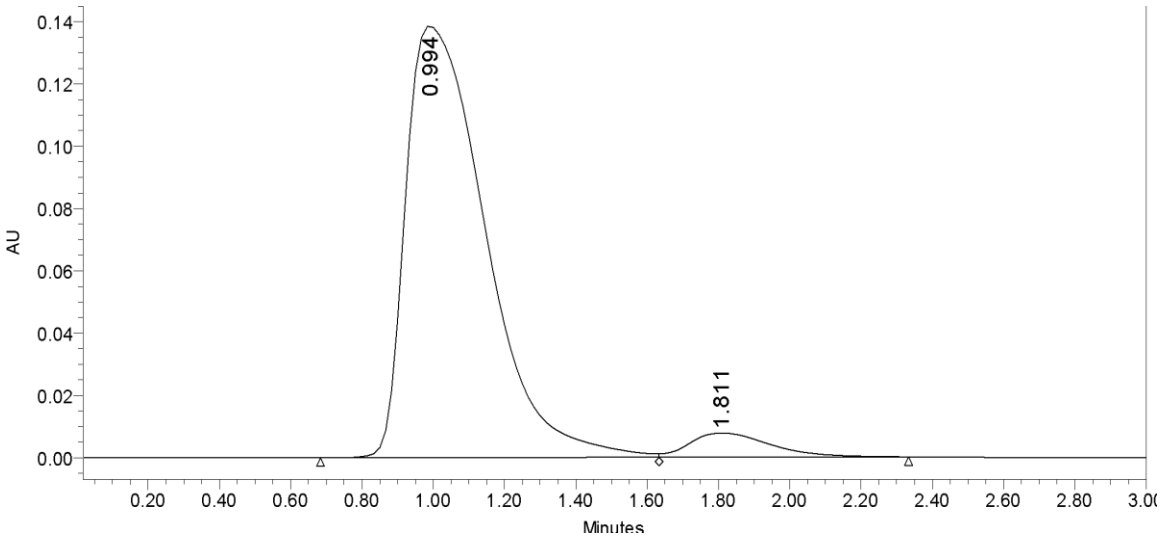


Figure 9. MsrA activity assays of A. ARPE-19 cells transfected with pcDNA3.1-FOXO3a, **B.** ARPE-19 cells transfected with pcDNA3.1, and **C.** bovine MsrA, as control. These assays were guided by Dr. Daphna Sagher.

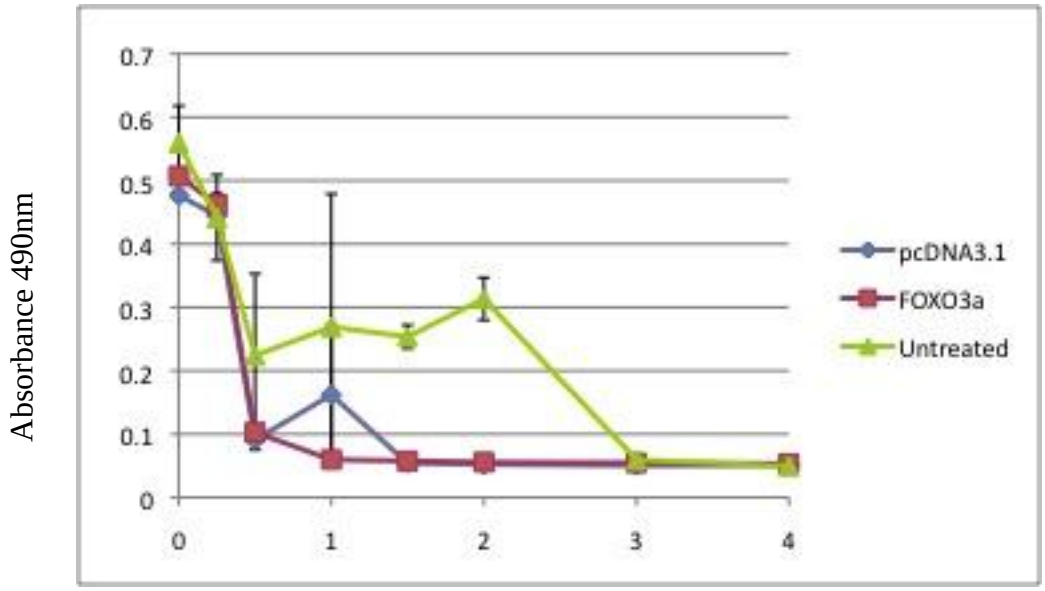


Figure 10. Cell viability assays of ARPE-19 cells. Effect of FOXO3a transfection in ARPE-19 Cells treated with hydrogen peroxide after transfection with either pcDNA3.1-FOXO3a or pcDNA3.1.

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