5-2024

Inhibition Of Foxo Induces Oxidative Stress In Osteosarcoma

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INHIBITION OF FOXO INDUCES OXIDATIVE STRESS IN OSTEOSARCOMA

THESIS

Presented in Partial Fulfillment of the Requirements for

the Degree Master of Science in the Graduate School

of Texas Southern University

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2024

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Redox homeostasis is a balance between reactive oxygen species (ROS) generation and neutralization by antioxidants. Oxidative stress (OS) results when the generation of ROS exceeds the neutralizing capacity of endogenous antioxidants. Reactive oxygen species are generated during cellular processes such as mitochondrial activity or metabolism of drugs. Expression of endogenous antioxidants is driven by redox-sensitive transcription factors; therefore, redox-sensitive transcription factors are essential for the maintenance of cellular redox homeostasis. There are several redox-sensitive transcription factors that contribute to the expression of endogenous antioxidants. These redox-sensitive transcription factors include nuclear factor erythroid 2-related factors (Nrf-2), hypoxia-inducible factor (HIF), activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB) and forkhead transcription factor class O (FOXO). The objective of this research project is to determine the contribution of FOXO to redox homeostasis in osteosarcoma. Osteosarcoma is the most common bone cancer with most osteosarcoma cases occurring in people under 30 years old. In this study, FOXO1 and FOXO3 were inhibited followed by assessment of

oxidative stress. Basal levels of select endogenous antioxidants were also investigated. The results of this study indicate that chemical inhibition of FOXO1 or FOXO3 increases oxidative stress in osteosarcoma. The results also indicate that different osteosarcoma cell lines express different levels of endogenous antioxidants. Collectively, the results of this study suggest that FOXO1 and FOXO3 contribute to redox homeostasis in osteosarcoma.

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LIST OF ABBREVIATIONS

AMPK	AMP-activated Protein Kinase
AP-1	Activator Protein-1
ARE	Antioxidant Response Element
CCHOSD	Human Metastatic Osteosarcoma Cell
DAF-16	FOXO Homolog
DCF	Dichlorofluorescein
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EAC.	Esophageal Adenocarcinoma
ECL	Enhanced Chemiluminescence
FOXO	Forkhead box transcription factor class O
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPx	Glutathione Peroxidase
HCC	Hepatocellular carcinoma
HIF	Hypoxia Inducible Factors
HSC	Hematopoietic Stem Cells
Hsp27	Heat Shock Protein 27
HOS	Human Osteosarcoma
JNK	Jun N-terminal Kinase
KEAP1	Kelch-like ECH-associated protein 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NQO1	NAD(P)H dehydrogenase [quinone] 1

NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
OS	Osteosarcoma
PI3K/Akt	Phosphoinositide 3-kinase/Protein Kinase B
PPP	Pentose Phosphate Pathway
Prx III	Peroxiredoxin III
PTMs	Post-translational Modifications
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD1	Superoxide Dismutase 1
TNF	Tumor Necrosis Factor
TBST	Tris-Buffered Saline and Tween 20

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ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to God. Without His guidance, grace, and gift of life, this opportunity would not be possible. Further, I would like to thank my thesis mentor and advisor, Dr. Mario Hollomon. With his invaluable advice, continuous support, and patience during my graduate academic studies, he has worked with me diligently to strengthen my skills, making me a better researcher. His immense knowledge and experience have encouraged me throughout my time during my research. I also could not have undertaken this journey without my defense committee members, Dr. Erica Cassimere, Dr. Hector Miranda, and Dr. Jahmario Williams, who generously provided knowledge and support.

Additionally, I am also thankful to the department of biology and all its members and staff for all the considerate guidance. Specifically, thank you to Mrs. Helen Pittman-Cockrell who has been supportive step-by-step throughout this journey. I would also like to give thanks to my classmates and office mates for their feedback sessions and moral support. Lastly, I cannot forget to thank my family, my mom, sisters, and friends for all the unconditional support within this very intense academic program. Their belief in me has kept my spirits and motivation high during this process. I would also like to thank my dog for all the entertainment and emotional support.

CHAPTER 1

INTRODUCTION

Redox Balance and Oxidative Stress

In redox reactions the oxidation number of a molecule, atom, or ion either increases or decreases from the loss or gaining of electrons. Redox reactions are common and vital to basic life functions such as in photosynthesis in plants and respiration in mammals. When there is an equilibrium between the products of redox reactions and the defense mechanisms to neutralize them, redox homeostasis is established. These redox reaction products, called free radicals, are atoms that contain partial outer shells with unpaired valence electrons (Phaniendra et al., 2014). Unlike atoms with full shells, free radicals are highly unstable and are quick to react with other substances to complete their outer shell by bonding with surrounding atoms. There is a subset of unstable free radicals that contain oxygen and these are referred to as reactive oxygen species (ROS). ROS radicals cause a chain of reactions converting attacked atoms into active radicals. The most common reactive oxygen species include hydroxyl radical (OH-), superoxide anion (O_2-), and hydrogen peroxide (H_2O_2) (Collin, 2019).

The system required to prevent excessive formation of ROS, contains a variety of proteins that prevent changes in redox status and realigns metabolic activities to restore balance. These proteins, called antioxidants, inhibit oxidation. Antioxidants can be either exogenous or endogenous. While normal levels of ROS are not hazardous, an

overaccumulation of ROS within the system can tip the balance between good and bad. Oxidative stress results when there is a disturbance in the normal redox cells causing a high concentration of free radicals and decreased antioxidants. Oxidative stress has the potential to cause many deleterious events that result in damage to nucleic acids, proteins, carbohydrates, and lipids, thereby it is critical in understanding and promoting redox homeostasis for maintaining health and disease prevention (Franco & Vargas, 2018).

Formation of Reactive Oxygen Species

The formation of reactive oxygen species can be stimulated through multiple processes. Environmental irritants such as pollution, tobacco and cigarette smoke, radiation, diet, and alcohol can cause increased ROS production (Juan et al., 2021). Reactive oxygen species formation is not limited to external factors. The main source of ROS production is through biological and metabolic pathways such as in the electron transport chain of aerobic respiration. The electron transport chain is a series of proteins and organic molecules found in the inner membrane of the mitochondria (Forrester et al. (2018). The membrane-embedded proteins and organic molecules in the electron transport chain are organized into four large complexes labeled I, II, III, IV, and V (Figure 1). The oxygen produced here can be used to form radicals and increase the formation of several additional reactive oxygen species such as H_2O_2 and OH-.

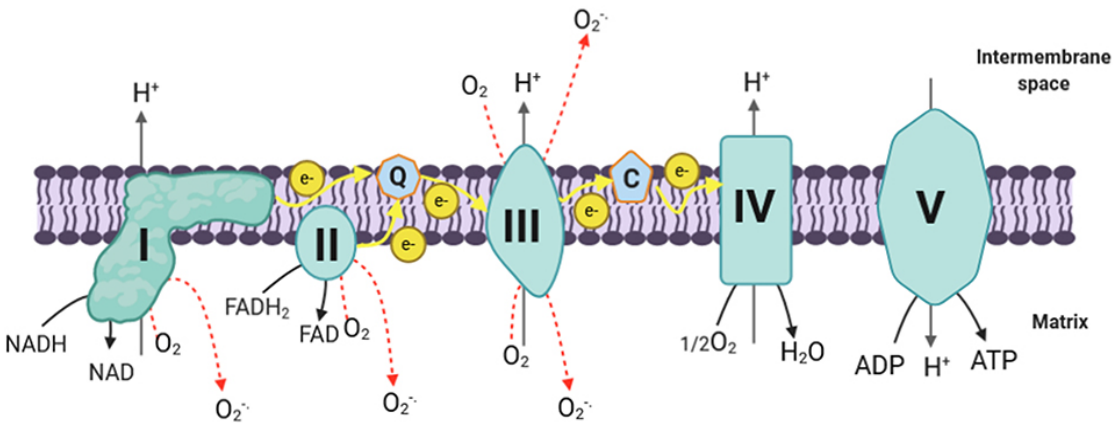

Figure 1: Mitochondria ROS Production via ETC

Leakage of electrons from complex I to II leads to O_2 production from reduced flavin. O_{2^-} and H_2O_2 byproducts are produced from complex II and III and generate in both the mitochondrial matrix and intermembrane space (Tirichen et al., 2021).

The NADPH oxidase (NOX) is an enzyme complex in the membrane that catalyzes ROS as its principal function (Checa & Aran, 2020). NADPH oxidase is found in various cell types, particularly phagocytes such as neutrophils, macrophages, and dendritic cells (Bedard & Krause, 2007; Prieto-Bermejo, 2017). In response to receptors of first messengers, recruitment of p67, p47, and Rac subunits activate NADPH oxidase. This sequence of events allows NADPH to transfer electrons to molecular oxygen (O_2), resulting in the formation of superoxide (O_2-) accumulation inside the cytoplasm.

Endogenous Antioxidants

Endogenous antioxidants prevent radical formation, scavenge, and neutralize active free radicals from the body, and repair damage caused by oxidative stress. The body contains several endogenous antioxidants some of which include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and there are many well-known

mechanisms against oxidative damage that have been largely studied. Additionally, there are antioxidant acting substances that provide an additional layer of protection against oxidative stress.

Superoxide Dismutase (SOD)

The main antioxidant within this category is the superoxide dismutase (SOD). SOD catalyzes the dismutation of O_2- radicals into ordinary oxygen and hydrogen peroxide, both of which are less damaging in the body. Three classes of SOD exist, all presenting distinct protein folds and catalytic metal ions. In humans, SOD1 is located on chromosome 21, encoded by the *SOD1* gene and found mostly in the cytoplasm of cells. Mitochondrial superoxide dismutase 2 (SOD2) is encoded by the *SOD2* gene located on chromosome 6 and codes for both MnSOD and FeSOD. SOD2 is found only in the mitochondrial matrix and actively detoxifies O_2- by forming a protein complex that binds and reduces superoxide. SOD3 is encoded by the *SOD3* gene. In comparison to other SOD isoforms, SOD3 is expressed less and is distributed less in the body. It is mostly localized in the extracellular space where it detoxifies O_2- (MUCHOVÁ et al., 2014; Lobo et al., 2010).

Catalase Enzyme

Catalase (CAT) is found in the peroxisomes of cells and is another important antioxidant enzyme. It is present in almost all aerobic organisms, but it is not abundant in the mitochondria. Catalase breaks down hydrogen peroxide molecules into oxygen and water in a two-step reaction. The first step of the reaction involves the binding of H_2O_2 to the heme group of CAT resulting in hydrogen peroxide reduction and the formation of an

iron-peroxide intermediate (Fe(III)-OOH). In the second step reaction, (Fe(III)-OOH) is cleaved leading to the formation of an oxyferryl species (Fe(IV)=O) and H_2O. Oxyferryl transfers an oxygen atom to H_2O_2. Both steps together result in H_2O_2 decomposition. Mutations in the CAT gene can increase the concentration of H_2O_2 to toxic levels and a deficiency is associated with many aging diseases (Heck, 2010).

NADPH/GPx Pathway

NADPH is a reducing agent produced in the pentose phosphate pathway (PPP). NADPH is used to restore redox balance by serving as a cofactor by donating electrons using the glutathione reductase (GR) enzyme. GR converts oxidized glutathione (GSSG) to a reduced glutathione (GSH) that is less harmful. GSH can then seek and attack multiple H_2O_2 radicals with the help of the glutathione peroxidase (GPx) enzyme. GPx is the general name of family enzymes that are produced in the body designed to remove damaging oxidants. GPx has several isomers ranging from GPx1-8 which varies in location and genetic coding however, GPx-1 is the most abundant and found in almost all mammalian tissues and works to convert H_2O_2 into H_2O and O_2 (Čapek & Roušar, 2021). The GSH and GPx enzymes have greater longevity and are quicker in reaction compared to other antioxidants and for this reason, they serve as an interest for possible future therapeutic approaches in disease prevention and treatments (Kersick & Willoughby, 2005). The NADPH/GPx pathway continuously supplies NADPH to regenerate steady amounts of (GSH) which is utilized by GPx to detoxify ROS, thus proving this pathway as essential in its role in protection against oxidative stress.

Redox-sensitive Transcription Factors

The ability to maintain optimal redox conditions is fundamental for preserving physiological functions and maintaining healthy conditions. Several redox-sensitive transcription factors are essential for the maintenance of redox homeostasis. These redox transcription factors include Nrf-2, NF-κB, and FOXO. Each of these transcription factors have shown significant evidence in redox maintenance with specific cellular events. These events result in enhanced transcription of antioxidant genes. The expression of these transcription factors is dependent upon the stress signal and are sometimes interlinked. Most transcription factors are located within the cytoplasm; however, some can be localized to the nucleus to participate in transcriptional activity.

Nuclear Factor Erythroid 2-related Factor 2 (Nrf-2)

Nuclear factor erythroid 2-related factor 2 (Nrf-2) is located on human chromosome 2 and encoded by the NFE2L2 gene. It is considered the paramount of redox-sensitive transcription factors. The main function of Nrf-2 is to activate a cellular response by inducing transcription for several antioxidant genes. Nrf-2 contains seven conserved homolog domains, Neh1-Neh7 and each domain is differentiated by its function (He et al., 2020). The Neh2 domain of Nrf-2 regulates the interaction of Kelch-like-ECH-associated protein 1 (keap1) and Nrf-2. Under unstressed, basal conditions, Nrf-2 is anchored in the cytoplasm. In response to ROS, Nrf-2 and keap1 dissociate in the cytoplasm and this allows Nrf-2 to stabilize and accumulate and translocate to the nucleus. Once inside the nucleus, the DNA binding domain of Nrf-2 and Neh1 domain recognizes the antioxidant response element (ARE) of target genes and facilitates the induction of numerous cytoprotective proteins (Rungratanawanich et al., 2018).

Nuclear Factor Kappa-light-chain-enhancer of Activated B cells (NF-κB)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a family of complex proteins that is found in almost all animal cell types. Each member has a highly conserved domain called a Rel homology domain which consists of a dimerization region, nuclear localization regions, and a DNA binding region (Ma, 2013). In response to stimuli, NF-κB activation results from two main signaling pathways: the classical, canonical pathway and an alternative pathway called the non-canonical pathway. The classical, canonical pathway is the most dominant pathway and consists of the NF-κB complex RelA and p50 (Ma, 2013). Under an inactive state, NF-κB remains bound to the inhibitory protein IκBα inside the cytoplasm. IκB kinase (IKK) is activated by phosphorylating the two serine residues of IκBα located in its regulatory domain which results in NF-κB activation and IκBα degradation. With the degradation of IκBα, the NF-κB complex is then free to enter the nucleus where it can facilitate transcription (Liu et al., 2017). Like Nrf-2, downstream binding of the NF-κB proteins to DNA regulates downstream transcriptional targets that express many antioxidants and protective genes. SOD2 and Gpx1 are two main antioxidants up-regulated by NF-κB in response to oxidative stress (Morgan & Liu, 2010).

Forkhead Box Protein (FOX)

FOX (forkhead box) proteins were first identified in the *Drosophila melanogaster* fruit fly. With the discovery of the forkhead gene, the DNA-binding domain was also discovered. This domain is conserved amongst each group within this family of transcription factors and in many species ranging from flies to humans. Like Nrf-2 and NF-κB, FOX proteins translocate to the nucleus to bind directly to specific DNA sequences of target genes. In mammals, The FOX family proteins are diversely grouped into subclass

types A to S based on sequence similarity within and outside of the conserved DNA-binding domain (Golson & Kaestner, 2016). The sequence variation is likely to differentiate the function of each protein. The forkhead homeobox transcription factor type O (FOXO) is a subclass of FOX proteins that are critical mediators of cellular responses to oxidative stress and have been implicated in various diseases, including cancer, autoimmune diseases, and neurodegenerative disorders. In mammals, there are four FOXO isomers, FOXO1, FOXO3, FOXO4, and FOXO6. FOXO1 and FOXO3 are found in nearly all tissues throughout the body and serve as a purpose for this study.

Regulation of FOXO Activity

FOXOs have a wide range of roles in various biochemical processes. Particularly, FOXO1 and FOXO3 are critical mediators of cellular responses to oxidative stress. FOXO1 and FOXO3 can activate or inhibit downstream genes that contribute to oxidative protection. In addition to antioxidant regulation, FOXO can regulate proteins that repair damage done to cells caused by oxidative stress (Figure 2). Additionally, FOXOs contribute to cell growth, aging, longevity, and metabolism. They also play dual roles in cancer development.

Figure 2: Various Biochemical Functions of FOXO

FOXO Roles Such as Cell Cycle Arrest, Apoptosis, DNA Damage Response, Cancer Metabolism, and Oxidative Stress Response (Hou et al., 2018).

In response to stress, FOXO activity is modulated by various post-translational modifications (PTMs). The most common PTM of FOXO includes methylation, ubiquitination, and phosphorylation (Du & Zheng, 2021). Phosphorylation of FOXO transcription factors depends heavily on the structure of FOXO. All members within the FOXO family have 3 key regulatory sites (Thr32, Ser253, and Ser315). Phosphorylation of all three sites is targeted by multiple kinases that modulate FOXOs activity (Figure 3). AMP-activated protein kinase (AMPK), c-Jun N-terminal kinase (JNK), and Mammalian Ste20-like kinase (MST1) are major cascade pathways that regulate FOXO under stressful conditions. Kinase phosphorylation disrupts the interaction between FOXO and 14-3-3 proteins and facilitates the nuclear export of FOXO resulting in increased transcriptional activity. This increased transcriptional activity promotes the expression of antioxidants such as CAT, SOD, and GSH. By inducing the expression of these proteins, FOXOs create cellular defenses and protect cells from oxidative damage (Storz, 2011; Thannickal &

Fanburg, 2000). Additionally, FOXO1 and FOXO3 have roles in glycogen metabolism and insulin regulation, acting as suppressors of mitochondrial biogenesis. FOXO1 helps regulate mitochondrial metabolism. On the other hand, FOXO3 plays roles in down-regulating mitochondrial respiratory activity in cancer cells. Dysregulation of FOXO PTMs can also be attributed to various diseases such as cancer and metabolic disorders (Brown & Webb, 2018). Due to FOXOs critical roles in gene regulation and disease pathology, FOXO proteins mechanisms are actively studied as potential therapeutic targets for various conditions.

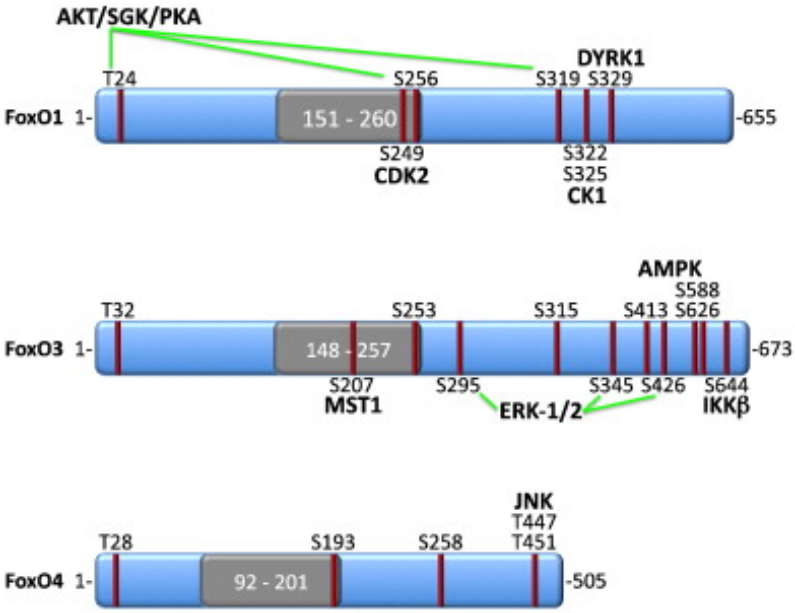

Figure 3: Post-translational Regulation of FOXO

Activity via phosphorylation by multiple Ser/Thr kinases FOXO proteins have nuclear import signal (NLS), a shared conserved forkhead domain, and three phosphorylation sites, including phospho-serine and phosphor-threonine sites. The first phosphorylation motif is located after the start codon, the second is within the conserved domain, and the third is located after the conserved domain. Phosphorylation sites of FOXO1 by AKT/SGK/PKA, FOXO3 by MST1, ERK, and IKK, and FOXO4 by JNK are illustrated. (Tzivion et al., 2011).

CHAPTER 2

LITERARY REVIEW

FOXO and Antioxidant Regulation

Numerous studies have investigated the efficacy of FOXO intervention in reducing oxidative stress by antioxidant induction. The interlink of FOXO and stress resistance was first observed in studies of mutants of *Caenorhabditis elegans*, *C. elegans* (Klotz et al., 2015). This study showed that the FOXO homology, DAF-16 targeted downstream genes that induce SOD expression and other antioxidant genes under oxidative conditions or reduced growth factor (Schmidt et al., 2002). Such studies suggests that the expression of genes coding for such antioxidant enzymes could be under similar control by FOXOs in humans.

Furthermore, FOXO3 specifically, has been studied to have a great effect on antioxidant expression (Marinkovic et al., 2007). Research done by (Chiribau et al., 2008) concludes that FOXO3A is needed for oxidative resistance by increasing the expression of SOD, CAT, and PrxIII following serum-deprivation in human cardiac fibroblasts. This study confirmed that FOXO3A depletion resulted in a significant decrease of PrxIII and increased cellular levels of H_2O_2 and apoptosis. Moreover, this study suggests FOXO3A regulation of PrxIII is a novel mechanism by which FOXO transcription factors modulate cellular redox state and oxidative stress response in the cardiovascular system.

Similarly, a study done by Akasaki et al., 2014 observed reduced FOXO activity and antioxidant expression in chondrocytes resulted in increased sensitivity to death

Induced oxidative stress. The data obtained from this study illustrates the comparison of FOXO mRNA expression in human osteoarthritis (OA) cartilage and normal cartilage. Normal cartilage was found to have a higher expression of FOXO1, FOXO3, and FOXO4 than OA cartilage and significantly damaged OA cartilage. Additionally, to investigate the effect of FOXO under stressful conditions, siFOXO cells that had undergone oxidative stress induction saw changes in antioxidant expression and cell viability that was dose dependent. Increased oxidative stress exposure and FOXO knock-down, resulted in an increase in ROS and GSSH generation. The combination of FOXO1 and FOXO3 knockdown measured an even greater decrease of GPx-1 and CAT expression, suggesting that FOXO downregulation can reduce redox defense by decreasing the expression of antioxidants (Akasaki et al., 2014).

FOXO Signaling Pathways

In response to increased presence of ROS, FOXOs can be activated by specific pathways to restore cellular redox balance. One of which includes a variety of post-translational modifications. Oxidative stress-induced phosphorylation of FOXO proteins by kinases such as JNK has been shown to promote nuclear translocation of FOXO and proven to be conserved across species (Eijkelenboom & Burgering, 2013). A study done by Bridge et al. (2010) examined the role of JNK on FOXO activity in the *Hydra vulgaris* species. The results from this study show that animals exposed to JNK inhibitors and heat shock showed significantly less nuclear localization of FOXO compared to heat control animals, thus proving JNKs role on FOXO. Additionally, JNK signaling was also studied in the *Drosophila melanogaster* to better understand its role on life longevity. A study done by Gan and colleagues (2016) showed that organisms with upregulation of JNK has

increased lifespan and higher stress tolerance due to increased NADPH used to detoxify ROS and reduce oxidative stress. Overall, JNK and FOXO activation was shown to induce the expression of many cytoprotective genes.

In addition to phosphorylation by JNK, a study done by (Lehtinen et al., 2006) confirmed FOXO3 and FOXO1 phosphorylation is mediated by mammalian Ste20-like kinase 1 (MST1). Unlike AKT, MST1 upregulates FOXO by promoting the nuclear translocation of FOXO. In mammalian neurons exposed to oxidative stress, MST was observed to phosphorylate FOXO at its serine 207 phosphorylation site which disrupts the interaction of the chaperone protein 14-3-3. This disruption allows for FOXO transcription activity to increase thereby, increased MST1 activation and FOXO was proven to regulate neuronal cell death. The role of MST1 on FOXO was also found to be conserved in nematodes by phosphorylating DAF-16 at is serine 196 phosphorylation site, which is the homology to FOXO3 serine 207. Additionally, advanced aging and reduced life span was observed in response to knockdown of the *C. elegans* MST1 ortholog, CST-1, and an overexpression of CST-1 was determined to promote life span and delays in tissue aging. These findings demonstrate that MST kinases play important roles in cellular responses to oxidative stress across multiple species.

FOXO and Cancer

FOXOs have been classified as cancer regulators for their ability to regulate oxidative genes, survival genes, and cell growth. While the understanding of FOXOs tumor suppressor mechanisms is vague, the inactivation of FOXO has been documented in many types of human cancer (Wang et al., 2014). A study done by Guan et al. (2016) studied FOXOs role in tumor progression in cholangiocarcinoma, bile duct cancer. Results from

this study showed that FOXO3 inhibition leads to decreased keap1 expression allowing nucleus translocation and hyperactivity of Nrf-2. Additionally, an increase in cytoprotective proteins such as HO-1 and NQO1 were observed. Following FOXO3 depletion, ROS levels were also investigated. FOXO3 depletion was proven to act as a protection for oxidative resistance to cells through Nrf-2 signaling, which in turn, indirectly increased the expression of target genes that are involved in cellular stress responses. Due to this, a large decrease of ROS presence was observed in multiple tumor cell lines. To further understand the impact of Nrf-2 and FOXO3 on ROS levels, a combination knockdown of Nrf-2 and FOXO3 resulted in an increase in ROS formation. The data from this study indicates that FOXO3 exerts a specific effect on redox homeostasis by regulating keap1/Nrf-2 pathway.

In support of the above study of FOXOs role in cancer, according to (Nogueira & Hay, 2013), increased ROS levels due to FOXO inhibition could contribute to tumor development but could also increase sensitivity to cell apoptosis. Due to unregulated cell growth in cancer cells, exceptionally high ROS byproducts are intracellularly generated by the upregulation of metabolic pathways. By this way, the PI3K/Akt signaling pathway becomes overactive. The hyperactivity of PI3K/Akt pathway in cancer cells has been documented to inhibit FOXO activity and promote downregulation of antioxidants proteins. Along with SOD and CAT, FOXO inhibition was shown to suppress Sestrin3 expression, a mammalian protein that has antioxidant properties and proven to activate AMPK and inhibit mammalian target of rapamycin complex 1 (mTORC1). Furthermore, FOXOs inhibition and suppression of Sestrin in cancer cells could increase ROS and activate mTORC1 thus, supporting previous studies that have suggested a complex

regulatory network involving mTORC1 and FOXO proteins that promote cell growth and proliferation.

While the above studies have reported FOXO proteins to be implicated in the regulation of longevity and lifespan in various organisms, other studies have found contrasting results, indicating that excessive or dysfunctional activation of FOXO may also contribute to age-related diseases, neurodegenerative disorders, and cancer. Additionally, FOXO1 overexpression has been observed in many cancers such as bladder, renal, breast, and prostate cancer, suggesting its potential role in tumorigenesis (Grupp et al., 2018). For example, a study done by (Grupp et al., 2018) proposed that FOXO1 overexpression was associated with poor prognosis in Esophageal Adenocarcinoma (EAC). In another study done by (Lu et al., 2019) it was found that overexpression of FOXO3 in the liver of hepatocellular carcinoma (HCC) patients induced considerable liver damage and elevated gene expression of several HCC-associated factors. This study also found a positive feedback effect which triggered increased ROS and DNA damage. Furthermore, the literature reviewed by (Jiramongkol & Lam, 2020), suggested that cancer cells can rely on alternative methods to promote survival by inducing JNK to activate FOXO to prevent cellular damages from oxidative stress.

Taken together, the findings of each of these studies underscore FOXOs effects on lifespan that could be linked to the regulation of genes involved in combating oxidative stress (Golson & Kaestner, 2016). Additional studies are needed to elucidate the underlying mechanisms of FOXO and their roles in cellular physiology and disease pathogenesis. While FOXO is relatively known to be a regulator in cellular homeostasis, it is paramount

to address such knowledge gaps to provide scientific insights. Such insights can be useful in the development of therapeutic and treatment innovative strategies.

CHAPTER 3

DESIGN OF THE STUDY

Antibodies and Reagents

FOXO3 antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). AS1842856, ML385 and carbenoxolone were purchased from ApexBio (Houston, TX). 2',7'-dichlorodihydrofluorescein (H2DCFDA) was purchased from Invitrogen (Carlsbad, CA). Ripa lysis buffer was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture media and supplement reagents were purchased from Invitrogen (Carlsbad, CA).

Cell Lines and Cell Culture

CCHOSD and LM7 are human osteosarcoma metastatic cell lines. HOS is a human osteosarcoma non-metastatic cell line. Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% FBS supplemented with antibiotic, non-essential amino acids. Cells were cultured in an incubator maintained at 5% CO_2 and 37°C. Cells were treated with drug as indicated in figure legends.

Western Blot

Cells were treated as indicated in the figures. Following drug treatment, the supernatant and cells were collected followed by centrifugation at 1000 rpm for 5 minutes at 4°C. Cells were removed from tissue culture dish with a cell scrapper. Collected cells were then lysed in RIPA lysis buffer (150 mM NaCl, 1.0% IGEPAL® CA-630,

0.5%sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) containing protease and phosphatase inhibitor cocktail for 30 minutes. Lysates were then cleared by centrifugation at 10,000 rpm for 10 minutes at 4°C and protein abundance was determined by Bradford assay. Cleared lysate was next added to 4X Laemelli buffer containing 2-mercaptoethanol then boiled for 3-5 minutes. Lysates were then subjected to SDS-polyacrylamide gels (SDS-PAGE) followed by transfer to nitrocellulose membranes. Membranes were blocked for 1 hour in 5% milk and then incubated over night with primary antibody indicated in blot. Membranes were washed three times in tris buffered saline containing 1% triton (TBST) for 30 minutes followed by incubation with secondary antibody for 1 hour. Membrane was next developed using ECL detection system.

Cell Viability/Death Determination

Cell death was determined by microscopic visualization of cells using an inverted microscope. Following drug treatment, pictures were taken of the cells to identify floating cells and cellular debris which are both indicative of cell death. Cells were treated as indicated in figure legends.

Determination of Oxidative Stress and Generation of Oxidative Stress

Flow cytometric measurement of 2',7'-dichlorofluorescein (DCF). Non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) upon oxidation is converted to fluorescent 2',7'-dichlorofluorescein (DCF) which is indicative of hydrogen peroxide (H_2O_2) levels. 2',7'-dichlorofluorescein fluoresces green.

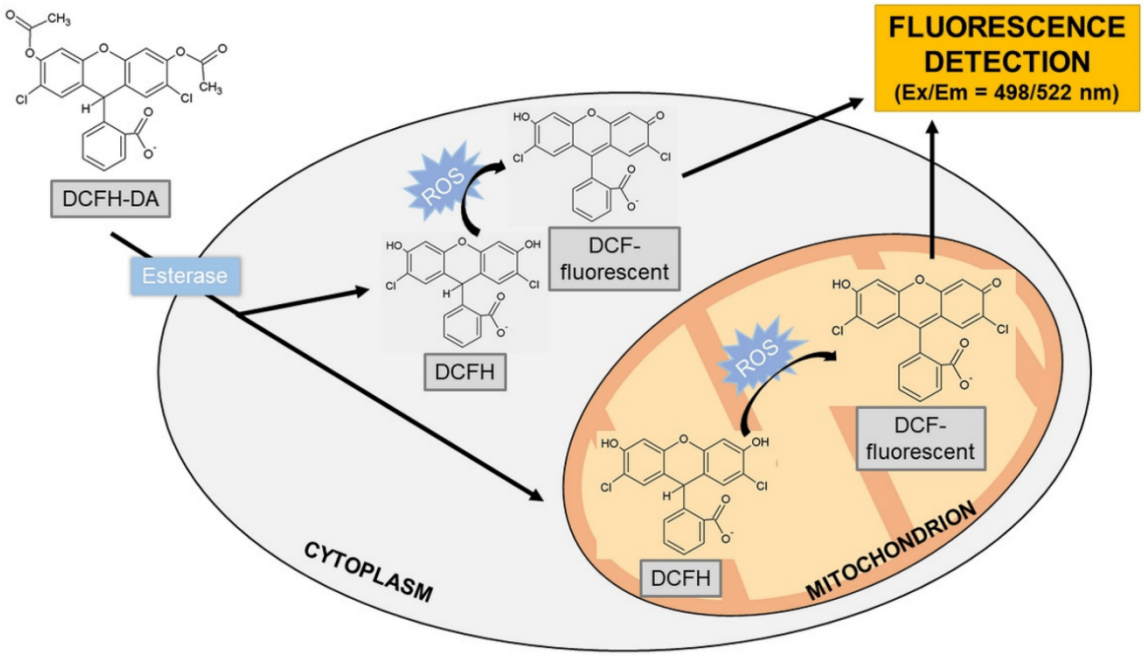

Figure 4: DCFH-DA Mechanism of Action

2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) detection mechanism of hydrogen peroxide presence. Abbreviations: DCFH = 2′,7′-dichlorodihydrofluorescein; DCF = 2′,7′-dichlorofluorescein; and ROS = reactive oxygen species (Čapek & Roušar, 2021).

CHAPTER 4

RESULTS AND DISCUSSION

Basal Level Expression of GPx1, hsp27 and keap1

To begin this study, we had to obtain three OS cell lines. Two metastatic cell lines, CCHOD and LM7, were collected while the other cell line, HOS, is nonmetastatic. Next, using the Western blot analysis, the basal expression levels of the antioxidant proteins GPx1, hsp27, and keap-1 in the presence of the target compound was determined (Figure 5). Due to high metabolic activity in cancer cells, we did hypothesize that expression of each antioxidant would be high in concentration across each OS cell line. It was found that the concentration of hsp27 was highly expressed in CCHOSD cells lines in comparison to HOS and LM7 cell lines. GPx1 expression was shown to be less expressed in all three cell lines compared to hsp27, with CCHOSD and HOS showing least presence than LM7. Keap1 was also identified at basal conditions for each cell line with less expression present in CCHOSD and LM7 cells. The Western blot analysis of each antioxidant in each cell under baseline expression was used to compare to the GAPDH loading control. Such results also allowed for the observation of redox changes following stimulus-induced expression of FOXO1, FOXO3, and Nrf-2 in each cell line.

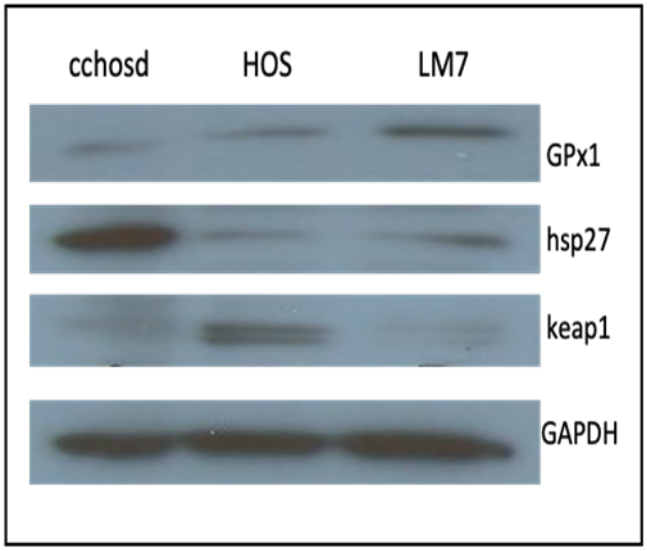

Figure 5: Western Blot Analysis of the Basal Level Expression of GPx1, hsp27 and keap1 Proteins.

Basal levels of GPx1, hsp27, and keap1 proteins in OS cells. GAPDH served as a protein loading control.

Moving forward in the study, we wanted to determine the basal level expression of NQO1, a multifunctional protein that aids in oxidation reduction. A Western blot analysis was performed (Figure 6) to identify its presence in each cell line. From this analysis, NQO1 presence was identified and observed in LM7 cell lines. NQO1 was observed to be higher in presence in LM7 compared to cell lines CCHOSD and HOS. A small observance of NQO1 was present in HOS cells but there is very little to no expression of NQO1 observed in CCHOSD cell lines.

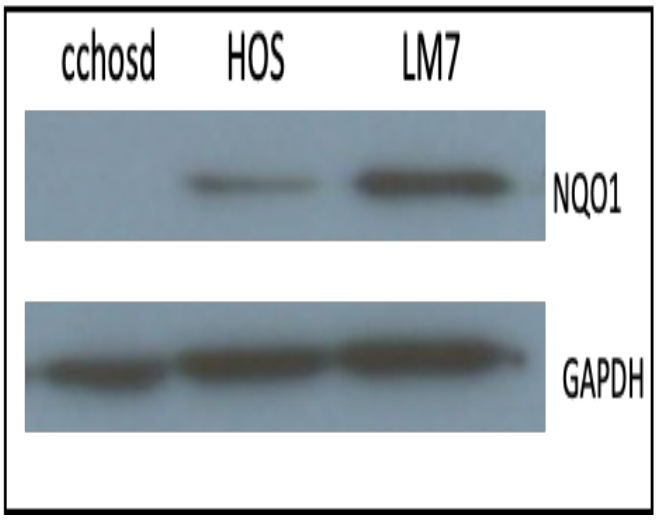

Figure 6: Western Blot Analysis of the Basal Level Expression of NQO1 Protein Expression.

Basal levels of NQO1 proteins in OS cells. GAPDH served as a protein loading control.

Following the identification of NQO1, an additional Western blot analysis was used to determine the presence of basal level expression of antioxidant acting protein, HO-1. (Figure 7). This analysis determined a high presence of HO-1 in CCHOSD and LM7 cells. Unlike CCHOSD and LM7, HOS cell line had very little to no expression of HO-1 protein.

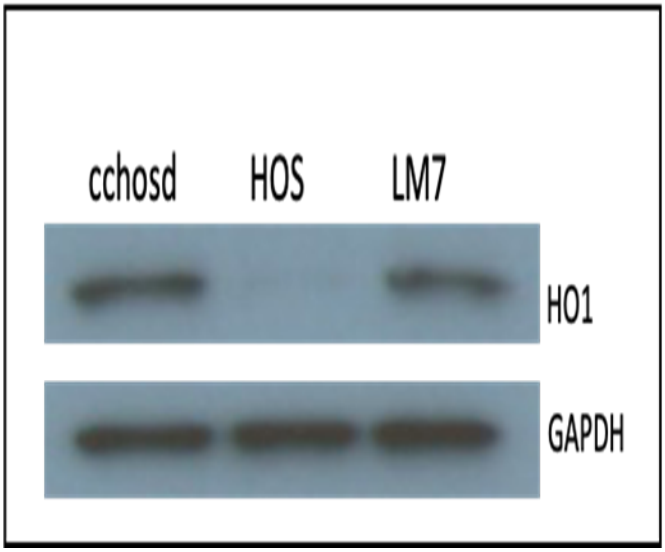

Figure 7: Western Blot Analysis of the Basal Level Expression of Heme oxygenase-1 (HO-1) Protein

Basal levels of HO-1 proteins in OS cells. GAPDH served as a protein loading control.

FOXO1 Inhibition Induces Oxidative Stress

Next, we needed to determine if FOXOs knockdown would induce oxidative stress in OS cell lines (Figures 8, 9, 10). We hypothesized that FOXO1 and FOXO3 deficient cell lines would result in a steady state of oxidative stress condition. After 24 hours, the sensitivity of cells after drug treatments were observed. AS1842856 inhibits the activity of FOXO1, carbenoxolone inhibits FOXO3, and ML385 inhibit Nrf-2. Following treatment, cells were treated with 1 uM DCFH-DA for 15min. Following this treatment, a flow cytometric was used along with 2′,7′-dichlorodihydrofluorescein diacetate (DCF). DCF is an H_2O_2-sensitive probe used to detect levels of hydrogen peroxide in treated cells.

Increased DCF expression indicates oxidative stress generation. In (Figure 8), DCF measurements were observed in all cell lines following FOXO1 inhibition by AS1842856 treatment. The drug dosage was also increased in each cell line, except for LM7 which did not get treated with 20 uM of AS1842856. In comparison to the control, following inhibition of FOXO, each cell line exhibited a significant increase of DCF expression. Amongst each cell line, HOS generated the highest DCF expression at 20 uM of AS1842856 treatment. The lowest DCF increase was observed at 10 uM of AS1842856 treatment in CCHOSD cells.

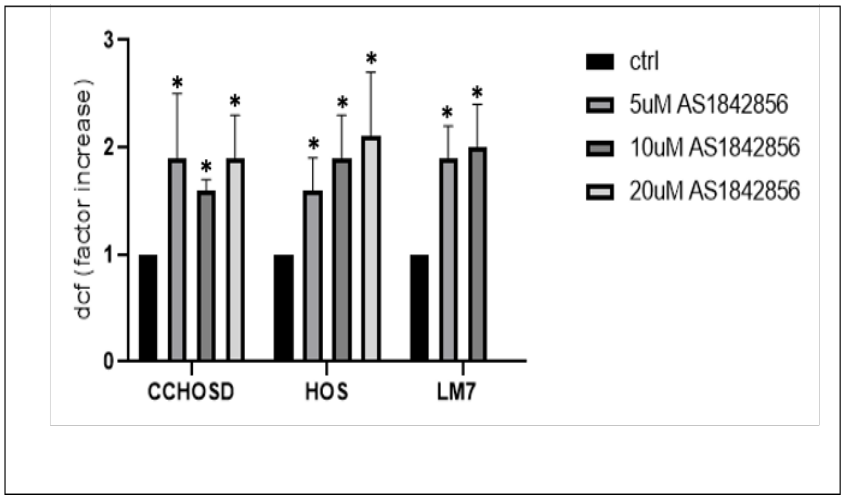

Figure 8: FOXO1 Induces Oxidative Stress

Inhibition drug treatment by three different concentrations of AS1842856, 5 uM, 10 uM, and 20 uM, respectively targeting FOXO1. DCF expression is measured by DCF assay.

In Figure 9, inhibition of FOXO3 is observed in all three cell lines. HOS cell line generated the highest DCF for carbenoxolone dosages of 100 uM and 200 uM. This indicates higher ROS presence in HOS compared to cell lines CCHOSD and LM7. While CCHOSD and LM7 did not show a significant change compared to the control in the initial dosage of 100 uM, there was a greater dose response at 200 uM in LM7 that resulted in an increase of DCF expression.

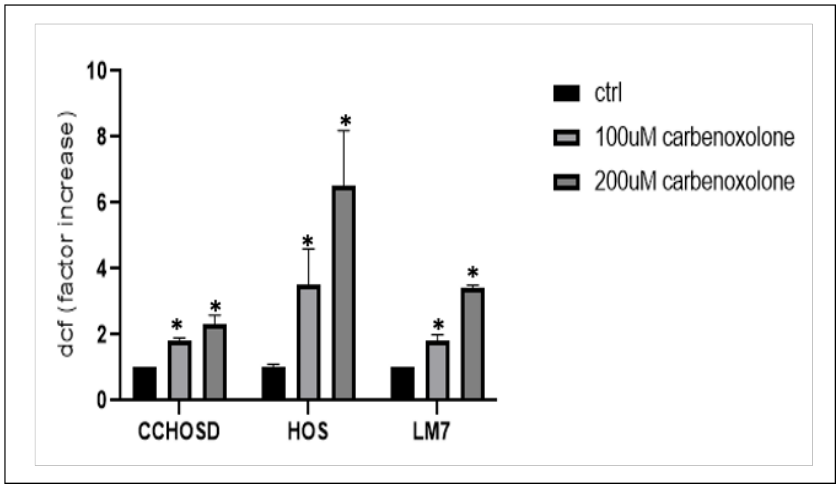

Figure 9: Inhibition of FOXO3 Induces Oxidative Stress

Inhibition drug treatment by 100 uM and 200 uM of carbenoxolone targeting FOXO3. Oxidative stress assessment via ROS generation is measured using DCF assay.

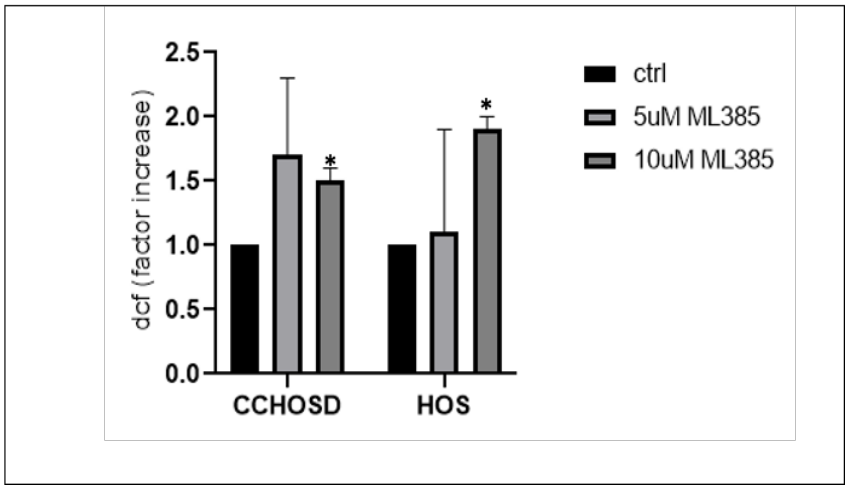

Figure 10: Inhibition of Nrf-2 Induces Oxidative Stress

Inhibition drug treatment by 5 uM and 10 uM of ML385 targeting Nrf-2. Oxidative stress assessment via ROS generation is measured using DCF assay.

Nrf-2 inhibition is observed in (Figure 10) following 5 uM and 10 uM of ML385 treatment in CCHOSD and HOS cell lines. In CCHOSD and HOS cell lines, there was an increase in DCF compared to the control group. Specifically, following drug treatment of 5 uM of ML385 in CCHOSD cells, an increase in DCF was observed, however an increase in dosage to 20 uM showed a slight decrease of DCF, indicating possible less detection of ROS. A change in DCF was also observed in HOS cell lines following drug treatment. The effect of drug treatment at 5 uM was lower in HOS cells but unlike CCHOSD, HOS had a greater dosage response at 20 uM resulting in a signification increase of DCF. This data aids in validating FOXOs role in redox maintenance.

Lentiviral Knockdown of FOXO3

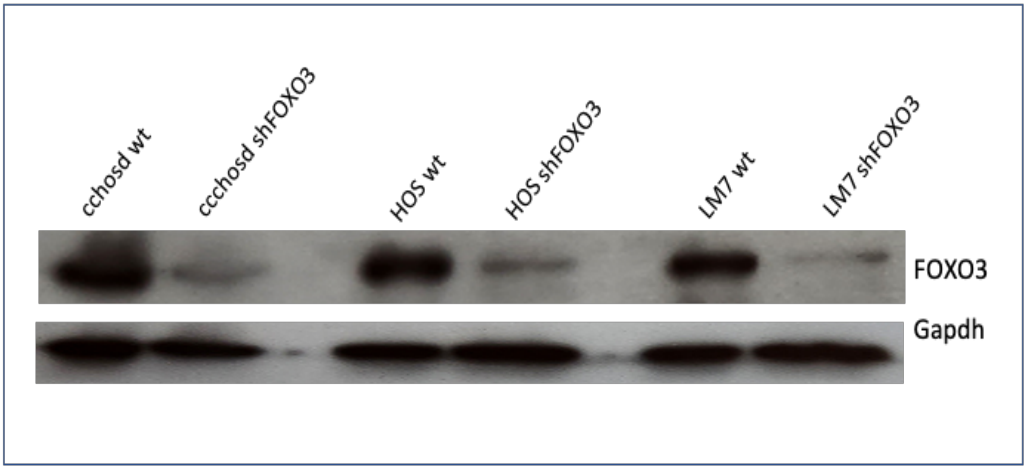

Figure 11: Knockdown Efficiency of lentiviral-mediated Small Hairpin RNA (shRNA) Targeting FOXO3 Expression in Human Osteosarcoma Cell Lines

Cells were infected with shRNA lentivirus targeting FOXO3 protein. Following infection, FOXO3 protein levels were determined by Western blot analysis. GAPDH served as protein loading control.

To confirm the effectiveness of FOXO3 knockdown, a Western blot was used to measure FOXO3 protein levels (Figure 11). FOXO3 knockdown was achieved by a lentiviral sRNA and was compared to CCHOSD wildtype, HOS wildtype, and LM7 wildtype. Additionally, GAPDH was used as a protein loading control.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

In summary, present research findings highlight the interplay between FOXO transcription factors and maintaining oxidative stress. While controlled ROS formation in the body is unharmful and is the result of constant processes of metabolism, an overaccumulation can generally be avoided. Minimizing toxin exposures such as smoke and pollutants can minimize the risk associated with oxidative stress. Such transcription factors such as Nrf-2, NF-κB, and FOXO sense alterations in the balance between ROS and antioxidants. By modulating the transcription of target genes involved in antioxidant defense, DNA repair, apoptosis, and other cellular processes, redox transcription factors help cells adapt to stress (Zhang et al., 2011). Several redox transcription factors have been identified, each with distinct structures, however, FOXOs are highly sensitive to redox status and have various roles in many cellular functions.

In this study we investigated FOXOs to better understand their role in redox homeostasis. Cells from three OS cell lines: CCHOSD, HOS, and LM7 were successfully cultured. We investigated the basal level expression of antioxidants HO-1, NQO1, keap1, GPx1, and hsp27 in each OS cell line. Our results indicated a low concentration of many of these antioxidant proteins at baseline levels, making OS cells sensitive to oxidative damage. Cell lines were also treated with inhibition drugs to suppress activity of FOXO1, FOXO3, and Nrf-2 to observe oxidative stress generation as determined using DCF fluorescence. Additionally, once drug treatment was introduced to each OS cell line, there

was a significant increase in oxidative stress by ROS determined by DCF increase. Furthermore, inhibition of Nrf-2 also resulted in an increase in DCF.

Results from this study indicate that the inhibition of redox transcription factors have a great effect on oxidative stress generation across all cell lines. While we did observe H_2O_2 presence after inhibition, 2-hydroxyethidium (2-HE) would have allowed us to observe the presence of superoxide in FOXO knockdown. This would have further shown the relationship of FOXO and ROS. Additionally, the results obtained indicated low basal level expression of HO-1, NQO1, keap1, GPx1, and hsp27 is OS cells, however a measure of expression for each antioxidant following drug treatment would emphasize FOXOs role in antioxidant and redox regulation. Furthermore, additional investigation of FOXOs role in response to oxidative conditions are needed, particularly in cancer cells.

Research studies have shown FOXOs ability to respond to several stimuli that can affect their transcriptional activity and expression. Exploring FOXO roles in redox balance can make therapeutic regulation of FOXO efficacious and relevant. Furthermore, FOXOs multiple roles have garnered increasing interest to implicate them in different areas such as in stem cell maintenance. Because of their targets, CAT, and SOD, FOXOs may protect hematopoietic stem cells (HSCs) from cell death and aid in self-renewal capacity (Marinkovic et al., 2007; Tothova, 2007). It is expected that better understanding of the modulatory mechanisms of FOXO will provide a basis for the discovery of molecular targets that can therapeutically lead to interventions in many stress conditions and diseases.

Moving forward, this study will investigate further effects of FOXO knockdown using a lentiviral. While our study did have a successful lentiviral knockdown of FOXO3,

FOXO1 lentiviral knockdown would be critical in confirming our data. Additionally, our study did not measure the basal levels of other antioxidants such as SOD and CAT. Lastly, we did not measure O_{2-} expression after FOXO1, FOXO3, and Nrf-2 inhibition, which could have provided further insight on ROS levels following drug treatment. This project will also be moved to additional cancer cell lines to further observe FOXOs role in oxidative stress and investigate a combination treatment of FOXO inhibitors.

REFERENCES

Akasaki, Y., Alvarez-Garcia, O., Saito, M., Caramés, B., Iwamoto, Y., & Lotz, M. K. (2014). FoxO transcription factors support oxidative stress resistance in human chondrocytes. *Arthritis & Rheumatology*, *66*(12), 3349–3358.

Bedard, K., & Krause, K.-H. (2007). The NOX family of ROS-Generating NADPH oxidases: Physiology and pathophysiology. *Physiological Reviews*, *87*(1), 245–313.

Bridge, D., Theofiles, A. G., Holler, R. L., Marcinkevicius, E., Steele, R. E., & Martínez, D. E. (2010). FoxO and stress responses in the cnidarian hydra vulgaris. *PLoS ONE*, *5*(7), e11686.

Brown, A., & Webb, A. E. (2018). Regulation of FOXO Factors in Mammalian Cells. *Current Topics in Developmental Biology*, *127*, 165–192.

Čapek, J., & Roušar, T. (2021). Detection of oxidative stress induced by nanomaterials in cells—the roles of reactive oxygen species and glutathione. *Molecules*, *26*(16), 4710.

Checa, J., & Aran, J. M. (2020). Reactive oxygen species: Drivers of physiological and pathological processes. *Journal of Inflammation Research*, *13*, 1057–1073.

Chiribau, C. B., Cheng, L., Ioan Cucoranu, Yu, Y., Clempus, R. E., & Sorescu, D. C. (2008). FOXO3A regulates peroxiredoxin III expression in human cardiac fibroblasts. *Journal of Biological Chemistry*, *283*(13), 8211–8217.

Collin, F. (2019). Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases. *International Journal of Molecular Sciences*, *20*(10).

Du, S., & Zheng, H. (2021). Role of FoxO transcription factors in aging and age-related metabolic and neurodegenerative diseases. *Cell & Bioscience*, *11*(1).

Eijkelenboom, A., & Burgering, B. M. T. (2013). FOXOs: signalling integrators for homeostasis maintenance. *Nature Reviews Molecular Cell Biology*, *14*(2), 83–97.

Forrester, S. J., Kikuchi, D. S., Hernandes, M. S., Xu, Q., & Griendling, K. K. (2018). Reactive oxygen species in metabolic and inflammatory signaling. Circulation Research, 122(6), 877–902.

Franco, R., & Vargas, M. R. (2018). Redox biology in neurological function, dysfunction, and aging. *Antioxidants & Redox Signaling*, *28*(18), 1583–1586.

Gan, T., Fan, L., h, L., Misra, M., Liu, M., Zhang, M., & Su, Y. (2021). JNK Signaling in Drosophila Aging and Longevity. *International Journal of Molecular Science*s, *22*(17), 9649.

Golson, M. L., & Kaestner, K. H. (2016). Fox transcription factors: From development to disease. *Development*, *143*(24), 4558–4570.

Grupp, K., Uzunoglu, F. G., Melling, N., Hofmann, B., El Gammal, A. T., Grotelüschen, R., Heumann, A., Bellon, E., Reeh, M., Wolters-Eisfeld, G., Ghabdan, T., Nentwich, M., Bachmann, K., Bockhorn, M., Bogoevski, D., Izbicki, J. R., & Kutup, A. (2018). FOXO1 overexpression and loss of pSerine256-FOXO1 expression predicts clinical outcome in esophageal adenocarcinomas. *Scientific Reports, 8*(1).

Guan, L., Zhang, L., Gong, Z., Hou, X., Xu, Y. B., Feng, X.-H., Wang, H., & You, H. (2016). FoxO3 inactivation promotes human cholangiocarcinoma tumorigenesis and chemoresistance through Keap1-Nrf2 signaling. *Hepatology, 63*(6), 1914–1927.

He, F., Ru, X., & Wen, T. (2020). NRF2, a transcription factor for stress response and beyond. *International Journal of Molecular Sciences, 21*(13).

Heck, D. E. (2010). Mechanisms of oxidant generation by catalase. *Annals of the New York Academy of Sciences, 1203*(1), 120–125.

Hou, T., Li, Z., Zhao, Y., & Zhu, W.-G. (2018). Mechanisms controlling the anti-neoplastic functions of FoxO proteins. *Semin Cancer Biol., 50*, 101–114.

Jiramongkol, Y., & Lam, E. W.-F. (2020). FOXO transcription factor family in cancer and metastasis. *Cancer and Metastasis Reviews,* 39(3), 681–709.

Juan, C. A., Pérez de la Lastra, J. M., Plou, F. J., & Pérez-Lebeña, E. (2021). The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. *International Journal of Molecular Sciences, 22*(9), 4642.

Kerksick, C., & Willoughby, D. (2005). The antioxidant role of glutathione and N-acetyl-cysteine supplements and exercise-induced oxidative stress. *Journal of the International Society of Sports Nutrition, 2*(2).

Klotz, L.-O., Sánchez-Ramos, C., Prieto-Arroyo, I., Urbánek, P., Steinbrenner, H., & Monsalve, M. (2015). Redox regulation of FoxO transcription factors. *Redox Biology, 6,* 51–72.

Lehtinen, M. K., Yuan, Z., Boag, P. R., Yang, Y., Villén, J., Becker, E. B. E., DiBacco, S., de la Iglesia, N., Gygi, S., Blackwell, T. K., & Bonni, A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell, 125*(5), 987–1001.

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy, 2*(17023).

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews, 4*(8), 118–126.

Lu, M., Hartmann, D., Braren, R., Gupta, A., Wang, B., Wang, Y., Mogler, C., Cheng, Z., Wirth, T., Friess, H., Jörg Kleeff, Hüser, N., & Yoshiaki Sunami. (2019). Oncogenic Akt-FOXO3 loop favors tumor-promoting modes and enhances oxidative damage-associated hepatocellular carcinogenesis. *BMC Cancer, 19*(1).

Ma, Q. (2013). Role of Nrf2 in oxidative stress and toxicity. Annual Review of *Pharmacology and Toxicology, 53*, 401–426.

Marinkovic, D., Zhang, X., Yalcin, S., Luciano, J. P., Brugnara, C., Huber, T., & Ghaffari, S. (2007). Foxo3 is required for the regulation of oxidative stress in erythropoiesis. *The Journal of Clinical Investigation, 117*(8), 2133–2144.

Morgan, M. J., & Liu, Z. (2010). Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Research, 21*(1), 103–115.

MUCHOVÁ, J., ŽITŇANOVÁ, I., & ĎURAČKOVÁ, Z. (2014). Oxidative stress and down syndrome. do antioxidants play a role in therapy? *Physiological Research, 63*(5), 535–542.

Nogueira, V., & Hay, N. (2013). Molecular pathways: Reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. *Clinical Cancer Research, 19*(16), 4309–4314.

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2014). Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry, 30*(1), 11–26.

Prieto-Bermejo, R. (2017). The importance of NADPH oxidases and redox signaling in angiogenesis. *Antioxidants, 6*(2), 32.

Rungratanawanich, W., Memo, M., & Uberti, D. (2018). Redox homeostasis and natural dietary compounds: Focusing on antioxidants of rice (Oryza sativa L.). *Nutrients, 10*(11), 1605.

Schmidt, M., Fernandez de Mattos, S., van der Horst, A., Klompmaker, R., Kops, G. J. P. L., Lam, E. W.-F., Burgering, B. M. T., & MedemaR. H. (2002). Cell cycle inhibition by FoxO Forkhead transcription factors involves downregulation of cyclin D. *Molecular and Cellular Biology, 22*(22), 7842–7852.

Storz, P. (2011). Forkhead homeobox type O transcription factors in the responses to oxidative stress. *Antioxidants & Redox Signaling, 14*(4), 593–605.

Thannickal, V. J., & Fanburg, B. L. (2000). Reactive oxygen species in cell signaling. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 279*(6), L1005–L1028.

Tirichen, H., Yaigoub, H., Xu, W., Wu, C., Li, R., & Li, Y. (2021). Mitochondrial reactive oxygen species and their contribution in chronic kidney disease progression through oxidative stress. *Frontiers in Physiology, 12*.

Tothova, Z., Kollipara, R., Huntly, B. J., Lee, B. H., Castrillon, D. H., Cullen, D. E., McDowell, E. P., Lazo-Kallanian, S., Williams, I. R., Sears, C., Armstrong, S. A., Passegué, E., DePinho, R. A., & Gilliland, D. G. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell, 128*(2), 325–339.

Tzivion, G., Dobson, M., & Ramakrishnan, G. (2011). FoxO transcription factors; regulation by AKT and 14-3-3 proteins. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(11), 1938–1945.

Wang, Y., Zhou, Y., & Graves, D. T. (2014). FOXO transcription factors: Their clinical significance and regulation. *BioMed Research International, 2014*, 1–13.

Zhang, X., Tang, N., Hadden, T. J., & Rishi, A. K. (2011). Akt, FoxO and regulation of apoptosis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(11), 1978–1986.