FOXO induces the Expression of Small and Large Heat Shock Proteins in Mammalian Cell Line

Senior Thesis

Presented to
The Faculty of the School of Arts and Sciences
Brandeis University
Undergraduate Program in Biology
Michael Marr, Advisor

In partial fulfillment of the requirements for the degree of Bachelor of Sciences

by
Yang (Katie) Li

April 2015

Copyright by
Yang (Katie) Li

Committee members (if applicable):

Name: Dr Michael Marr
Signature:

Name: Dr Nelson Lau
Signature:

Name: Dr Paul Garrity
Signature:
Abstract
Senior Thesis
Presented to
The Faculty of the School of Arts and Sciences
Brandeis University

FOXO is a family of transcription factor that is involved in many vital cellular processes; one of its functions is modulating the cellular response to oxidative stress. Heat Shock Proteins (Hsp) are molecular chaperonins that are upregulated in response to protein denaturation and aggregation that can result from oxidative stress. The FOXO ortholog in C. elegans and Drosophila are both capable of inducing expression of Hsp in response to oxidative stress. Unlike invertebrates, mammals have four FOXO proteins, FOXO1, FOXO3 and FOXO4. I investigate whether these FOXOs are capable of inducing expression of small and/or large Hsps in mammalian cells and if FOXO induces Hsp expression in response to oxidative stress. The treatment of HEK 293T cells with Paraquat, an herbicide that introduces oxidative stress, was sufficient to increase the expression of Hsps. When FOXO1, FOXO3 or FOXO4 was overexpressed in 293T cells, there was an increase in expression of both small and large Hsps examined (Hsp 70A, Hsp 22 and Hsp 27). All of the FOXO proteins caused the induction of at least two fold in each of the Hsp, however the level of induction differs between FOXOs. This suggests that perhaps the effect of FOXO induction might vary among Hsps.
Introduction

FOXO Proteins

Forkhead box class O (FOXO) is part of the forkhead super family of proteins. Forkhead box protein (FOX) was first discovered in Drosophila and when mutated results in a head structure that resembles a fork (Wiegel et al 1989). The FOX family of proteins function as transcription factors and their defining feature is a DNA binding domain known as the fork head domain (Lai et al 1993). This domain is a hundred amino acids long and is highly conserved. It consists of three alpha helices and two large loop regions, resulting in a structure resembles butterfly wings and the FOX proteins are sometime referred to as winged helix proteins (Clark et al 1993). The domain binds DNA at a consensus motif of 5’-TTGTTTAC-3’ (Furuyama et al 2000).

The FOXO proteins were first identified in humans due to their presence at chromosomal translocations that led to rhabdomyosarcomas and Acute Myeloid Leukemia (Hillion et al 1997, Parry et al 1994). FOXO proteins are conserved from Caenorhabditis elegans to mammals. Invertebrates have just one FOXO protein. It is known as DAF-16 in C. elegans and dFOXO in Drosophila melanogaster (Burgering et al 2002). Mammals have four FOXO genes- FOXO1, FOXO3, FOXO4 and FOXO6 (Anderson et al 1998, Jacobs et al 2003). The mammalian FOXO proteins share the same DNA binding domain and have overlapping transcriptional targets. Nonetheless, they also have certain distinct biological functions. In mice, the lack of FOXO1 is embryonic lethal and mice that are null for FOXO1 do not survive past embryonic day 10.5(Furuyama et al 2004). FOXO3 null mice do survive, but display lymphoproliferation, organ inflammation and for females, age dependent infertility (Castrillon 2003). Mice null for FOXO4 and FOXO6, on the other hand, show no detectable adverse phenotypes (Hosaka et al 2004).
Low levels of all FOXO proteins are expressed in almost all mammalian tissues, however the particular level of each FOXO proteins shows tissue specificity. FOXO1 expression is highest in adipose tissue, uterus and ovaries. FOXO3 is expressed at particularly high levels in the brain, spleen and the heart. High FOXO4 expression is found in both the cardiac and skeletal muscles. FOXO6’s expression is almost exclusively in the brain (Hookman et al 2006). The differential expression of FOXOs might contribute to the difference in the null phenotypes.

FOXO Functions

FOXO proteins (FOXOs) are involved in a wide range of cellular processes. This includes metabolism, DNA damage repair, cell cycle arrest, apoptosis, stress resistance and longevity (van der Horst 2007). FOXOs play an important role in metabolism through both the control of food intake and glucose metabolism. FOXOs modulate appetite by regulating the expression of orexigenic Agouti-related peptide (AgRP) and Neuropeptide Y (NPY) as well as anorexigenic Pro-opiomelanocortin in the hypothalamus (Hong et al 2012). In the liver, FOXOs regulate the transcription of glucose-6-phosphatase (G6Pase) and phosphoenopyruvate carboxykinase (pepck), two enzymes involved in gluconeogenesis (Schmoll et al 2000 70, Hall et al 2000).

FOXOs aid in the repair of DNA damage by up-regulating genes that sense this damage, such as Damage-specific DNA binding protein 1 (DDB1) and Growth Arrest and DNA damage 45 protein (GADD45) (Tran et al 2002). FOXOs have tumor suppressor functions through their regulation of cell cycle arrest, apoptosis and autophagy. FOXOs induce cell cycle arrest by increasing the expression of Cyclin-dependent kinase inhibitors p27 and p21, both of which function as cell cycle inhibitors (Stahl et al 2014)). FOXOs can also reduce the expression of D-type cyclins D1 and D2, which are needed for CDK4/6 activation. CDK4/6 inhibits pRB, a
transcriptional repressor of G1-S progression (Schmidt et al 2002). Depending on the circumstances, FOXOs can also promote apoptosis. FOXOs can induce the expression of BCL-2 protein Bim, a pro-apoptotic protein of the Bcl-2 family in the intrinsic pathway (Dijkers et al 2000). They can also increase the transcription of FasL and TRIAL, which are death cytokines (Zhang et al 2011). FOXOs induce autophagy by inhibiting the localization of mTORC1 complex to the lysosome. mTORC normally functions to block autophagy through the inhibition of ULK1/2 complex (Lin et al 2013).

FOXOs also help the cell mediate response to a variety of environmental stresses such as nutrient deprivation and oxidative stress, and play a role in longevity. FOXO is downstream of the Insulin signal pathway and can function as a transcriptional feedback control of the insulin receptor. FOXO in both *Drosophila* and mammals are responsible for activating transcription of the insulin receptor (Puig and Tjian 2005). In time of nutrient deprivation, decreased signaling through the insulin receptor leads to activation of the FOXOs, which in terms increase the amount of insulin receptor available. The newly synthesized insulin receptor can then accumulate in the cell membrane, rendering the cell more sensitive to insulin and insulin like peptides (Puig and Tjian 2005). FOXOs also help cells combat oxidative stress by increasing the transcription of manganese superoxide dismutase (SOD2) and catalase, two enzymes that neutralizes Reactive Oxygen Species (Kops et al 2002). FOXO has been implied in longevity as well. In *C. elegans*, exposure to hypoxic (low oxygen state) and nutrient deprivation leads to the formation of the dauer stage, where the animal is long lived and stress resistant. However, when FOXO is inhibited, this increase in lifespan is no longer observed, suggesting that FOXO is needed for the formation/maintenance of the dauer state (Junger 2003). This reinforces the idea
that downstream targets of FOXO include genes that help the cell deal with stress and protein damage.

**FOXO regulation**

Due to its numerous functions, FOXO proteins can be regulated by multiple pathways. Post-translation modifications such as phosphorylation, acetylation, and methylation can modulate FOXO activities by altering their cellular localization (Calnan and Brunet 2008). Under normal conditions, FOXOs are inactive within the cell. The insulin/insulin-like growth factor-1 (IGF-1) signaling pathway mediates this inactivation. Insulin and IGF-1 bind the insulin receptor and acs through PI3K to increase the level of phosphatidylinositol-3-phosphate (PI3P). PI3P is a second messenger that recruits protein kinase B (PKB), also known as Akt. Akt is activated by PDK and translocates to the nucleus where it phosphorylates FOXO proteins at three residues. This phosphorylation leads to increased binding of FOXOs to regulator 14-3-3 and its localization to the cytoplasm (van der Horst and Burgering 2007). As transcription factors, FOXOs are unable to modulate gene expression when excluded from the nucleus. This export can also be regulated by glucocorticoid-regulated kinase (SGK), casein kinase 1 (CK1), and DYRK1A (Monsalve et al 2011). The cytoplasmic FOXOs are polyubiquitinated and targeted for proteosomal degradation (Eijkelenboom and Burgering 2011).

In addition, FOXO proteins can be phosphorylated by Cyclin-dependent kinases (CDKs), which can lead to either activation and inhibition depending on the CDK involved (Huang et al 2006, Yuan et al 2008). In response to DNA damage, MAPK-activated protein kinase 5 activates FOXOs through phosphorylation (Kress et al 2011). Phosphorylation by AMP-activated protein kinase (AMPK) also activates FOXOs, though does not change their cellular localization (Greer et al 2007). Acetylation and deacetylation by histone acetyltransferase such as SIRT and HDAC
might play a role in regulating what downstream genes are activated by FOXOs (Frescas et al 2005). FOXOs can also be methylated. PRMT1 (Arg N-methyltransferase) methylates FOXOs at an Arg residue within the binding site for Akt and might prevent phosphorylation by Akt (Yamagata et al 2011). Methylation by SSET9, on the other hand, inhibits FOXOs function by adding a methyl group within the DNA binding domain (Xie et al 2012). O-linked-D-N Acetylglucosamine addition can also occur on FOXOs and might oppose phosphorylation at these sites. (Kuo et al 2008)

**FOXO and oxidative stress**

FOXO plays an important role in modulating cellular resistance to oxidative stress. Oxidative stress results from the build up of Reactive Oxygen Species due to metabolic processes and is connected to both aging and disease. FOXO1, FOXO3 and FOXO4 are all involved in the response to oxidative stress (Tothova et al 2007). However, they might have different mechanisms of activation. Under condition of oxidative stress, the activation of FOXO proteins allows them to translocate from the cytoplasm to the nucleus even in the presence of insulin and/or growth factors. In both mice and human cells, mammalian Ste20-like kinase (MST1) forms a physical complex with FOXO1/3 when exposed to peroxide. MST1 phosphorylates FOXO1/3 at the serine 207 position, allowing FOXO1/3 to be released from the 14-3-3 regulator proteins that keeps it in the cytoplasm and leads to nuclear accumulation (Lehtinen et al 2006). FOXO4 is activated by oxidative stress through a Ral-mediated, JNK dependent pathway.

Treatment with peroxide activates Ral, a small GTPase. Ral is capable of activating stress kinase JNK. JNK phosphorylates FOXO4 on the threonine 447 and threonine 415 residues. The phosphorylated FOXO4 then translocate back into the nucleus where it activates among other genes further transcription of FOXO4 (Essers et al 2004).
Silent information regulator 2 (Sir2), a NAD-dependent deactylase also appears to play a role in the activation of FOXO proteins. SIRT1, the mammalian homolog of Sir2, deacetylates FOXOs and overrides the nuclear export induced by growth factors and insulin (Brunet et al 2004). When stimulated with peroxide, FOXOs form a complex with cAMP response element-binding protein (CREB)-binding protein (CBP). CBP is an acetylase that acts as a coactivator of FOXO and helps mediated transcription through histone acetylation. It also recruits pre-initiation complex containing RNA Polymerase II to the promoter of apoptosis inducing genes. However, CBP also acetylates FOXOs, mitigating their function as transcription factors (Wang et al 2007). SIRT1 reverses the effect of CBP by deacetylating FOXOs allowing them to function. SIRT2’s effect on FOXO induced transcription is two fold, it inhibits FOXO activation of pro-apoptotic genes while stimulates the expression of FOXO genes involved in cell cycle arrest and resistance to oxidative stress (Daitoku et al).

One of the possible downstream targets of FOXOs that’s activated in response to oxidative stress are the Heat Shock Proteins (Hsps). Hsps are a family of highly conserved proteins that are ubiquitously expressed at normal conditions (Kalmer and Greensmith 2009). They were originally discovered to aid in cellular response heat shock but are also upregulated in response to multiple other stresses. They function mainly as chaperones, and they help mitigate oxidative stress by inhibiting both apoptosis and inflammation (Morimoto 2008). Hsps are divided into many subfamilies based on their molecular weight. There are small Hsps that range from 16-30 kDa, and the large Hsps which are the Hsp 40, 70, 90 and 110 families (Tower 2009). Hsps are characterized as either constitutive or inducible. Constitutive proteins are expressed at a certain level consistently in the cell. Inducible Hsp are normally expressed at a low level in the cell but their expression increases dramatically in response to various form of cellular stress. The
inducible Hsps can be activated by Heat Shock Factor 1 (HSF1). HSF1 activates transcription by binding to the Heat Shock response elements (HSEs) in the promoters of Hsp genes (Voellmy 2004). Hsps such as Hsp 32 and Hsp 72 are redox sensors and are the first to detect oxidative stress within the intracellular compartments (Jakob et al 1999). This increase in oxidation causes the denaturation or misfolding of cellular proteins, leading to protein aggregation and precipitation. It reveals hydrophobic residues that are sensed by and serves as binding sites for Hsp70 and Hsp 90. This has a two-fold effect. Hsp 70 and 90 has chaperone functions which involve refolding of denatured proteins and targeting damaged proteins for proper degradation(Freeman and Morimoto 1996). This also allows for the activation of HSF1. Normally, HSF1 is inactivated due to its binding with Hsp70 and 90. With Hsp 70 and 90 performing their chaperon functions, the free HSF1 can form a trimer and translocate to the nucleus where it activates the transcription of Hsp proteins (Morimoto and Santoro 1999). This leads to an overall increase in Hsp expression to combat oxidative stress. FOXOs appear to be another way of activating inducible Hsps.

Research Question

FOXO proteins have been shown to increase the expression of inducible Hsps in both C. elegans and Drosophila melanogaster in response to oxidative stress. DAF-16 in C. elegans activates only the small Hsps (Kuo et al 2013). dFOXO in Drosophila activates both small (Hsp 22, 23 and 26) and large (Hsp 70) Hsps (Marr and Donovan, unpublished). I investigated whether the FOXO family of proteins also induces the expression of Hsp in mammalian cells and whether FOXO is inducing Hsp expression in response to oxidative stress. It is important to note that in the mammalian system there are four FOXOs, and it seems likely that there would be both small and large Hsps. Human Embryonic Kidney (HEK) 293T cells were used as the model
mammalian cell line. Oxidative stress is induced through the addition of paraquat, a herbicide that produces intracellular reactive oxygen species.

HEK 293T endogenously express low levels of FOXO proteins. The 293T cells were either left untreated or treated with varying concentration of paraquat to determine if there is an increase in level of Hsps. Paraquat treatment induced expression of both small and large Hsps. FOXO proteins were then overexpressed in 293T cells to determine if FOXOs are capable of inducing Hsp expression. When plasmids for FOXO1, FOXO3 and FOXO4 genes were transfected into 293T cells in the absence of any paraquat, there is an increase in expression of both small and large Hsps, Lastly, to determine if Hsps are still induced in response to paraquat without the presence of FOXO proteins, each individual FOXO is selectively knocked down using CRISPR-cas9.

Results

Paraquat induces expression of Hsps in 293 T cells

Oxidative stress induced by the presence of Reactive Oxygen Species leads to protein misfolding and aggregation; this leads to upregulation of cellular chaperon such as Heat Shock Proteins (Hsps). The expression level of Hsps is dramatically increased in response to heat treatment, however oxidative stress can also induces their expression. (Kalmar and Greensmith, 2009) the induction by oxidative stress usually takes longer and is not as prominent. To determine if FOXO plays a role in inducing Hsps in response to oxidative stress in mammalian cell lines, oxidative stress must be able to reproducibly introduced to 293T cells and this stress needs to lead to an increase in Hsp expression. Paraquat is an herbicide known to be toxic to kidney cells. It has been shown that paraquat is reduced in the mitochondrion and forms intracellular oxygen species, thus leading to oxidative stress in 293T cells (Kim et al 2008).
However, it is important to determine if paraquat treatment actually causes a change in Hsp expression. I treated 293T cells with varying concentrations of paraquat and used RT-qPCR results to determine if there is an increase in inducible Hsp expression. Confluent 293T cells were treated with 1 mM paraquat for 4 hours or 100μM paraquat over 24 hours. I examined the expression of Hsp 70 (representative of a large Hsp) and Hsp 22 (representative of a small Hsp). Relative quantification was determined by normalizing the expression level of Hsps against tubulin, a gene whose expression is constant between control and paraquat treated cells. For each biological replicate, triplicates were run and the average and standard deviation computed from the technical replicates. Both paraquat treatments lead to a higher expression of Hsp 70 and Hsp 22 when compared to the control, cells that were left untreated (Fig 1). With 1 mM paraquat over 4 hours, both Hsp 70 and Hsp 22 showed a 6 fold higher expression level than in the control. With 100 μM paraquat over 24 hours, there is a smaller increase in expression, however expression level of both Hsps was at least 3 fold higher. This could be explained by the fact even though the 100 μM treatment is longer, the concentration of paraquat used is nonetheless lower, thus there would be less oxidative stress induced. The experiment was repeated and the result remain consistent. This shows that the oxidative stress caused by paraquat is sufficient to induce the expression of both large and small Hsps in 293T cells.
Figure 1. Induction of Hsp expression in response to Paraquat treatment. 293T cells were treated with 1 mM of Paraquat for 4 hours or 100 uM of Paraquat for 24 hours. Expression level of Hsp 70A (a) and Hsp 22 (b) in Paraquat treated and control Non Treated (NT) were determined by RT-qPCR. Expression level of Hsp were calculated using relative quantification and normalized to Tubulin (Tub2). Samples were run in triplicates and standard deviation calculated from technical replicates. The Figure is representative of 2 biological replicates.
**FOXOs induces the expression of large and small Hsps**

HSF1 is a known regulator of Hsps, it induces Hsps expression by binding to Heat Shock Elements (HSE) in their promoters. In both *C. elegans* and *Drosophila* FOXO has been shown to be capable of also inducing Hsps transcription. In *Drosophila* in particular, both small and large Hsps are induced (Marr and Donovan, unpublished results). Mammalian cells have four FOXO proteins, of which FOXO1, FOXO3 and FOXO4 are known to be involved in oxidative stress. I overexpressed FOXO1, FOXO3 and FOXO4 proteins in 293T cells to determine whether FOXO is capable of inducing Hsp expression in mammalian cells. If FOXO is capable of Hsps induction, then overexpression of FOXO should lead to increase in Hsp expression without paraquat treatment. I transfected 2 ug of plasmid DNA into a 6 mm dish of confluent 293T cells along with Polyethylenimine. The plasmid expresses constitutive active FOXOs. GFP was also transfected and used to determine transfection efficiency and as a control of no FOXO overexpression. Cells were harvested 24 hours post transfection and RT-qPCR was again used to determine the expression of Hsp 70 (isoforms 70A and 70 A/a B/b), Hsp 22 and Hsp 27. Western Blot analysis was used to confirm FOXO overexpression. The constitutively active FOXO1 and FOXO3 express a FLAG tag. Using Anti-FLAG antibody as well as Anti-Tubulin as a control, normalization to the GFP control shows both FOXO1 and FOXO3 are being exogenously expressed (Fig2a). FOXO1 expression results in a 4 fold higher FOXO1 expression when compared to the GFP control while FOXO4 overexpression leads to a 3 fold higher exogenous expression when compared to the control (Fig 2b). The data for FOXO4 was inconclusive. For all the Hsps examined, all three of FOXO proteins can increase caused an increase in expression when compared to the control. The two Hsp 70 isoforms were nearly identical in their changes in expression levels (Fig 3a and b), Hsp 70A refers to primers that only targets 70A while 70 A/a
A/b targets more than one Hsp 70 isoforms. Hsp 70 expression was increased 25 fold when FOXO1 is overexpressed, 15 fold when FOXO3 was overexpressed and 3 fold when FOXO4 is increased. Hsp 22 and 27 did not show as dramatic an increase expression as Hsp 70 but there was a minimal a two fold increase in expression in response to each of the FOXO overexpression. Similarly to Hsp 70, Hsp 22 expression was increased the greatest with FOXO1 overexpression at 5 fold increase, followed by 3 fold increase with FOXO3 overexpression and 2 fold increase with FOXO4 overexpression. In contrast, Hsp 27 showed the greatest increase in expression, 5 fold, when FOXO4 is overexpressed; its expression is increased by 3 fold when FOXO3 is overexpressed and 2 fold when FOXO1 was overexpressed. The experiment was repeated three times for biological replicates and showed consistent result to Fig 3. All three of the FOXO proteins are capable of inducing the expression of both large and small Hsps. However, each FOXO proteins causes a different level induction of each of the Hsps.

<table>
<thead>
<tr>
<th>Name</th>
<th>antibody</th>
<th>Signal</th>
<th>Area</th>
<th>Background.</th>
<th>FLAG/Tubulin signal</th>
<th>Normalized expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV-GFP</td>
<td>anti-FLAG</td>
<td>890000</td>
<td>1092</td>
<td>260</td>
<td>0.468421053</td>
<td>0.9999999999</td>
</tr>
<tr>
<td>CMV-FOXO1</td>
<td>anti-FLAG</td>
<td>2950000</td>
<td>1092</td>
<td>746</td>
<td>2.286821705</td>
<td>4.881978918</td>
</tr>
<tr>
<td>CMV-FOXO3a</td>
<td>anti-FLAG</td>
<td>2730000</td>
<td>1092</td>
<td>299</td>
<td>1.761290323</td>
<td>3.760057989</td>
</tr>
<tr>
<td>CMV-GFP</td>
<td>anti-tubulin</td>
<td>1900000</td>
<td>810</td>
<td>187</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV-FOXO1</td>
<td>anti-tubulin</td>
<td>1290000</td>
<td>810</td>
<td>611</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 2. Overexpression of FOXO1 and FOXO3 proteins. 293T were transfected with FOXO1 plasmid (lane 2), FOXO3 plasmid (lane 3) and GFP (lane 1) a. Western Blot analysis probed with Anti-FLAG (for FOXO expression) and Anti-tubulin. b. Quantification of FOXO protein expression in FOXO1 and FOXO3. The same size rectangle was drawn in all three lanes and the Anti-FLAG and Anti-Tubulin signal obtained. The FLAG/Tubulin signal indirectly measures protein expression and FOXO overexpression was normalized to GFP. The figure is representative of three biological replicates.
Figure 3. Induction of Hsp expression by FOXO overexpression. 293T cells were transfected with FOXO1, FOXO3, FOXO4 and GFP plasmid for overexpression. Expression level of Hsp 70A (a), Hsp 70A/a B/b (b), Hsp 22 (c) and Hsp 27 (d) in FOXO overexpressed and GFP control cells were determined by RT-qPCR. Expression level of Hspd were calculated using relative quantification and normalized to Tubulin (Tub2). Samples were run in triplicates and standard deviation calculated from technical replicates. The Figure is representative of 3 biological replicates.
FOXO’s role in induction of Hsp in response to oxidative stress

Since FOXOs are capable of inducing Hsp expression, and Hsp expression is increased when mammalian cells are exposed to oxidative stress, it would be of interest to determine whether FOXO is responsible for inducing Hsp expression in response to oxidative stress. To determine if FOXO is necessary, I knocked down FOXO1, FOXO3 or FOXO4 expression using CRISPR-cas9 in 293T cells to see if there is any change in Hsp expression in response to paraquat treatment. CRISPR-cas9 is a technique that uses a guide RNA and a nuclease (cas9) that makes double stranded breaks for genome editing. In this case, the guide RNA is complementary to the FOXO genes, and the formation of a double stranded break by Cas9 leads to nonhomologous end joining. This introduces random indels in the FOXO genes leading to knockout of specific FOXOs. The plasmid used for CRISPR-cas9 mediated knockdown also confers puromycin resistance. 24 hour post transfection, the 293T cells were put in media with 2 ug/uL of puromycin. This was done in an attempt to increase the frequency of knockdown by selecting for only the cells that have taken up the plasmid. The 293T cells were grown in puromycin media for four days. The cells were then either treated with 1 mM paraquat for 4 hours or left untreated as control. Preliminary data from RT-qPCR seems to show that there is a decrease in Hsp induction in response to paraquat treatment in FOXO knockdown cells. 1 mM paraquat treatment over 4 hours has consistently increase the expression level of Hsp 70 and Hsp 22 by around 6 fold (Fig1). In FOXO1, FOXO3 and FOXO4 knockdown cells, there does not appear to be any significant increase in Hsp expression when compared to the control (Fig 4). However the experiment has not yet been repeated. Western blot analysis for FOXO expression proved inconclusive. Additional experiments will be needed before a conclusion can be made.
Figure 3. Induction of Hsp expression in response to Paraquat with FOXO knockdown. FOXO1, FOXO3 or FOXO4 was knocked down in 293T cells using CRISPR-cas9. Expression level of Hsp 70A (a) and Hsp 22 (b) with FOXO knockdown and either 1 mM of Paraquat for 4 hours or Not Treated (NT) were determined by RT-qPCR. Expression level of Hsp were calculated using relative quantification and normalized to Tubulin (Tub2). Each sample were run in triplicates and the standard deviation calculated from technical replicates.
**Discussion**

The induction of both large and small Hsps by Paraquat provides a experimental method of examining Hsp response to oxidative stress in mammalian cell line in culture. Both of the Hsps examined showed the same level of increase in expression when treated with paraquat, and this increase in expression is dependent on paraquat concentration. From examination of cells under the microscope, the paraquat does not appear to cause cell death, thus the treatment is not toxic enough to induce apoptosis. While Hsp’s role in response to Heat Shock is well studied, its role in oxidative stress is less well known. This partially due to the fact heat shock can easily be induced through heating the cells. Paraquat treatment thus provides a way to easily introduce oxidative stress that can induce Hsp expression and allow for further experimentation on Hsps functions. For future experiments, it would be interesting to see if paraquat is capable of inducing all the Hsps and whether the level of induction is consistent or Hsp specific.

FOXO1, FOXO3 and FOXO4 overexpression all leads to an increase in expression of both large and small Hsps in 293T cells. This confirms that FOXO is capable of inducing of Hsps and this capability is conserved from *C. elegans* to mammals. Mammalian FOXO appears to be more similar to dFOXO in that they are capable of inducing both small and large Hsps. This is of interest as HSF has traditionally been viewed as the only transcription factor responsible for Hsps activation. FOXOs thus represent another way for cells to modulate Hsp expression. The different FOXO proteins induce expression to a different levels in different Hsps. The overall level of induction appears higher in Hsp 70 isoforms than in small Hsps, Hsp 70A expression can be as high as 25 fold higher than the control. This is in contrast with the
paraquat treatment, where Hsp 70A and Hsp 22 showed the same increase in expression. It is also interesting to note that each FOXO induces Hsp expression differently, and the overexpression of none of FOXOs consistently leads to the highest induction in every Hsp. While FOXO1 overexpression leads to the greatest increase in expression for Hsp 70 and Hsp 22, FOXO4 overexpression leads to the greatest increase in expression for Hsp 27. This suggests that perhaps each FOXO preferentially induce a particular Hsps, eg FOXO1 is the FOXO primarily responsible for Hsp70 induction. Since mammals have multiple FOXOs, they might have a unique division of labor among the FOXOs that’s not possible in other organisms. Also since all FOXOs lead to at least a 2 fold increase in expression of all the Hsp, it would be of interest to see if there is a loss of one of the FOXOs, can the remaining FOXOs compensate for its induction of Hsps. Future experiments would look at how FOXO overexpression affects the expression of other Hsps. This would help determine if there is redundancy in FOXO induction of Hsps and whether each Hsps respond to FOXO induction differently.

Further experiments are necessary to conclude whether FOXO is required for Hsp expression in response to oxidative stress. The knockdown of FOXOs needs to be confirmed by Western Blot analysis to ensure that any changes seen in RT-qPCR data is due to loss of FOXO expression. 293T cells without any knockdown treated with paraquat should be used a positive control to ensure that paraquat treatment is working. If future experiment show that FOXO is not necessary for the induction of Hsp in response to oxidative stress, it would be of interest to see in mammalian cells when does FOXO normally induce Hsp expression.

Material and Methods

Cell Culture
HEK 293T cells are a mammalian cell line originally derived from human embryo kidney cells. 293T cells were cultured in 60mm or 100 mm plates with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (vol/vol), 1% Penicillin/Streptomycin and 1% Amino Acids. The cells were incubated at 37 °C with 5% CO\textsubscript{2}. The cells were split 1:20 every three to four days to avoid overgrowth.

Paraquat Treatment

A confluent 100 mm dish of 293T cells were split 1:6 into a 6 well plate the day before paraquat treatment. One well served as the control and was not treated with any paraquat. The other wells were treated with 1mM of paraquat for 4 hours or 100uM of paraquat for 24 hours. The cells were incubated at 37 °C with 5% CO\textsubscript{2} for the duration of the treatment. The cells were harvested after 4 or 24 hours using a cell scraper and washed with PBS.

Plasmid Purification

1 uL of plasmid (FOXO1, FOXO3, and FOXO4) were transformed into Z-competent E.Coli cells. The mixture was then plated on warm Carb plates overnight. A single colony was selected from the CARB plates and cultured in 10 mL 2YT media overnight at 37 °C in the shaker. The plasmids were then purified using PEG plasmid prep. E. Coli cells were pelleted at 4000 rpm for 10 min and the pellet resuspended in buffer P1 (50 mM Tris 8.0, 10 mM EDTA and .1 mg/ml RNAse A). Buffer P2 (.2 N NaOH and 1% SDS) was added for lysis for 5 minutes followed by buffer N3 (4.2 M guanidine hydrochloride, .9 M potassium acetate pH 4.8) on ice for 15 minutes. The mixture was centrifuged at > 20,000 xG for 30 min at 4 °C with the resulting supernatant containing the plasmid DNA. The plasmid DNA was precipitated using isopropanol. The plasmid DNA was treated with Proteinase K (.2 mg/ml) for 30 min at 37 °C and then extracted using Phenol: Chloroform: Isoamyl Alcohol (PCI). 5M LiCl and 50 mM EDTA was used to
precipitate and remove RNA. The resulting DNA was resuspended in TE and precipitate using PEG precipitation solution at 4°C overnight. The DNA was extracted again using PCI and precipitated using 100% ethanol. The resulting plasmid DNA was resuspended in PE.

Transfection

A confluent 100 mm dish was split 1:20 into a 60 mm dish the day before transfection. On the day of transfection, the media on the cells was changed and 2 mL of complete DMEM media was added per dish or per plate. 2 ug of plasmid DNA (FOXO overexpression plasmid or CRISPR-cas9 plasmids and a GFP plasmid as control) was diluted into 200 uL of serum-free DMEM. Polyethylenimine was added at a 3:1 ratio to the DNA. The mixture was vortexed for 10 seconds and incubated for 15 minutes at room temperature. The mixture was then added to the cells. 24-hours post transfection, the plate transfected with GFP was visualized under microscope to confirm transfection efficiency (usually > 90%). For the overexpression experiment, the plasmid carries FLAG-tagged consitutively active FOXOs. The cells were harvested after 24 hours.

For the CRISPR-cas9 experiment, the plasmid carries the guide RNA (sequence complementary to the targeted FOXO), gene coding for the nuclease Cas9, a PAM sequence that’s required for Cas9 cutting and confers puromycin resistance. The guide sequence would target the Cas9 to make double strand DNA break in the FOXO gene, where repair via Non-homolougs end joining would introduces random indels. In most of the cases, indels leads to knockout of the gen. 24 hours post transfection, each 60 mm dish was split 1:6 into a 6 well plate with total volume of 3 mL. After the cells adhere, puromycin was added was added at a concentration of 2 ug/mL. Puromycin selects for cells that have at least uptaken the CRISPR-Cas9 plasmid. CRISPR-Cas9 never has 100% efficiency, and this selection increases the likelihood that the cells used for the
Paraquat treatment has FOXO knockdown. The cells were allowed to grow in puromycin for three days. On the third day, media was changed to remove cells that cannot survive in puromycin. The next day, paraquat treatment was performed and cells harvested after 24 hours. A well of cells transfected with the plasmid but not treated with paraquat serves as a negative control.

**RNA isolation and cDNA synthesis**

RNA was isolated using Tri-reagent following standard RNA extraction protocol. Harvested cells were mixed with Tri-reagent, homogenized and stored at -80°C overnight to improve lysis. Chlorophenol was then added and mixture span for 15 min at max speed, resulting in two separate layers. RNA was then precipitated from the clear upper phase using isopropanol by centrifuging at max speed for 30 min at 4°C. The precipitated RNA was washed with 70% ethanol, resuspended in pH 7.5 TE buffer and stored at -20°C. Before cDNA synthesis, RNA sample was treated with DNAse I enzyme for at least 30 minutes at 37°C and then neutralize by 50 mM EDTA at 70°C for 10 min to eliminate presence of DNA. 1 ug of RNA was used for each cDNA synthesis reaction. RNA sample was incubated with 50 uM anchored oligodT at 70°C for 10 min. 10 mM dNTP mix, 10X RT buffer (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂), 10 mM DTT and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) were added followed by PCR to synthesis cDNA. The resulting cDNA was was diluted 1:5 with TE pH 8.0 buffer and stored at 4°C.

**qPCR**

qPCR was conducted in white 96 well plates using synthesized cDNA. qPCR mix consisted of 4 uL cDNA, and 16 uL qPCR mix (1X ThermoPol buffer, 200 uM dNTP mix, 1 uM primer mix, .5X SYBR Green, 1.075 M ethylene glycol and 1U/20 uL Taq polymerase). Samples were
running in duplicates or triplicates. Relative quantification was calculated using Tubulin 2 as a gene whose expression does not change due to experimental conditions. Ct values (threshold cycle value) from qPCR was linearized using the formula \(1/2^{Ct}\). The resulting value for each Heat Shock Protein was divided by the linearized value for Tubulin 2 run in the same qPCR reaction for normalization. The average and standard deviation were calculated from technical replicates. The experiments were repeated three times resulting in biological replicates.

*Western Blot*

Whole cell protein extracts were obtained from the harvested cells through the addition of RIPA buffer (1X PBS, 1% Igepal-630, 1% SDS, 10% glycerol and .5% DOC). 1X protease inhibitor and 1U/uL benzonase were added fresh to RIPA buffer right before use. The proteins were resolved using a SDS- polyacrylamide gel electrophoresis with 8% resolving gel and 5% stacking gel. APS and TEMED were used to induce gel formation and improve polymerization. The proteins extracts were resuspended in 5X SDS gel loading dye and boiled at 95°C for 5 minutes. 15 uL of samples were loaded into each well along with 3 uL of ladder. The running buffer was 1X Tris-Glycine Buffer (25 mM Tris, 250 mM glycine and .1% SDS). The gel was run at room temperature at 120 V until dye runs off gel (approximately 90 minutes). The proteins were then electrotransferred to nitrocellulose membrane in cold Transfer Buffer (20 mM Tris, 250 mM glycine and 10% methanol). Transfer was run at 100V for 1 hour at 4 °C. The resulting membrane was blocked with 10% (w/v) non fat dry milk in 1X PBS for 1 hour at room temperature on shaker. The membrane was incubated with primary antibody in 1% milk in PBST (1X PBS with .1% Tween-20) over night on shaker at 4 °C. The membrane was then washed 3X in PBST and blocked with secondary antibody for a hour at room temperature followed three more washes in PBST. For the overexpression experiment, primary antibody used were: anti-
FLAG (1:5000) for FOXO1 and FOXO3, anti-Tubulin (1:1000) for Tubulin and anti-FOXO4 (1:1000) for FOXO4. For knockdown experiment, anti-FOXO1 (1:1000), anti FOXO3 (1:1000), and anti-tubulin (1:1000) was used. The secondary antibodies were Goat anti-mouse (1:10,000) for FLAG and tubulin, and Goat anti-rabbit (1:10,000) for FOXO1, FOXO3, and FOXO4. The membrane was visualized using a Licor Odyssey Scanner. Quantification of expression was calculated by taking the anti-FLAG (or FOXO1/3) signal, divided by the Anti-Tubulin signal (determined by drawing the same size rectangle in all the lanes). The expression was then normalized to the GFP control to determine if there was overexpression or knockdown.

Acknowledgment

I want to give special thanks to Dr Michael Marr for allowing me to work in his lab and advising me on all aspect of my research; I am extremely grateful for this opportunity and I have learned so much from my time at his lab. I want to give special thanks to Marissa Donovan for providing me with day-to-day guidance on every single step of this project and without whom I would not be able to complete this project. I also want to thank rest of Marr lab: Nicholas Clark, Drew Sawyer and Michael Spellberg for being so willing to assist me with any questions I had. Thank you all very much.
References
Freeman, B. C., & Morimoto, R. I. (1996). The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. The EMBO journal, 15(12), 2969.


