HSF1 is not activated by MEK in OSCA-40

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http://hdl.handle.net/10456/42775

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HSF1 is not activated by MEK in OSCA-40

By
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Biochemistry Program

A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

William Christopher Tippins
Pledge

Dr. Ann Kleinschmidt
Dr. Shaun Murphree
April 3rd, 2017
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Abstract

Osteosarcoma (OSA) is a form of highly aggressive bone cancer associated with a poor prognosis that occurs in high rates of both humans and canines. Heat shock proteins (HSPs) are thought to assist in stabilizing certain forms of cancer, allowing for resistance to abnormal cellular environments and chemotherapeutics. Heat shock factor 1 (HSF1) is the master regulator of HSPs and is a downstream target of the mitogen-activated protein kinase kinase (MEK) pathway and, inhibition of HSF1 has shown to decrease tumor size and formation. In this study, basal levels of HSF1 mRNA were examined through RT-PCR analysis in three oncological canine cell lines (OSCA 32, OSCA 40, and Diesel) with three endogenous controls (HPRT1, RSP18, and ATP5B) to determine if non-metastatic and metastatic tumor cells similar or different HSF1 mRNA levels. Treatments of a MEK inhibitor (AZD6244) and quercetin were conducted to examine cellular proliferation as well as relative mRNA levels of HSF1 and HSP70. It was found that while AZD6244 had no significant effect, quercetin caused a significant decrease in proliferation. Upon treatment with both the MEK inhibitor and quercetin HSF1 and HSP70 mRNA levels showed no significant change. These results indicate that MEK does not play a role in proliferation nor HSF1 activation in OSCA 40. Furthermore, the results indicate that HSF1 is not activated by MEK in OSCA 40.

Introduction

Osteosarcoma (OSA) is a form of bone cancer that originates through the transformation of osteoblasts to form tumor cells. OSA is known to be very aggressive. It usually originates in local bone sites, and often goes unnoticed until metastasis happens and the cancer migrates throughout the body, eventually spreading to the lungs. OSA typically occurs during periods of bone growth and during adolescence. OSA is known for its low prognosis. Once metastasis occurs, five-year survival levels fall from 80% to 30% (Mirabello et al. 2009). Fortunately, OSA is rare in humans, and accounts for less than 1% of all cancers diagnosed in the United States. Unfortunately, OSA occurs at much higher rates in canines (OSCA), specifically larger dog breeds. In order to combat low survival rates, efforts are being made to advance early detection as well as treatment options once metastasis occurs. A way to accomplish these goals is to look at heat shock proteins (HSPs) as both therapeutic targets and early cancer indicators.
HSPs are known as molecular chaperones. In normal, noncancerous cells, HSPs are a part of the cytoprotective mechanism known as the heat shock response. HSPs bind to exposed hydrophobic parts of denatured proteins and assists in stabilizing as well as refolding. Some HSPs even completely encase proteins to further assist the refolding process (Vabulas et al., 2010). The heat shock response occurs when a cell undergoes a higher-level temperature increase to where proteins begin to denature and misfold. In mammals, this temperature is usually around 37 °C to 40 °C (Issels 2008). While called the heat shock response, this cytoprotective mechanism can be activated by a number of other cell stress factors including, but are not limited to metallic stressors, hypoxia, changes in intracellular pH, and oxidative damage (Jolly and Morimoto, 2000). It is not uncommon to find all of the above cell stressors, especially oxidative damage, in cancer cells along with high levels of HSPs.

Elevated levels of HSPs are thought to be critical to the stability and survival of cancer cells. In knockdowns of HSP70 and HSP27 proteins in cancer cells, spontaneous apoptosis is observed. Inhibition of HSP60 via silencing RNA (siRNA) has shown to decrease amounts of HSE expression as well as activation of the apoptotic pathway in canine OSCA (Calderwood et al., 2006; Ciocca and Calderwood, 2005; Selvarajah et al., 2013; Romanucci et al., 2011). The interesting thing about the heat shock proteins is that under normal or non-fever temperatures, HSPs are not needed to maintain cellular viability. In fact, it has been observed that in heat shock factor 1 (HSF1) knockout mice, HSF1 being the main transcription factor for HSPs, the mice did not only remain viable, but showed decreased tumor volume as well as higher survival rates when exposed to hot spot mutations (Dai et al., 2007). These findings broaden treatment options to include the method of creating an environment wherein mutated cells can not survive but normal cells remain unaffected.
Fig 1. Mechanism of HSF1 activation and nuclear transport. Upon the stress response HSF1 breaks from its inactive state, bound to HSPs, and is phosphorylated by MEK. When phosphorylated, HSF1 becomes active and trimerizes before being imported into the nucleus. P300 binds to assist in the stability of HSF1. Once in the nucleus, HSF1 binds to HSEs to produce HSF1 and HSP mRNA (Pan et al., 2016).

As a way to decrease HSP levels in cancer cells, research has suggested looking at heat shock factors, specifically HSF1. Heat shock factor 1 is the main transcription factor in mammalian cells and is critical to the continuation of a heat shock response. While there are over 20 known, different HSFs discovered, only four of those are used in mammals. These four HSFs are HSF1-HSF4; HSF2 is used in development, HSF3 is only expressed in avian cells, HSF4 is found exclusively in the nucleus and negatively interacts with HSF2. HSF1 is approximately 529 amino acids longs, consisting of five main molecular domains, a DNA-binding domain, leucine zipper 1-3 (LZ1-3), a regulatory domain, leucine zipper 4, and the C-terminal transactivation domain. At cellular conditions without any stressors, HSF1 is present as an inactive monomer bound to HSP70 and HSP90 in the cytosol. HSP90 and HSP70 act as autoregulators for HSF1 repression (Naidu and Dinkova-Kostova, 2017).
Upon activation of the heat response, HSF1 breaks from bound HSPs, and is phosphorylated on the serine 326 site by MEK (Mitogen-activated protein kinase) (Fig. 1). There are over 15 known phosphorylation sites on HSF1, most of which are on the regulation domain and are inhibitory. Serine 326 phosphorylation allows for several additional post-translational modifications to occur as well as for a trimer to be formed with two other newly phosphorylated HSF1 molecules at the LZ1-3 domain (Fig. 2; Naidu and Dinkova-Kostova, 2017; Dai and Sampson, 2016). The trimerization allows for increased nuclear transport and increased binding affinity to the HSE.

Once trimeric, Death-associated protein 6 (DAXX) binds to HSF1 to assist in transcription and HSF1 is imported into the nucleus. HSF1 recognizes and binds to nGAAAn sequences in the major groove of heat shock elements (HSEs) within DNA (Boellmann et al., 2004; Vujanac et al., 2015). When bound to the appropriate HSEs, transcription of HSP mRNA and HSF1 mRNA is conducted by RNA polymerase II. The newly formed mRNAs are transferred out of the nucleus to the ribosomes and synthesized into proteins. In most cases, the proteins carry on to refold denatured proteins and downregulate HSF1.
Fig 2. RAS/MEK signaling cascade phosphorylates HSF1. RAS is activated through a growth factor receptor which swaps GDP for GTP. Once activated RAS activates the RAF kinase which phosphorylates and activates MEK. MEK then activates ERK and phosphorylates the Serine 326 (S326) site in the regulatory domain of HSF1, activating HSF1. Through a negative feedback loop, ERK inhibits MEK by means of Threonine 292 (T292) and Threonine 386 (T386) phosphorylation. HSF1 goes on to travel into the nucleus and bind to HSEs. AMPK is also implicated in Serine 121 (S121) inhibitory phosphorylation on the DNA-binding domain of HSF1 (Dai and Sampson, 2016).

As the main regulator of HSPs in mammals, HSF1 has become a target for oncologic chemotherapeutics to decrease HSP levels. Inhibition of proteins that produce activating phosphorylation events for HSF1 or increasing inhibitory phosphorylation events of HSF1 are the most common routes used. Cantharidin treatment in colorectal cancer cells was observed to block HSF1 from binding to the HSP70 promoter, preventing HSP70 expression and inducing
apoptosis (Kim et al., 2013). In breast adenocarcinoma and hepatocellular carcinoma cells, quercetin blocked HSP70 expression through inhibition of HSF1, increased tumor destruction/endpoint survival was observed in vivo (Yang et al., 2016). In breast cancer with mutations in the human epidermal growth factor receptor-2 (HER2), HSF1 knockdown p53 negative nude mice when compared to p53 negative mice were shown to be unable to have tumor growth from xenografts (Meng et al., 2010). In p53 negative and HSF1 negative mice, lymphoma development saw a large decline and increase to tumor free survival when compared to just p53 negative mice (Min et al., 2007). MEK inhibition has been shown to decreased chemotherapeutic resistance in patients who have melanoma with a V600E BRAF mutation, through the limitation of the cytoprotective responses of oncological cells (Flaherty et al., 2012).

Fig 3. Chemical structure of Selumetinib (AZD6244) and Quercetin.

This study attempts to inhibit HSF1 activation phosphorylation in two ways, though treatment with a MEK1 inhibitor AZD6244 and with a known HSF1 inhibitor quercetin (Fig. 3). AZD6244 is a highly selective MEK1 inhibitor that is currently undergoing clinical trials for cancer treatment. MEK1 is one of the kinases that phosphorylates the Serine 326 site on HSF1 for activation, inhibition of MEK1 has been found to diminish active HSF1 levels in human
kidney cells. Without the phosphorylation event, HSF1 activation decreases and HSP levels follow, allowing for destabilization of oncological cells (Tang et al., 2015).

Quercetin is a flavonol found in many fruits and vegetables, it has been found to negatively regulate active HSF1 levels through reduction of phosphorylated HSF1 (Nagai et al., 1995). Quercetin has also been found to play a role in MEK1 and MEK2 activity, leading to apoptosis in lung cancer cells (Nguyen et al., 2004). More so, quercetin has been found to inhibit MEK1 by formation of a hydride bond with the backbone of amide of Serine 212 within MEK1, stabilizing the inactive conformation that prevents activation of MEK1 (Lee et al, 2008).

I hypothesize that quercetin inhibits HSF1 via the MEK pathway, specifically preventing Ser326 phosphorylation of HSF1 by inhibiting MEK. The goal of this study is to determine if HSF1 is required for OSCA 40 proliferation and to gain evidence for the argument that quercetin functions though the MEK pathway to inhibit HSF1 by using MEK1 and HSF1 inhibitors.

Methods

Cell lines and Cell Culturing

Cell pellets were obtained from Dr. Kleinschmidt. OSCA 40 was cultured in high glucose medium (Gibco ® Dulbecco’s Modified Eagle Medium (DMEM 1X), [+] 4.5 g/L D-Glucose [+] L-Glutamine [-] Sodium Pyruvate, with 25 mM FBS, HEPES and primocin). Cell incubation was done at 37°C at 5% CO₂.

Chemical Treatments

Selumetinib (AZD6244) (MedChem Express) and quercetin (Cayman Chemical Company) stocks were dissolved in DMSO at and stored at -20°C. Proliferation treatments were conducted over 72 hours at concentrations at various concentrations. Concentrations were made
through serial dilution of a 10 mM stock. qPCR treatments were conducted over 18 hours at 0µM and 10µM.

**Cell Proliferation**

Cell proliferation was conducted with CellTiter 96 AQUeous One Solution Cell Proliferation Assay. The reagent used was MTS Tetrazolium with PES electron coupling reagent. Wells were seeded with a density of 5.0*10³ cells in high glucose medium (Gibco Dulbecco’s Modified Eagle Medium (DMEM 1X), [+] 4.5 g/L D-Glucose [+] L-Glutamine [-] Sodium Pyruvate, with FBS, HEPES and primocin) for 2 hours then treated with selumetinib (0.5µM, 1.0µM, 2.0µM, and 10µM) and quercetin (10µM, 25µM, 50µM and 75µM) for 72 hours. For treatments, 100 µL of medium with twice the final concentration of chemical was added to the 100 µL of medium in the well. Prior to reading, 20µL of proliferation reagent was added, rocked for 5 minutes and incubated for 2 hours. The absorbance was measured using Thermo Electron Corporation Original Multiskan Ex Plate Reader at 492nm. Quercetin treatments were conducted by KayLynn Carver. (Appendix A)

**RNA Isolation and cDNA Synthesis**

Diesel, OSCA 32, and OSCA 40 RNA was previously isolated and obtained from Dr. Kleinschmidt. RNA isolations for OSCA 40 chemical treatments were done using RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Once isolated RNA was stored at -80°C prior to use. cDNA synthesis was performed with 200 ng of RNA in a final volume of 10µL and incubated with 3µL of Invitrogen SuperScript VILO master mix (2µL 5X VILO™ Reaction Mix [SuperScript™ III RT, RNaseOUT™ Recombinant Ribonuclease Inhibitor, and a proprietary helper protein] and 1µL 10X SuperScript® Enzyme
Mix [random primers, MgCl₂, and dNTPs]). Initial denaturation was done at 25°C for 10 minutes, held at 45°C for 1 hour, then 85°C for 5 minutes, and finally cooling down to 4°C. (Appendix B)

**Real-Time PCR**

qPCR was designed using StepOne Software. SYBR ® Green Reagent, Primers, and RNA were used in the 48 well Applied Biosystems StepOne ™ Real-Time PCR System. PCR cycle was held at 95°C for 10 minutes, cycled 40 times at 95°C for 15 seconds to 60°C for 1 minute. For the melt curve the sample was brought to 95°C for 15 seconds to 60°C 1 minute to 95°C for 15 seconds. (Appendix C)

**Table 1. Primers used for RT-PCR.** Primers were designed using BLAST software and were ordered from Eurofins Genomics.

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1 Forward</td>
<td>GATGAAGGAAATGGGAGGCC</td>
</tr>
<tr>
<td>HPRT1 Reverse</td>
<td>GTCCCCCTGGTGGACTGGTCAT</td>
</tr>
<tr>
<td>HSF1 Forward</td>
<td>TACAGCAGCTCCAGCTCTCTAC</td>
</tr>
<tr>
<td>HSF1 Reverse</td>
<td>GCTCCTCCTTGACGCGTACCT</td>
</tr>
<tr>
<td>RPS18 Forward</td>
<td>CGTGAAGACCTGGAGAGACTG</td>
</tr>
<tr>
<td>RPS18 Reverse</td>
<td>CTTCTTGGACACACCCACCGG</td>
</tr>
<tr>
<td>ATP5B Forward</td>
<td>GGTTGTGGATCTGTGGTC</td>
</tr>
<tr>
<td>ATP5B Reverse</td>
<td>CACCAACACCCAGCACCAGAG</td>
</tr>
<tr>
<td>HSP70 Forward</td>
<td>ACGTGGCCCTCACCAGACACC</td>
</tr>
<tr>
<td>HSP70 Reverse</td>
<td>GTAGCCTACCTGCACCTTGG</td>
</tr>
</tbody>
</table>

**Data Analysis**

RT-PCR fold difference was calculated with \(2^{-\Delta C_t}\) with \(\Delta C_t\) being the the \(C_t\) of the gene of interest minus the \(C_t\) of the control. Error bars were calculated from standard deviation. Statistical analysis for significance was conducted via random block Anova, \(P>0.05\) were considered significant.

**Results**
**HSF1 mRNA Levels Between Cell lines**

To determine whether there are differing levels of HSF1 mRNA in a non-cancerous tumor cell line, or two lines with different metastatic potential (OSCA 32 low, OSCA 40, high), a reverse transcription, quantitative PCR analysis was conducted. The endogenous controls were also compared to give the lowest change and highest amounts of mRNA levels between cell lines. The lowest change between cell lines would produce the best control, Table 2. RSP18 gave the highest amounts of relative mRNA. Diesel and OSCA 40 produced similar levels of HSF1 mRNA for RPS18 and ATP5B (Fig. 4).

**Table 2. C<sub>t</sub> Values for the endogenous controls between cell lines.**

<table>
<thead>
<tr>
<th></th>
<th>Diesel</th>
<th>OSCA 32</th>
<th>OSCA 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1</td>
<td>23.2 ± 1.2</td>
<td>20.6 ± 0.5</td>
<td>20.1 ± 0.4</td>
</tr>
<tr>
<td>RPS18</td>
<td>21.9 ± 1.4</td>
<td>20.5 ± 0.8</td>
<td>21.7 ± 0.4</td>
</tr>
<tr>
<td>AT5B</td>
<td>20.0 ± 0.2</td>
<td>18.0 ± 0.4</td>
<td>18.2 ± 0.5</td>
</tr>
</tbody>
</table>

**Proliferation of OSCA 40 cells is decreased upon treatment with quercetin but not AZD6244**

AZD6244 is a known MEK inhibitor and quercetin is a known HSF1 inhibitor. Canine OSCA 40 cells were treated with increasing concentrations of AZD6244 for 72 hours prior to measuring absorbance for proliferation. Proliferation decreased moderately as concentration increased, but the decrease was not significant (Fig. 5). Canine OSCA 40 cells were treated with increasing concentrations of quercetin for 72 hours prior to measuring absorbance for proliferation. Proliferation decreased as concentration increased, 75uM was found to have a significant decrease (Fig. 6).
Treatment with Quercetin or AZD6244 Does Not Decrease HSF1 or HSP70 mRNA levels

Canine OSCA 40 was treated with quercetin (75 uM) and AZD6244 (10 uM) for 21 hours prior to RNA isolation and PCR analysis. Treatments with quercetin produced a slight, but not significant decrease HSF1 mRNA levels. When compared to HSF1, HSP70 mRNA showed higher expression across all treatments (Fig. 7).

![Relative HSF1 mRNA Levels Between Cell Lines](image)

**Fig. 4. Relative HSF1 mRNA Levels Between Cell Lines.** Relative HSF1 mRNA levels of OSCA 32, OSCA 40, and Diesel were obtained through RT-PCR. Controls were HPRT1, RPS18, and ATP5B; averaged standard deviation between cell lines were 1.504, 1.076, and 0.9111 respectively. HPRT1 and RPS18 were done in quadruplicate, ATP5B was done in triplicate.
Fig. 5. AZD6244 Treatment Has No Effect on Proliferation. OSCA 40 was treated with AZD6244 for 72 hours. Proliferation was measured at 492 nm with the CellTiter 96 AQ ueous One Solution Cell Proliferation Assay. Random Block Anova was used to determine significance P<0.05.

Fig. 6. Quercetin Treatment Decreases Proliferation. OSCA 40 was treated with quercetin for 72 hours. Proliferation was measured at 492 nm with the CellTiter 96 AQ ueous One Solution Cell Proliferation Assay. Random block Anova was used to determine significance P<0.05, 75uM was found to be significant. Data was collected by KayLynn Carver.
Fig. 7. Treatment with Quercetin and AZD6244 Does Not Decrease HSF1 or HSP70 mRNA levels. Cells were treated at the concentrations above for 21 hours, relative mRNA levels of HSF1 and HSP70 were measured using RT-PCR. Endogenous control was RPS18. Random black Anova was used to determine significance $P<0.05$, no significance detected.

Discussion

The heat shock proteins have the ability to stabilize denatured proteins and assist in refolding, however this stabilization is thought to allow for cancer survival as well (Calderwood et al., 2006). HSF1 is the main transcription factor involved in regulating heat shock proteins and can be activated by MEK1 via Serine 326 phosphorylation. However, in this study, I found that using the selective MEK1 inhibitor (AZD6244) does not decrease HSF1 mRNA levels, more so in OSCA 40 cells 10 uM treatment of AZD6244 does not produce a significant decrease in cell proliferation. While quercetin treatments do produce a significant decrease to proliferation, no significant decrease to either HSF1 mRNA levels nor HSP70 mRNA levels in OSCA 40 cells was observed. Between cell lines HSF1 mRNA expression also varied (Fig. 4). These results indicate that HSF1 is not activated by MEK in OSCA 40.

HSF1 is thought to be the main transcription factor of the heat shock response. One way HSF1 can be activated is by phosphorylation of Serine 326 by MEK1 from the MEK pathway.
Inhibition of MEK1 using AZD6244 has been found to decrease HSF1 protein and mRNA levels in human embryonic kidney via prevention of HSF1 Serine 326 phosphorylation (HEK293T) (Tang et al., 2015). In melanoma, mutations of MEK1 and MEK2 have been found to constitutively activate both MEK1 and MEK2, which may lead to increased HSP levels (Nikolaev et al, 2011). Treatment of AZD6244 at 10 uM has also decreased proliferation in melanoma (Villanueva et al., 2010). However, no known mutations have been found to the MEK pathway in OSA, but inhibition of MEK using PD98059 has been found to reduce migration activity of OSA cells (Huang et al., 2009). Prior to this study no published research has been conducting in measuring the proliferation impact of MEK1 inhibition in OSCA or OSA in general. I found that there is no significant impact from AZD6244 treatment on proliferation in OSCA 40 (Fig. 5). This indicates that in OSCA 40 the MEK1 inhibition does not affect proliferation. Further, since there are no known mutations of the MEK pathway in OSCA nor OSA, MEK1 inhibition may have no effect on OSA in non-heat shock conditions.

While phosphorylation of Serine 326 in HSF1 by MEK1 has been found to be an activator of HSF1, there are also 15 known inactivating phosphorylation sites on the regulatory domain of HSF1 which are active during normal cellular conditions (Naidu and Dinkova-Kostova, 2017). The lack of change in HSF1 and HSP70 mRNA indicates that MEK may not activate HSF1 in OSCA-40 in non-heat shock conditions (Fig. 7). More so, without a mutation to the MEK pathway, HSF1 may already be inhibited by normal cellular inhibitors bound to HSF1’s regulatory domain.

An alternative to MEK1 phosphorylation, the p38 mitogen-activated protein kinase (MAPK) can also phosphorylate the Serine 326 site of HSF1 (Naidu et al., 2016). Quercetin treatments have been linked to inhibition of the p38 MAPK (Li et al., 2016). There was no significant
decrease in HSF1 nor HSP70 mRNA levels upon 75 uM quercetin treatment for 21 hours observed. Another explanation is that Quercetin may be working to inhibit MAPK phosphorylation of the Serine 326 site in HSF1 (Fig. 7).

HSF1 inhibition is not the only target of quercetin, AMP-activated protein kinase (AMPK) has been found to upregulated by quercetin via the downregulation of Cyclooxygenase-2 (COX-2). The down regulation of COX-2 is known to decrease proliferation and cause apoptosis in breast as well as colon cancer cells (Lee et al., 2008). Quercetin was found to significantly decrease proliferation upon 72-hour treatment at 75 uM (Fig. 6). Activation of AMPK has also been linked to inhibitory phosphorylation of the Serine 121 site of HSF1 (Dai et al., 2015). The AMPK activation may be a contributing cause to the lack of HSF1 mRNA expression observed.

HSP70, while being a heat shock protein, is not exclusively used in cellular survival. HSP70 is also used in everyday protein folding and thus does not need HSF1 to be expressed. The CCAAT-Box-Binding transcription factor is another transcription factor that binds to the HSP70 promoter to cause HSP70 mRNA expression (Morgan et al., 1987). The nuclear phosphoprotein, p53 has been found to interact with the CCAAT-Box promoter to decrease hsp70 transcription (Agoff et al., 1993). The p53 tumor suppressor gene is also known to be mutated and inactive in OSCA (Johnson et al., 1998). Thus, this p53 mutation may be causing HSP70 to remain highly expressed while HSF1 mRNA levels remain low as observed (Fig. 7).

Based off the findings and explanations above, I conclude that HSF1 is most likely not activated by MEK in OSCA 40 and that the expression seen by HSP70 is mediated through CCAAT-Box activation due to a p53 mutation in OSCA 40. The lack of MEK activation of HSF1 may be extended to OSCA or even OSA in general. Further investigations could be
conducted to determine MEK1 expression levels and to look at actual protein levels to confirm the assumption that mRNA levels are proportional to protein levels. An analysis of HSF1 phosphorylation in OSCA 40 would also prove useful to determine which sites on HSF1 are in play. Exposure to heat shock could also useful to assist in determining if the cells are already in a cytoprotective response.
Appendix

**A. Cell Proliferation**

Cell proliferation assays is a way of detecting the number of living cells present in a sample. This is done measuring the amount of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to Formazan by the cell through absorbance at 492 nm. MTS is converted to Formazan through the protonation of a nitrogen to break the center five-member ring. The amount of formazan is proportional to the number of viable cells. The phenazine ethosulfate (PES) enhances chemical stability which allows it to be combined with MTS to form a stable solution.

**B. cDNA Synthesis**

cDNA synthesis serves as a way to synthesize first strand complementary DNA from RNA for use in qPCR. This is accomplished by adding a Master Mix that consists of an Enzyme Mix and Reaction Mix. Enzyme Mix consists of SuperScript™ III, RNaseOUT™, recombinant ribonuclease inhibitor, and a proprietary helper protein. The Reaction Mix includes random primers, MgCl₂, and dNTPS in a buffer. The random buffers are oligonucleotides with random base sequences, the goal of these are to anneal to any RNA species in the sample. After adding the Master Mix, the reaction goes through the RT-Synth program on the thermal cycler (See methods above for details).

**C. Real-Time PCR**

Real time PCR (qPCR) is a way to measure targeted DNA amounts in DNA samples. This is accomplished through a polymerase chain reaction (PCR) in which specific sequences of
complementary DNA (cDNA) that are combined with target primers are amplified in the presence of the intercalating chemical, SYBR green that fluoresces when bound to DNA, and after each round of amplification the level of fluorescence is measured. The PCR is cycled 40 times, each time measuring the change in relative fluorescence and plotting the fluorescence on a graph. The point in which the reaction curve intersects with the threshold line becomes the $C_T$ value. The threshold line is the point where the signal becomes statistically significant over the calculated baseline signal. The $C_T$ value is used to determine the fold difference then compared between the target gene and the endogenous control to produce a reading of the relative amount of RNA.
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