The effects of Phosphatase and Tensin Homolog (PTEN) overexpression on longevity of cultured Human Umbilical Vein Endothelial Cells (HUVEC).

By

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Abstract

Ageing, the deterioration of organs and tissues and their ability to respond effectively to stressors, is a major cause of illnesses, disease and death. This is due largely to the shortening of telomeres resulting in cells undergoing senescence and apoptosis. This affects stem cells, without which organs cannot repair damage adequately. By removing oxidants that cause DNA and telomere damage the rate which telomeres shorten is reduced, resulting in a longer cellular lifespan. This can be accomplished by increasing the autophagic and antioxidant levels to remove oxidants and other toxic chemicals.

Phosphatase and Tensin Homolog (PTEN) has been shown recently to be able to increase an organism's lifespan by up-regulating pathways involved in DNA-damage repair and autophagy/antioxidants. Along with PTEN's properties in metabolic increases, protection from insulin resistance and cancer prevention, PTEN is a good potential candidate to extend human longevity. The purpose of this thesis was to investigate whether these effects, having only been tested in animals, would have similar effects on humans. The goal was to explore the effects that overexpression of PTEN in Human Umbilical Vein Endothelial Cell (HUVEC) cultures would have on cell longevity via an increase in antioxidant potential.

This was achieved by transfecting HUVEC cultures with PTEN plasmids using lipofection. Three doses of plasmids were tested: 0.01µg per ml, 0.1µg per ml and 1µg per ml. An Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed to verify the success of the transfection and to quantify the levels of PTEN in each culture. The antioxidant potential of the cultures was assessed by a total antioxidant potential assay, expressing the total antioxidant potential in µM Copper Reducing Equivalents. The cultures were maintained until all cells achieved senescence and apoptosis in order to determine longevity. The results of each assay was then compared and correlated with each other and with the cells' longevity in order to observe any patterns.

The ELISA results showed a successful transfection. The transfected cultures showed a significant increase in PTEN levels compared with the non-transfected cultures ($P < 0.001$). The AntiOxidant Potential-450 assay (AOP-450) results showed significant increases in total antioxidant potential between the transfected and non-transfected

cultures ($P < 0.001$). However, there was no significant difference between the 0.01 µg set and the Control set (P-value = 0.0957). This was due to a great degree of variance amongst the transfected cultures, ten times that of the non-transfected cultures. The cell culturing showed the transfected cultures had significantly longer lifespans than the non-transfected cultures ($P < 0.001$). Correlation between cell longevity and PTEN levels was 0.8727; and correlations between cell longevity and antioxidant potential was 0.6564.

This thesis demonstrated that there is a potential for PTEN to be used to extend human longevity. The results show that the successful transfection of PTEN lead to an increase in PTEN levels, an increase in antioxidant potential and an increased cellular longevity. The higher correlation between cell longevity and PTEN than antioxidant shows there is a definitive link between increased levels of PTEN and longevity but there may be underlying factors which have influenced longevity independently of antioxidants.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Name: **Izak Schalk Tait**

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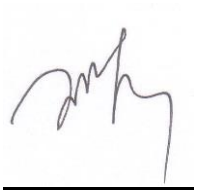
Co-Authored Works

The literature review is drawn in large parts from the review article titled "PTEN, Longevity and Age-Related Diseases", published in *Biomedicines* in 2013 (doi: 10.3390/biomedicines1010017) that I co-authored with Dr. Jun Lu and Dr. Yan Li (Tait, 2013).

It was researched and written by myself, under guidance from Dr. Lu and Li, and edited by Dr. Lu and Dr. Li.

Dr. Jun Lu



Dr. Yan Li

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1 Introduction

1.1 Background

There is an age-old proverb that says only two things in life are certain: death and taxes. A more recent one states that life is a sexually transmitted disease with a 100% mortality rate. While both are amusing, they are undoubtedly true. While you can escape death by infectious diseases; avoid diseases caused by poor health and diet; be fortunate enough not to die in any number of accidents or be privileged not to have been the target of a homicide; eventually you will age, your tissues and organs will start to atrophy and fail, and you will die. Beyond figures in myths, legends, or religions, there have never been a recorded case of a human that did not age.

On a more sombre note, ageing in a biological fashion can be described as "an overall decline in the functional capacity of various organs to maintain baseline tissue homeostasis and to respond adequately to physiological needs under stress" (Sahin & Depinho, 2010). The symptoms of this decline are as many and varied as they are well documented, and ultimately linked to this functional inability to adequately respond to various stresses. The most marked and observable symptoms due to the decline of ageing include sarcopenia (loss of muscle mass) (Thomas, 2010); decreased musculoskeletal strength, mobility (Leveille, 2004), coupled with arthropathies and osteoporosis (Tung & Iqbal, 2007); and the most common sign: wrinkles caused by thinning and reduced elasticity of skin. Progressive normocytic anaemia is a well-known symptom in the ageing haematopoietic system (Balducci, 2010) as well as greatly reduced immune function in the face of infectious diseases (Goronzy & Weyand, 2012). Other healing mechanisms such as proliferative stress responses and wound repair are universally impaired (Ashcroft, Horan, & Ferguson, 1995; Ashcroft, Mills, & Ashworth, 2002; Emmerson & Hardman, 2012; Raekallio, 1977). Tissues such as the liver and kidneys exhibit a reduction in mass and a diminished capacity to metabolise compounds (Grizzi et al., 2013; Schmitt & Melk, 2012); while the cardiovascular system shows a decreased heart rate and lung capacity (Knight & Nigam,

2008). Lastly, and most importantly, the brain's cognitive abilities deteriorate and it can be afflicted by numerous age related diseases such as dementia, Alzheimer's, Parkinson's to name but a few (Carroll, Hewitt, & Korolchuk, 2013; Hung, Chen, Hsieh, Chiou, & Kao, 2010).

While each of the above mentioned systems and diseases can, and have, served as the basis of its own thesis and report, it merely shows the intricateness and complexity of the decline that organs and tissues face as they age. The myriad biological causes of this decline are as intricate, complex and contentious as the symptoms themselves. However, the scale of this thesis will not focus on organs and tissues, but rather at the scale of genes and proteins. In this vein, a review on the genetics of ageing in 2010, Kenyon showed that merely increasing telomeres, as were the traditional thought (Kipling & Faragher, 1999), were not the sole method of preventing ageing. There were many genes, proteins and hormones involved in ageing and that each played a necessary part, ranging from caloric restriction, antioxidant capability, reproductive regulation and epigenomic transformation (Kenyon, 2010).

In saying this, however, telomeres have been found to be a key indicator of ageing (Jiang, Ju, & Rudolph, 2007; Sahin & DePinho, 2012) and thanks to the Telomere Hypothesis (Harley, Vaziri, Counter, & Allsopp, 1992) we know that telomere shortening can have an adverse effect on cellular ageing and this has been the basis for work done in the past twenty years regarding telomeres' effect on humans as a whole organism. This has, one could say, culminated the paper published in 2011 that showed that telomerase had the capacity to not only stop, but actively reverse tissue degeneration (Jaskelioff et al., 2011), once again opening up the question of whether telomerase was the answer to extended longevity.

In light of this, telomere deserves further investigation. Telomeres are sequences of repetitive TTAGGG nucleotides that cap chromosomes, preventing damage to them from being recognised as DNA damage (S. R. Chan & Blackburn, 2004). The major contributor to telomere shortening, beyond regular shortening during mitosis, is oxidative damage (von Zglinicki, 2002) caused by metabolic processes and environmental elements. At a critically short length, telomeres cease to function as chromosome terminus caps and any further shortening of the DNA is then recognised by proteins such as p53 and the cell undergoes senescence (Herbig, Jobling, Chen, Chen, & Sedivy, 2004). Further DNA-damage then results in apoptosis, cell death

(Mondello & Scovassi, 2004). It has been hypothesised that this simple process is one of the major culprits in tissue and organ deterioration.

Beyond the correlation between telomere shortening and cellular ageing, there have been many studies that have shown a correlation between ageing (as a whole organism) and telomere shortening. Dyskeratosis congenita (DC) is a congenital disorder that, similar to progeria, resembles premature ageing (Blasco, 2005). Patients with DC carry mutations in Telomerase RNA Component and Telomerase Reverse Transcriptase (TERC and TERT) that results in poor protein stability leading to increased telomere shortening (Nelson & Bertuch, 2012). While it is dangerous to extrapolate from rare congenital disorders to general conceptions of ageing, the association between telomere shortening and symptoms of ageing does support telomeres' (perhaps central) role in the ageing process.

As have been mentioned above, the decline of ageing is due to the organs and tissues losing the regenerative properties needed to offset the continual deterioration, leading to tissue (and organ) degeneration and atrophy (Jaskelioff et al., 2011). Evidence of this can be found in the decreased production of myogenic fibres in the muscular system (Cerletti, Shadrach, Jurga, Sherwood, & Wagers, 2008); the lower numbers of naive B and T cells in the haematopoietic system (Linton & Dorshkind, 2004) and a decreased production of neurones in the central nervous system (Maslov, Barone, Plunkett, & Pruitt, 2004). The common theme amongst these are that they are all, or derive from, stem cells and it is these stem cells which decrease in number and production as one ages (Sahin & Depinho, 2010). The cause for this has been found to be, in large part, the shortening of telomeres (Blasco, 2007). As with other cell types, stem cells are not immune to the ageing effects of shortened telomeres.

Another critical feature of ageing that have been shown to have an impact on telomeres is autophagy, the ability of cells to catabolise unneeded, unwanted or dysfunctional elements within them. One of these elements that autophagy is responsible for catabolising is ROS, which have been documented to cause DNA-damage (Wang, 2008) and shorten telomeres (von Zglinicki, 2002). Studies involving autophagy showed that increasing the rate of autophagy reduces the shortening of telomeres and can extend longevity (Fleming, Noda, Yoshimori, & Rubinsztein, 2011; Harris & Rubinsztein, 2012; Rubinsztein, Marino, & Kroemer, 2011) and that the

reverse is also true: by inhibiting autophagy, the lifespan of an organism is reduced (Lee et al., 2010).

To capitalise on this knowledge, a method by which one potentially could increase the lifespan of humans would be through the increased effectiveness of telomeres, and their corresponding enzyme: telomerase, to reduce their impact on ageing and age-related complications; and of autophagy, to prevent damage to DNA and to the telomeres. Whilst Phosphatase and Tensin Homolog (PTEN) may not have an effect on telomeres directly, the literature review below will show how it has an effect on autophagy, to reduce telomeric damage and thereby reduce telomere shortening, delaying the inevitable.

PTEN has significant implications for extending human longevity through its actions on DNA-damage reduction, antioxidant activity, caloric restriction, inhibition of replication and tumour suppression. The importance cannot be overstated as PTEN overexpression can assist a variety of maladies including weight-related diseases such as diabetes to age-related diseases such as Alzheimer's and Parkinson's. Its function as a tumour suppressor can maintain an anguish-free life. It is because of this variety and necessity of function that PTEN is vital for further research.

1.2 Purpose of the Study

This thesis follows in theme on recent papers such as Kenyon; Jaskelioff, et al., and Ortega-Molina, et al., which showed the strides that anti-ageing research have made over recent years (Jaskelioff et al., 2011; Kenyon, 2010; Ortega-Molina et al., 2012). PTEN has the potential to play a crucial role alongside these other studies as, beyond its documented ability to extend longevity, its function as a tumour repressor is vital to any lasting extended longevity to prevent the rise of tumours often associated with extended longevity.

These mentioned studies, and those that follow in the literature review show that PTEN has been extensively studied for its cancer prevention properties in humans and animals. Its capacity to extend longevity, however, has only been studied in the laboratory in animals. The purpose of this thesis is to bridge this gap in the literature, to

apply the knowledge surrounding PTEN's capacity to extend longevity and to determine whether PTEN can extend human cellular longevity.

One can say that this thesis will serve as a proof of concept of PTEN's ability to extend human cellular longevity, to pave the way forward for future studies to look at the avenues of PTEN that can, with or without other medicinal treatments, potentially extend the human lifespan. Research has shown how PTEN can have positive effects on the tissues and organs of animals, but to investigate these effects on humans, there must first be a solid basis of PTEN's effectiveness. That is the overarching purpose of this thesis.

Previous research has suggested that the method by which PTEN is most likely to extend cellular longevity is by raising the levels of antioxidants, thereby removing potentially toxic oxidants and preventing damage to DNA and reducing the shortening of telomeres. It is this indirect route to extended cellular longevity that this thesis investigated, to determine this effect would apply equally to human cells as it does to animal cells.

1.3 Overview of the Thesis

Chapter 2 explores the previous research that has been done on PTEN. It discusses the pathways that regulate, positively or negatively, PTEN and those pathways that are regulated by PTEN. It is then followed by the cellular functions of PTEN, both in the cytoplasm and in the nucleus, such as its effect on the PI3K pathways and p53. Lastly in this chapter, the properties of PTEN involving cancer is discussed, including the mutations of PTEN that can lead to cancer and its effects that can prevent it.

Chapter 3 describes the first and most delicate stage of the experiment: the transfection of the HUVEC cultures with PTEN plasmids. This is followed by the Enzyme-Linked ImmunoSorbent Assay (ELISA) used to verify the transfection and to capture data that was then used in Chapters 4 and 5.

Chapter 4 describes the methodology used to test the total antioxidant properties of the cultures to determine if the transfected PTEN had any influence thereon. This was

done using the AntiOxidant Potential-450 assay (AOP-450). The results of this assay are then compared with the results from Chapter 3.

Chapter 5 discusses the means by which the cultures themselves were grown, passaged and maintained throughout the time period of the experiment. This is followed by the results of the number of cell divisions the cells have undergone as a measure of their longevity. The results from Chapters 3, 4 and 5 are then correlated to observe any patterns.

In Chapter 6, the main results from Chapters 3 to 5 are summarized. This is followed by the problems that were experienced over the course of the thesis and the caveats that follow the results. Lastly, future directions of research are discussed that can be based on this thesis' results.

1.4 Overview of the Methodology

The purpose of this thesis was to discover what effect overexpression of PTEN had on the longevity of HUVEC cultures, and as such, the design of the thesis (shown in Figure 1) was made to reflect the most practical approach to achieve this solution. This most practical approach was decided on due to the considerations of the prices of the assays and reagents involved as well as the timeframe in which a thesis must be completed. Preliminary trials had shown that culture period, running from the thawing of the cells to the end of their longevity, could take upwards of two months.

The design can be broken down into six key steps as reflected in Figure 1. The first step would be the initial culturing to see the stability of the acquired cells. It was during this time that master stocks were prepared and frozen and the preliminary trials were run. Due to various incidents beyond the author's control, several cultures were started from the frozen stock before transgenesis took place. This allowed the author to familiarise him with HUVEC cultures and the culturing process.

The most crucial step in the design would be the transgenesis. Before this took place, the cultures were split into three separate culture groups: a Test group, a Negative control group, and a Control group. While the purpose of the test and control groups may be apparent, the purpose of the negative control group was to exclude the

possibility that the vector in which the PTEN plasmid did not have any effect on the cultures that would present as a false positive. As such, the negative control group was transfected with an empty vector only.

Each culture group consisted of a twelve well plate, meaning a total of 36 discreet samples were available for analysis. The test group plate was further divided into three areas regarding the amount of PTEN that was added. The initial purpose was to determine which amount would yield the best rate of transfection, but after it was found all three ratios delivered a satisfactory transfection, the differing wells were kept as such to see which dose would have a better effect on longevity.

As seen in Figure 1 each culture was subjected to both assays. The reason for this was so that the control group could act as baseline as no standard curve was used. As the purpose of this thesis was to determine what effect added PTEN would have on cultures, this was decided to be the best course of action as it would provide a direct contrast between those cultures with transgenic PTEN and those without. The results of the assays would also provide a good correlation to the longevity of each culture group.

As well as correlating with the longevity of each culture group, the two assays served a secondary purpose. For the ELISA test, this was to determine if the transfection was successful; if the Test group showed a significantly higher concentration of PTEN, this would indicate a successful transfection. ELISA was chosen instead of Western Blot due to its more quantifiable results. Because a key method by which PTEN can extend lifespan is through its antioxidant capabilities, the AOP-450 was used to determine if this was the case. If the Test control group produced a higher antioxidant potential as well as an increased longevity, then it would support this hypothesis.

The last step of the experiment is the final culturing. This was done to determine the longevity of each culture group following standard culturing practices.

The statistical analysis reflected the design of the study. The purpose of the study was to determine whether the exogenous PTEN had any positive effect to the longevity of the cultures. As such, this means a comparison between the transgenic cultures and the control cultures. Thus, the bulk of the statistical analysis was to transform the data so that *t*-tests comparing each culture's longevity to the control could be carried out. As a difference between the transgenic cultures was observed, *t*-tests between them were also done.

Beyond simply analysing the longevity on its own, it was important to determine whether there were any correlation between an elevated PTEN and the longevity, and similarly an elevated antioxidant potential and longevity. More so than a test of significance of the longevity, these correlations would show whether the increased longevity were due to the exogenous PTEN or to another, unknown, factor.

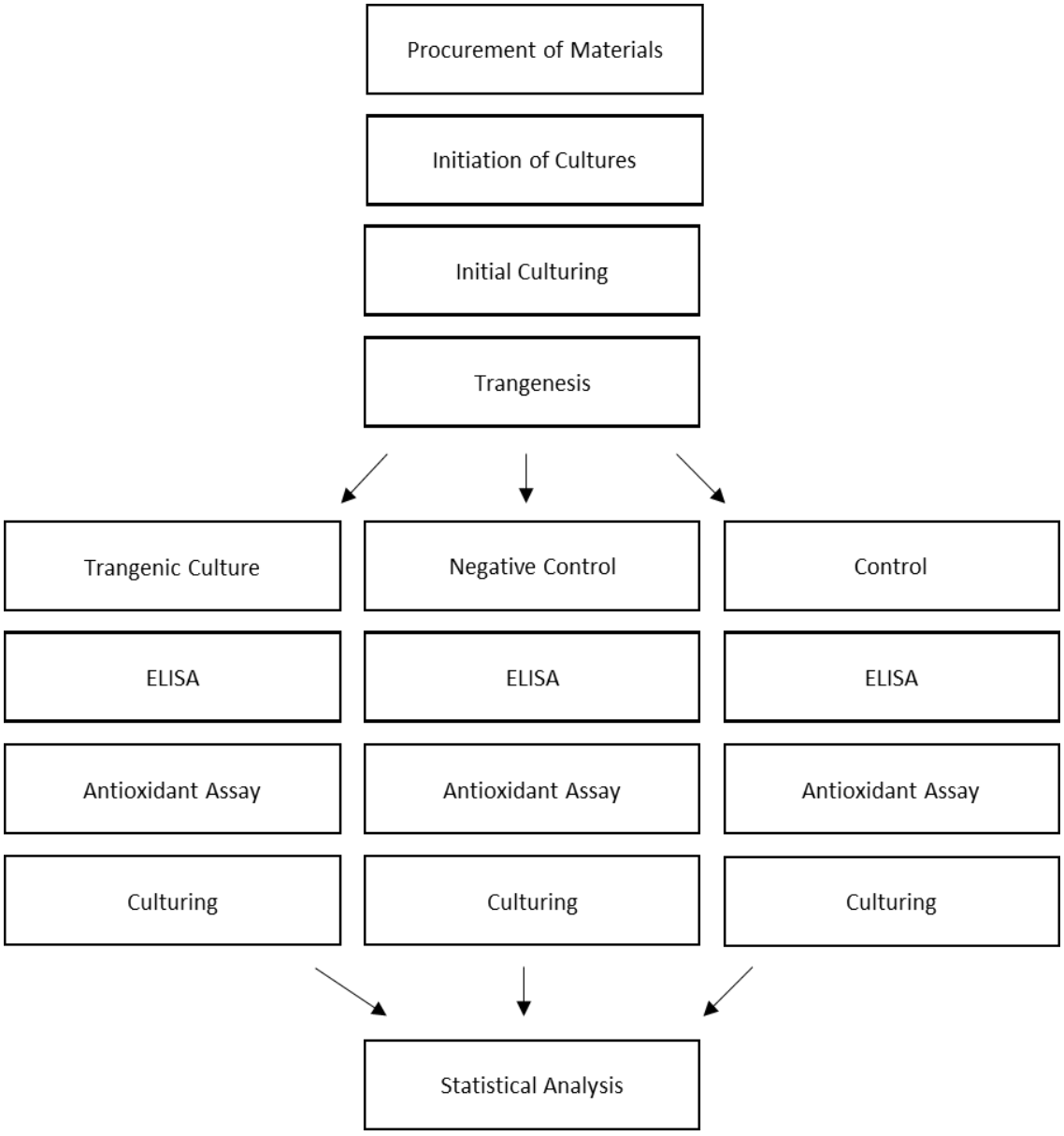

Figure 1: Design of the thesis. Beginning with the procurement of the materials, then the initial culturing before the cultures are split into three groups for the transgenesis: The Test Culture with the transgenic PTEN; a Negative Control with only an empty vector; and the Control Culture. Each culture group underwent two assays, an ELISA to determine the levels of PTEN in each group, and an Antioxidant assay to determine the total antioxidant potential. Culturing was then done to see the lifespan of each culture before statistical analysis was done.

2 Review of the Literature

2.1 Introduction

PTEN, also known as MMAC1 and TEP1, was first discovered in 1997 by two independent groups and recognised as the long sought after tumour suppressor gene frequently lost on human chromosome 10q23 (J. Li et al., 1997; Steck et al., 1997). This locus is highly susceptible to mutation in human cancers: the frequency of mutations have been estimated to be 50 - 80% in sporadic tumours such as glioblastomas, prostate cancers and endometrial carcinomas; and 30 - 50% in lung, colon and breast tumours. PTEN is often associated with advanced cancers and metastases (Ali, Schriml, & Dean, 1999), due to loss of PTEN having been observed at its highest frequency in late stages of cancers.

Together with p53, Ink4a and Arf, PTEN makes up the four most important tumour suppressors in mammals (Ortega-Molina et al., 2012) as evidenced by their overall high frequency of inactivation across a variety of cancer types. Because of this, it is vital to understand the mechanisms of how PTEN functions.

The gene that encodes PTEN is non-redundant and expressed in all eukaryotic cells (Sulis & Parsons, 2003). While there is only one homologue of PTEN found in fungi and lower animals, several orthologues have been discovered in mammals, namely TPTE, PTEN2 and TPIP (H. Chen et al., 1999; Walker, Downes, & Leslie, 2001; Y. Wu et al., 2001). Unlike PTEN, however, these orthologues are not ubiquitously expressed in all tissues. TPTE and PTEN2 are only expressed in the testis, while TPIP is expressed in the testis, brain and stomach. Whilst the orthologues share PTEN's major function, that of a phosphatidylinositol (3,4,5)-triphosphate (PIP3) phosphatase (as will be discussed later), analyses have shown that the orthologues are limited to the Golgi apparatus and endoplasmic reticulum, while PTEN is expressed in the nucleus, cytoplasm and at the cellular membrane. The difference in localisation demonstrates that the PTEN orthologues do not regulate PIP3 in the same manner as PTEN, reinforcing the non-redundancy of PTEN.

The PTEN protein contains 403 amino acids and several domains (J. Li et al., 1997). The crystal structure of PTEN shows a 179 residue N-terminal domain and a 166 residue C-terminal domain (J. O. Lee et al., 1999). The N-terminal domain contains a protein tyrosine phosphatase (PTP) signature motif that is similar to dual specificity protein phosphatases. The C-terminal domain contains the C2-domain that is responsible for its recruitment to phospholipid membranes. The PTEN homologue found in fungi and lower animals lack this C2 domain (J. Li et al., 1997).

While the functions of PTEN will be discussed in depth in this review, in brief the main function of PTEN is to antagonize the PI3K/AKT pathway (Maehama & Dixon, 1998), thereby opposing the pathway's cell proliferative response and, more important to longevity, opposing AKT's down-regulation of antioxidant genes and proteins (M. Ashcroft et al., 2002). In concert with this function, PTEN has been reported to bind with another antioxidant gene, p53, and arresting the cell cycle whilst positively regulating proteins involved in DNA-damage repair (Chang et al., 2008). These functions serve not only to extend cellular longevity but also prevent deleterious DNA-damage that can lead to malignant tumours (Ming et al., 2011).

2.2 PTEN Regulations

2.2.1 Regulates PTEN

2.2.1.1 Mutations

Among the varied sources that can act as regulators upon PTEN (as summarised in Table 1), mutations can have a much more significant impact on the structure and function of PTEN as it appears before transcription and translation have occurred. PTEN haploinsufficiency (where only a single functional allele remains) have been shown to contribute to tumour progression and even minor deficiency of function can aid tumour development (Kwabi-Addo et al., 2001). This is evident in many cancers and syndromes (to be discussed in depth further on) such as Cowden syndrome, whose C2-domain mutations may retain partial or full PTEN lipid phosphatase functionality, as seen in biochemical assays (Han et al., 2000; Waite & Eng, 2002). By truncating phosphorylated residues on the C-terminal, mutations such as these can affect PTEN

stability, phosphorylation, protein interactions and proper localisation (Trotman et al., 2007). Mutation of Lys289 has also been found to alter PTEN localisation.

The Ser380, Thr382 and Thr383 cluster on the C-terminal (referred to as the STT (Salmena, Carracedo, & Pandolfi, 2008)) have been shown to negatively affect PTEN stability while positively affecting phosphatase activity when mutated (Vazquez, Ramaswamy, Nakamura, & Sellers, 2000), despite the fact that mutation of the major sites for phosphorylation, Ser370 and Ser385, have been shown to have little effect on PTEN function. Leslie and Downes suggests STT phosphorylation renders PTEN into a "closed" state while mutation renders it "open", increasing PTEN's interaction with binding partners, making it more unstable by rendering it more susceptible to proteolysis (Leslie & Downes, 2004). Because of this, it has been proposed that PTEN's basal state is that of a phosphorylated, inactive state, being activated by dephosphorylation of the STT cluster to foster conformational changes. Further strengthening this hypothesis is the fact that tumour-derived C-terminal mutants of PTEN are highly susceptible to proteolysis, indicating the C-terminus to be responsible for protein stability (Vazquez et al., 2001).

Mutations on the N-terminal are also thought to affect the stability of the PTEN protein whilst not inactivating phosphatase activity (Han et al., 2000).

The evidence of mutational regulation of PTEN is further supported by the PTEN hamartoma tumour syndromes (PHTS) (Pilarski & Eng, 2004) a group of autosomal dominant syndromes identified by developmental disorders, hamartomas, neurological deficiencies and an increased risk of cancer. This shows that loss, or mutation, of an allele directly leads to a deficiency of PTEN function thereby resulting in PHTS and an increased cancer risk.

2.2.1.2 Transcriptional Regulation

Despite the fact that the exact nature of PTEN regulation is still unclear, there are a number of factors, shown in Table 1, that have been demonstrated to up-regulate PTEN transcription. One of these is the transcription factor peroxisome proliferation-activated receptor γ (PPARγ), a regulator of glucose metabolism and fatty acid storage. PPARγ up-regulates PTEN via its selective ligand rosiglitazone on peroxisome proliferator

response elements (PPRE) identified on the PTEN promoter region (Patel et al., 2001). PPARγ is itself regulated by oleamide, a fatty acid primary amide that has been implicated in sleep induction and hypolocomotion (Dionisi, Alexander, & Bennett, 2012), giving an indication of the complex network that surrounds PTEN. In addition, ligand-activated PPARδ up-regulates PTEN and suppresses the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Kim et al., 2011). Knockdown of PTEN with siRNA abrogated the effects of PPARδ on cellular senescence and on generation of ROS in angiotensin II treated vascular smooth muscle cells.

The early growth-regulated transcription factor-1 (EGR-1) up-regulates PTEN messenger RNA, and thus protein, expression leading to increased levels of apoptosis (a function of PTEN) (Virolle et al., 2001). It accomplishes this by binding to a functional GCGGCGGCG Egr-1-binding site on an untranslated region at the 5′ end. Much like PTEN, EGR-1 is involved in growth restriction and apoptotic processes (Sperandio et al., 2009) and it has been hypothesized that the interactivity of both proteins is a result of the similarity of their functions, that part of PTEN's apoptotic function is due to EGR-1. This is supported by Virolle, et al.'s experiment which showed that the introduction of exogenous EGR-1 restored PTEN stimulation in EGR-1 -/- cells in vitro.

EGR-1 can also be stimulated by IGF-2 in a negative feedback loop (Moorehead et al., 2003). According to Moorehead, et al.'s study, IGF-2 regulates and is regulated by EGR-1, and while EGR-1 stimulates PTEN, IGF-2 stimulates the opposing pathway: the AKT pathway (Codina et al., 2008). Introduction of IGF-2 has been found to increase AKT levels in the short term while increasing PTEN levels in the long term. This is suggestive of a negative feedback loop to counter the cell-proliferative properties of the AKT pathway.

Ras, a small GTPase, regulates the passage of extracellular signals to intracellular pathways by acting as a molecular switch (Marshall, 1996). Ras can act as an anti-apoptotic/cell-survivalist regulator by inducing the Ras-Raf-MEK-ERK pathway, down regulating PTEN via the transcriptional factor c-Jun (Hettinger et al., 2007), and inducing the AKT-PI3K-NFκB pathway, thereby down regulating p53 and forkhead transcription factors (FoxO) genes (Vasudevan, Gurumurthy, & Rangnekar, 2004). It is unsurprising, therefore, to discover that Ras is a key oncogenic protein.

Epigenetic effects may inhibit PTEN expression (J. M. Garcia et al., 2004) such as promoter hypermethylation in various types of cancer (Goel et al., 2004; Kang, Lee, & Kim, 2002; Mirmohammadsadegh et al., 2006). Caution must be taken when interpreting epigenetic silencing regarding PTEN as a PTEN-pseudogene exists with a promoter also shown to be methylated (Zysman, Chapman, & Bapat, 2002) although there is doubt about the expression of the pseudo-gene (Hamilton et al., 2000).

PTEN is negatively affected by MicroRNAs (miRNAs) which are short, single-stranded endogenous RNAs approximately 22 nucleotides in length that suppress mRNA translation. Specifically, it was shown that PTEN is inhibited by miR-21 (Meng et al., 2006; Meng et al., 2007), one of the most frequently found miRNAs to be up-regulated in cancer to promote cell proliferation and to inhibit apoptosis (J. A. Chan, Krichevsky, & Kosik, 2005; Si et al., 2007; Volinia et al., 2006). This suggests that its oncogenic effect is due, at least in part, to its suppression of PTEN. The oncogenic effects of PTEN inhibition will be discussed in detail later on.

2.2.1.3 Post-Translational Regulation

By far the greatest number of regulatory effects on PTEN occurs post-translation (as can be seen in Table 1), by the interaction of other proteins and chemicals on the PTEN protein. The various post-translation modifications that may regulate PTEN include phosphorylation, acetylation, oxidation and ubiquitination.

For example, it has been reported that PTEN stability is subject to various post-translational modifications such as phosphorylation of specific residues on its C-terminal tail, as done by protein interacting with carboxyl-terminus tail 1 (PICT1) for instance (Okahara, Ikawa, Kanaho, & Maehama, 2004), that have been associated with increased stability (Georgescu, Kirsch, Akagi, Shishido, & Hanafusa, 1999; Torres & Pulido, 2001; Vazquez et al., 2000) while phosphorylation of other sites such as Thr366 destabilize PTEN (Maccario, Perera, Davidson, Downes, & Leslie, 2007). A total of six phosphorylation sites (at Thr366, Ser370, Ser380, Thr382, Thr383, and Ser385) have been shown to regulate PTEN tumour suppressing function, by modulating protein stability. Phosphorylation also results in decreased catalytic activity toward lipid substrates, resulting in a decreased ability to interact with membranes, a key function of PTEN.

Table 1: Regulators of PTEN and their effects

PTEN regulators	Effects
Haploinsuffiency	Reduced PTEN function, higher susceptibility to tumours (Kwabi-Addo et al., 2001)
C2-domain mutations	Negatively affect PTEN stability, phosphorylation, protein interactions and proper localization (Han et al., 2000; Trotman et al., 2007; Waite & Eng, 2002)
N-terminal mutations	Negatively affect protein stability (Han et al., 2000)
PPARγ	Up-regulation of PTEN transcription (Patel et al., 2001)
EGR-1	Up-regulation of PTEN mRNA leading to apoptosis (Virolle et al., 2001)
Ras	Down-regulates PTEN via c-Jun, induces PI3k-AKT pathway (Hettinger et al., 2007)
Promoter hypermethylation	Possible inhibition of PTEN expression (J. M. Garcia et al., 2004)
STT phosphorylation	Significantly negatively affect PTEN stability while positively affecting phosphatase activity (Leslie & Downes, 2004; Vazquez et al., 2000)
mir-21	Down-regulates PTEN transcription (J. A. Chan et al., 2005; Meng et al., 2006; Meng et al., 2007; Si et al., 2007; Volinia et al., 2006)
PICT1	Phosphorylates PTEN C-terminal, increases stability (Georgescu et al., 1999; Torres & Pulido, 2001; Vazquez et al., 2000)
Phosphorylation of T366, S370, S380, T382-3, S385	Affects protein stability and decreases PTEN's ability to interact with membranes (Maccario et al., 2007)
Chk1	Induces CK2-mediated phosphorylation of PTEN, recovers stalled cell cycle (Martin & Ouchi, 2008; Torres & Pulido, 2001)
ROCK	Phosphorylates PTEN to increase PTEN localization to membrane (Z. Li et al., 2005)
PEST-sequences	Destabilizes PTEN (Torres & Pulido, 2001)
NEDD4-1	Ubiquitinates PTEN via L13, L289 (X. Wang et al., 2007)
PCAF	Promotes PTEN acetylation to decrease PTEN catalytic activity (Okumura et al., 2006)
ROS	Decreases PTEN catalytic activity (S. R. Lee et al., 2002)
NHERF & MAGI-2	Regulates PTEN localization and recruitment to the membrane (X. Wu et al., 2000)
NFκB	Down-regulates PTEN transcription (Xia et al., 2007)

Depletion of serine/threonine-protein kinase (Chk1), a signal transducer in the cell cycle pathway (Martin & Ouchi, 2008), decreases phosphorylation and levels of PTEN. Chk1 and PTEN are linked in the cell cycle regulatory pathway as phosphorylation of both Chk1 and PTEN recovers the cell cycle after DNA replication has stalled. An ATR-Chk1-CK2-PTEN pathway also exists as ATR phosphorylates Chk1 at Ser137, which in turn induces casein kinase 2 (CK2)-mediated phosphorylation of PTEN at Thr383 (Torres & Pulido, 2001). This is vital for recovery of the cell cycle and illustrates the roles of Chk1 and PTEN in DNA damage response.

Rho-associated protein kinase (ROCK), a protein involved in cellular membrane activities, has been shown to increase PTEN localisation to the plasma membrane by phosphorylating sites Ser229, Thr322, Thr319, and Thr321 in the C2 domain, in contrast to the above mentioned phosphorylation of site Thr366 (Li et al., 2005). Phosphorylation of PTEN has also been attributed to glycogen synthase kinase 3β (GSK3β) (Al-Khouri, Ma, Togo, Williams, & Mustelin, 2005; Miller, Lou, Seldin, Lane, & Neel, 2002).

PTEN contains two PEST-sequence (proline, glutamic acid, serine, and threonine) motifs which are normally found on short-lived, unstable proteins which are degraded by ubiquitin mediation of the proteasome. While PTEN is, under normal circumstances, a stable and long lived protein, studies have shown that PTEN may be regulated by the PEST-sequence domains so that the half-life of PTEN is increased during proteasome inhibition (Torres & Pulido, 2001). It was also shown that exposure to zinc-ions initiated ubiquitin-dependent, proteasomal degradation of PTEN (Wu et al., 2003). Wu, et al. found that proteasome inhibition prevented PTEN degradation in response to zinc-ions, suggesting that zinc-ions activate the degradation process either through the proteasome or through ubiquitin.

In contrast to this Tang and Eng found that p53 may destabilize and degrade PTEN in cells with proteasome dysfunction (Tang & Eng, 2006).

Lys13 and Lys289 are two conserved sites identified for PTEN ubiquitination and Trotman et al. showed that ubiquitin conjugation to these sites is highly important for the shuttling of PTEN between nucleus and cytoplasm (Trotman et al., 2007). Wang et al. isolated E3 ubiquitin-protein ligase 4-1 (NEDD4-1) and showed that overexpression

of NEDD4-1 mediated mono- and poly-ubiquitination of PTEN through physical interaction (X. Wang et al., 2007).

The nuclear histone acetyltransferase-associated PCAF protein promotes PTEN acetylation at Lys125 and Lys128 during PTEN interaction to decrease the catalytic activity of PTEN (Okumura et al., 2006). The catalytic activity can also be down-regulated by oxidation of reactive oxygen species (ROS) which causes the formation of a disulfide bond between Cys124 and Cys71 (S. R. Lee et al., 2002). This has been seen to either take the form of hydrogen peroxide (H_2O_2) or endogenous ROS produced in macrophages in response to cellular and metabolic stress and is associated with oxidant-dependent downstream signalling (J. Kwon et al., 2004; Leslie et al., 2003).

Proteins such as Na^+-H^+ exchanger regulatory factor (NHERF) as well as membrane-associated guanylate kinase inverted 2 (MAGI-2) can regulate PTEN localisation and recruitment to the membrane through the 3 aa C-terminal region on PTEN (Georgescu et al., 1999; X. Wu et al., 2000). These PDZ domain interactions can be negatively modulated by phosphorylating PTEN on its C terminus (Takahashi, Morales, Kreimann, & Georgescu, 2006; Vazquez et al., 2001; Vazquez et al., 2000); however, deletion of the 3 aa amino acids does not alter the tumour suppressive activity of PTEN (Georgescu et al., 1999).

2.2.2 Regulated by PTEN

2.2.2.1 In the Cytoplasm

Of the various molecules that are regulated by PTEN (summarised in Table 2), the most well documented is the phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3]/Protein Kinase B pathway, otherwise referenced as the PIP3/AKT pathway, which PTEN antagonizes (Maehama & Dixon, 1998; Stambolic et al., 1998). PTEN dephosphorylates PIP3 by removing the D3 phosphate from the inositol ring, resulting in PIP2 (Maehama & Dixon, 1998). PIP3 is responsible for the recruitment of proteins containing pleckstrin homology domains to the cellular membranes, including the AKT isoforms and PDK1 as evidenced upon PTEN inhibition. The AKT pathway promotes cell survival and proliferation and this effect upon longevity will be discussed further on.

A study done by Vivanco, et al, showed that the Jun-N-terminal Kinase (JNK) pathway to be activated upon PTEN loss, suggesting it is down regulated by PTEN (Vivanco et al., 2007). Further investigation led the authors to discover this was through the Phosphatidylinositide 3-kinase (PI3K) family of proteins of which PIP3 is a part of. While PIP3, as mentioned above, is a well-known regulator of AKT, the activation of JNK was AKT independent. The exact process how PIP3 activates JNK is as yet unclear.

Xia, et al., showed how JNK, through its upstream protein mitogen-activated protein kinase kinase-4 (MEKK4), down-regulates the transcription of PTEN by activating the transcription factor NFκB whereby it binds to the promoter sequence of PTEN (Xia et al., 2007). This suggests a negative feedback loop between PTEN and JNK.

2.2.2.2. In the Nucleus

A p53 binding sequence has been identified on the promoter sequence of PTEN and a survival mechanism that only functions through the transcription of PTEN (Stambolic et al., 2001). PTEN has also been reported to bind to p53's C-terminus directly via its C2 domain, increasing its stability and transcription and therefore increasing p53 protein levels. This is further demonstrated by a study showing the drastically reduced half-life of p53 in PTEN-null mouse cells (Freeman et al., 2003) independently of PTEN phosphatase activity, suggesting it is the binding of PTEN that gives this result. Contrasting studies have shown, however, a marked up-regulation of p53 in the absence of PTEN (Z. Chen et al., 2005). In addition, PTEN-loss induced senescence is associated with enhanced p53 translation (Alimonti et al., 2010).

Li, et al., showed another method how PTEN can influence p53: by the interaction of PTEN with the transcriptional co-activator p300/CBP (CREB-binding protein) (A. G. Li et al., 2006). By forming a complex with PTEN, p300 acetylates p53 on sites Lys 373 and Lys 382. This results in stabilization and tetramerisation of p53. This in turn allows PTEN to bind to p53 and, as described above, further stabilising the p53-p300 complex allowing a more efficient acetylation of p53. The net result in all of this is a maximum activation of p53 DNA binding and transcription. Li, et al., also showed an increase in this process during radiation, implying this process is in response to DNA damage.

It has been suggested that PTEN has a role in maintaining chromosomal integrity as extensive centromere breakage and chromosomal translocations observed in PTEN null cells (Puc & Parsons, 2005; Shen et al., 2007). Puc and Parsons showed this to be due to AKT-phosphorylation of CHK1 that leads to sequestration of CHK1 from the nucleus and, as explained above, induces phosphorylation and levels of PTEN. The regulation of centromere stability is done by PTEN physically associating with the centromere binding protein Centromere Protein C (CENP-C) (Shen et al., 2007). This is done in a phosphatase independent manner but requires a functional C-terminus on PTEN's behalf. Shen, et al., also showed that PTEN positively regulates Rad51, a protein involved in repairing double stranded breaks, through the transcription factor E2F-1 (Shen et al., 2007).

Table 2: The regulatory effects of PTEN

Regulated by PTEN	Effects
PIP3/AKT pathway	Increased longevity (Partridge & Bruning, 2008), reduced insulin signalling (Carracedo & Pandolfi, 2008), tumour suppression (Scanga et al., 2000), reduced DNA damage (Y. Wang, 2008).
JNK pathway	Down-regulated via phosphorylation PI3K (Vivanco et al., 2007).
p53	Increased stability and transcription of p53 (Freeman et al., 2003).
CENP-C	Binds with PTEN, maintains chromosomal integrity (Shen et al., 2007).
Rad51	Positively regulated via E2F-1, repairs DSB (Shen et al., 2007).

2.3 Cell Functions

The regulatory effects of PTEN are synonymous with its cellular functions as PTEN operates by affecting a variety of pathways. While the review above focused on the mechanics of PTEN regulations, the review below will focus on the effects of this and how it relates to longevity.

2.3.1 PI3K

The most prominently reported function of PTEN is to antagonize the PI3K signalling pathway (Chalhoub & Baker, 2009; Maehama & Dixon, 1998; Stambolic et al., 1998). PI3Ks can be stimulated by growth stimuli or insulin stimuli (Ortega-Molina et al., 2012), whereupon class proteins I of the PI3K family such as PIP2 are catalysed to convert to PIP3, a second messenger protein that stimulates growth, proliferation, survival and inhibition of apoptosis (M. Ashcroft et al., 2002; Backman et al., 2001). PIP3 is responsible for recruiting proteins containing a pleckstrin homology domain to cellular membranes, including the AKT isoforms. Once there, AKT isoforms are phosphorylated at two residues: on Thr308 by PDK1 (Cho et al., 2001) and Ser473 by mTOR kinase complex 2 (mTORC2) (Alessi et al., 1996) which is required for full activation. As such, termination of AKT activity is brought about by dephosphorylating AKT isoforms on Ser473 (Brognard, Sierecki, Gao, & Newton, 2007; T. Gao, Furnari, & Newton, 2005).

AKT is responsible for phosphorylating many cellular substrates to promote survival, growth, proliferation and metabolism such as mouse double minute 2 homolog (MDM2), glycogen synthase kinase 3 (GSK3), FoxO, Bcl-2-associated death promoter (BAD), Caspace-9, and p27 (Manning & Cantley, 2007). It also activates mammalian target of rapamycin complex 1 (mTORC1) through phosphorylating the TSC tumour suppressor complex and activates Ras-homolog-enriched-in-brain (RHEB), a Ras-related GTPase (Guertin & Sabatini, 2007). There are several feedback loops incorporated into this cascade, one negative feedback loop being mTORC1 which works to inhibit AKT activation (Manning, 2004) as well as a positive feedback loop: the inhibition of FoxO transcription factors by AKT which, when activated, suppresses insulin signalling to initiate the PI3K/AKT cascade (Carracedo & Pandolfi, 2008; Puig & Tjian, 2005).

A significant role of the PI3K/AKT cascade is to inhibit apoptosis. One method is by phosphorylating pro-caspase 9 on residues Ser196 and Thr125 (Datta et al., 1997). Dephosphorylated pro-caspase 9 binds to, cleaves and activates caspases 3 and 7, which target key regulatory and structural proteins for proteolysis, resulting in cell death (Cardone et al., 1998). Another method is by phosphorylating Mdm2, resulting in its nuclear localisation leading to the subsequent export and degradation of p53 (Mayo & Donner, 2001; B. P. Zhou et al., 2001), a key aspect of PTEN's pathways as mentioned earlier.

Another role of the AKT pathway is the dual regulation of the FoxO forkhead transcription factors and NF-κB, the former involved in longevity and the latter in antagonising the ageing process (Adler et al., 2007; Partridge & Bruning, 2008; Salminen & Kaarniranta, 2009; Salminen et al., 2008). By phosphorylating the FoxO proteins, AKT ensures their retention in the cytosol in a complex with the 14-3-3 protein. With reduced insulin signalling, enacted by various methods including a negative feedback induced by FoxO, FoxO proteins translocate to the nucleus whereby they form complexes with co-activators (Calnan & Brunet, 2008; Huang & Tindall, 2007; Van Der Heide, Hoekman, & Smidt, 2004). These complexes then allows FoxO to induce the expression of several antioxidative enzymes, stress resistance inducers (Calnan & Brunet, 2008; van der Horst & Burgering, 2007), and the regulation of immunosenescence (Peng, 2008) and oxidative stress. Conversely, NF-κB is responsible for down-regulating FoxO and antioxidative proteins (Adler et al., 2007; Salminen & Kaarniranta, 2009; Salminen et al., 2008).

The importance to longevity here is twofold. Firstly, by inhibiting the PI3K pathway, FoxO proteins are indirectly overexpressed leading to increased antioxidant levels, stress resistance and immune activity. The enhanced antioxidant levels would result in a reduction of DNA-damage and telomere damage, for example the single strand and double strand break lesion induced by reactive oxygen species (ROS) (Y. Wang, 2008) whilst the increased stress resistance and immune activity would result in an increased capability to fight off disease.

The second impact that inhibition of the PI3K pathway can have on longevity is by the inhibition of its cell proliferative abilities (Scanga et al., 2000). Beyond the decreased risk of cancer that curtailing excessive cell proliferation would bring, apoptosis contains and disposes of cellular toxins that have the potential to damage tissues.

2.3.2 Membrane

Das, et al., discovered, through the use of a fluorescent mutant version of PTEN, that PTEN is expressed at the plasma membrane (Das et al., 2003). This was in contrast to previous studies showing PTEN to be expressed only in the cytoplasm. More recent

immunohistochemical studies have shown the distribution of PTEN varies between tissues: in epithelial cells such as in the skin and colon, PTEN is mostly found in the cytoplasm (Deichmann et al., 2002; McMenamin et al., 1999); in neurons, fibroblasts, adrenal medulla, and thyroid cells most PTEN is in found in the nucleus (Gimm et al., 2000; Lachyankar et al., 2003; Sano et al., 1999) and polarized MDCK cells show that PTEN localizes at the membrane in cell-cell tight junctions (X. Wu et al., 2000).

A possible explanation for the different localisation patterns may involve the 50 amino acid C2 domain on the C-terminus of PTEN. As mentioned earlier, this region contains multiple residues for phosphorylation, such as by CK2, and also a PDZ-binding domain that can bind to MAGI2 (Das et al., 2003; X. Wu et al., 2000), a protein localized at the cellular membrane. At the membrane, PTEN has been implicated to exert effects on the cytoskeleton and suppress cell migration (Tamura et al., 1998). This has been indicated by an increased migration in PTEN null embryonic fibroblasts. Elevated PIP3 in these cells activates Rac1 and Cdc42, small GTPase mediators of cellular migration (Liliental et al., 2000). Raftopoulou, et al., showed that phosphorylation of the Thr383 residue on the C2 domain inhibited PTEN's effect on migration (Raftopoulou et al., 2004).

2.3.3. Nucleus

2.3.3.1 Localisation

It was initially assumed that PTEN was strictly a cytoplasmic protein. This was due to the reported lipid-binding domain, an absence of a nuclear localisation signal (NLS) on PTEN, overexpression studies, and PTEN antibodies that were exclusively in the cytoplasm (J. O. Lee et al., 1999; D. M. Li & Sun, 1997; Whang et al., 1998). It is now known, however, to be present and functional in the nucleus (Lian & Di Cristofano, 2005).

More recent studies done with reliable PTEN antibodies have produced immunocytological and immunohistochemical data that demonstrates the presence of PTEN in the nucleus of primary differentiated cells such as neurons (Lachyankar et al., 2000), pancreatic cells (Perren et al., 2000), vascular smooth muscle cells (Deleris et al., 2003), thyroid tissue (Gimm et al., 2000), and in the intestinal mucosa (Trotman et al.,

2007). Ginn-Pease and Eng showed that the concentration of nuclear PTEN mirrors that of cytoplasmic PTEN during the cycle (Ginn-Pease & Eng, 2003). The highest concentration of nuclear and cytoplasmic PTEN is during the G0-G1 phase when the cell is quiescent or undergoing protein synthesis. In contrast, rapidly cycling cell lines have shown a marked decrease in PTEN localisation (Ginn-Pease & Eng, 2003; Lachyankar et al., 2000; Perren et al., 1999) indicating that the nuclear localisation of PTEN may not only be dependent on cycle stage but also on differentiation status.

Mechanisms by which PTEN enters the nucleus includes diffusion, active shuttling, cytoplasmic-localisation-signal-dependent export and monoubiquitylation-dependent import (Planchon, Waite, & Eng, 2008). PTEN has also been associated with the Major Vault Protein (MVP), a nuclear-cytoplasmic transport protein which was found to mediate PTEN nuclear importation. This is dependent on NLS-like residue sequences on PTEN: NLS4 (amino acids 65-269KKDK), NLS2 (amino acids 60-164 RTRDKK) and NLS3 (amino acids 233-237 RREDK) (Chung, Ginn-Pease, & Eng, 2005). NLS4 is necessary for PTEN importation but requires either NLS2 or NLS3 for proper function. These allow MVP to mediate PTEN, and Chung, et al., found this process to be independent of PTEN phosphorylation and of its phosphatase activity (Chung et al., 2005).

It was more recently reported that nuclear cell cycle dependent transportation of PTEN is regulated by the PI3K/AKT/mTOR/S6K signalling cascade (Liu et al., 2007), specifically the export through S6K phosphorylation of PTEN and a CRM1-dependent mechanism mediating its export . Activation of S6K initiates a negative feedback loop by inhibiting IRS-1, slowing the PI3K/AKT cascade, but not stopping it due to activation by growth factors. As PTEN is implicated in cell cycle arrest, it has been suggested that this export is a method of controlling this arrest.

2.3.3.2 Nuclear Interactions

PTEN has been implicated to play a role in a variety of nuclear functions such as chromosome stability, DNA repair, cellular stability and the aforementioned cell cycle arrest. A study by Chang, et al., found that oxidative stress retards the nuclear exportation of PTEN due to the dephosphorylation of Ser380, thus accumulating PTEN in the nucleus (Chang et al., 2008). This, the authors found, allows PTEN to bind to p53

(as discussed above) and enhance p53-mediated functions, which showed a decrease in cellular reactive oxygen species (ROS) in a p53-dependent manner and an increase in the p53 downstream antioxidant gene Sestrin.

PTEN was also found to arrest the cell cycle via p53 during the G1 phase, suggesting that the cause of this was for DNA repair (Chang et al., 2008). It was also found PTEN could arrest the cell cycle at the G1 phase by suppressing the transcription of cyclin D1 through phosphorylation of the mitogen-activated protein kinases (MAPK) pathway (Gil et al., 2006) or by limiting its nuclear accumulation (Radu, Neubauer, Akagi, Hanafusa, & Georgescu, 2003).

PTEN was shown to increase chromosomal stability by binding to CENP-C to associate with the centromere and by increasing the transcription of Rad51 to repair double-strand breaks (DSB), independent of its phosphatase functions (Puc & Parsons, 2005; Shen et al., 2007). The importance of stable and integral chromosomes cannot be understated. Poor chromosomal stability due to DSB has been found to lead to loss of heterozygosity leading to the formation of tumours (as reviewed in van Gent, et al. (van Gent et al., 2001)). Defects in the centromere, acting as the locus to which the spindle microtubules bind (Cleveland, Mao, & Sullivan, 2003), would disrupt the process of correct segregation of chromosomes to each daughter cell during mitosis.

When all of these functions are taken together, it can be argued that the reasons for the cell cycle arrest during the G1 phase is to reduce DNA damage by reducing oxidation, to repair DSB, and to enhance centromere integrity. It is surprising then to find that the most reported function of PTEN, that of down-regulating the PI3K/AKT pathway, is not involved in these functions; in fact it has been reported that only cytoplasmic PIP3 is sensitive to PTEN (Lindsay et al., 2006). In saying that, however, it was found that enforced nuclear PTEN expression can reduce cellular levels of phosphorylated AKT (Trotman et al., 2007).

2.3.4 DNA Damage

If ageing can be described as the increase in entropy of life sustaining systems, then that increasing entropy is due in large part to the accumulation of damage to DNA. In this review thus far, one has seen the effects that make PTEN a crucial factor regarding

longevity. It can aid in caloric restriction, preventing an abundance of the sources of ROS from entering the cell; it enhances p53's antioxidant capabilities, preventing the accumulation of ROS already in the cell; in a complex with p53 it helps repair DNA damage and through suppression of the PI3K/AKT and other pathways it prevents the emergence of tumours, malignant or otherwise. Through all these functions, PTEN promotes longevity. But one of, if not the key factor in longevity is DNA damage. Beyond the prevention of tumours, this prevents aberrant proteins and cells accumulating in tissues and further increasing the deterioration of the life sustaining systems.

The deterioration within liver tissues due to accumulation DNA damage is a robust biomarker for ageing (Matheu et al., 2007; C. Wang et al., 2009) and Ortega-Molina, et al., found through immunofluorescence that PTEN transgenic mice showed a significantly reduced level of DNA damage in the liver compared to control mice. This effect was more pronounced the older the mice were. The older transgenic mice also performed significantly better during exercise tests, suggestive of good health.

Whether through exogenous (radiation) or endogenous (ROS via metabolic processes) agents, cellular DNA is constantly under stress which can result in errors occurring during replication or mutations and other lesions. There are several DNA repair mechanisms which can effectively, in most cases, repair such lesions. These include nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), DNA double strand break repair (DSBR) and postreplication repair (PRR) (Cline & Hanawalt, 2003; Friedberg, 2003; Hoeijmakers, 2001). The NER and BER pathways are most typically activated in response to damage done to individual bases. Single strand breaks (SSBs) and double strand breaks (DSBs) on the other hand require repair through more complex mechanisms such as homologous recombination (HR), non-homologous end joining (NHEJ) or single strand annealing (SSA) (Branzei & Foiani, 2008; Seviour & Lin, 2010).

Of these, NER is the most versatile and flexible repair mechanism which has been conserved in most organisms, especially eukaryotes (Cleaver, 2005; Cleaver, Lam, & Revet, 2009; Sugasawa, 2006; Sugasawa et al., 1998) and is crucially important in repairing lesions caused by UV (Niggli & Rothlisberger, 1988; Vink, Berg, de Gruijl, Roza, & Baan, 1991). This pathway involves proteins, such as downstream proteins of p53, which can detect, unwind and remove damaged DNA. There are two forms of

NER: if the damage is linked to transcription there is transcription-coupled repair (TCR); if it is found to be linked to the genome in a general sense there is global genome NER (GG-NER). Linked to the NER pathways are the DNA damage response (DDR) pathways (Cimprich & Cortez, 2008) which are activated to regulate cell cycle transitions, DNA repair and replication and apoptosis, processes which involve PTEN either indirectly or directly.

In studies using low suberythemal UV radiation, mice with down regulated PTEN in epidermal cells showed a predisposition to skin tumours (Ming et al., 2011). PTEN has been found to be significantly down-regulated in human skin malignant and premalignant lesions. As NER has been linked to UV based DNA damage repair, this strongly implicates PTEN in NER-related activation.

PTEN has also been indicated to positively regulate GG-NER activation through the promotion of XPC transcription in keratinocytes. XPC in turn is impaired via PTEN loss, crippling GG-NER. This occurs due to the AKT/p38 pathway which is critical for regulating XPC levels, through the increased nuclear translocation of the transcription repressor p130 (Ming et al., 2010). Thus, PTEN is indicated to positively regulate GG-NER through the suppression of AKT following DNA-damage (Ming et al., 2011).

As stated previously, PTEN has a positive effect on DSBR through the up-regulation of Rad51. Various organisms studied with PTEN loss have shown evidence of DSBs and defective DSBR (Shen et al., 2007). This has been disputed, however, by Gupta et al., 2009, who found that the initial phase of DNA damage sensing and modification that has been associated with DSBs is similar in cells with or without PTEN. As the authors used different cell lines than previous studies, this may suggest that PTEN's role in DSBR is tissue specific.

Chk1 and Chk2, as well as regulating PTEN, play a significant role in DNA repair, activated by ATR (E. J. Brown & Baltimore, 2003), by activating p53 among other effectors (Chehab, Malikzay, Appel, & Halazonetis, 2000; Yoon & Smart, 2004).

2.3.5 Stem Cells

PTEN's effect on stem cells originates from its influence on the regulation of cell growth and proliferation through the inhibition of PIP3. Three studies (Backman et al., 2001; Groszer et al., 2001; C. H. Kwon et al., 2001) found that PTEN deficiency in neuronal stem cells provided a strong proliferative response and promoted a greatly enhanced capacity for self-renewal. This enhanced self-renewal capacity was discovered to be due to PTEN not being present to arrest the G_0-G_1 cell cycle, and a decreased growth factor dependency of PTEN null neural/stem progenitor cells. This was discovered through the deletion of PTEN in murine brains that led to macrocephaly (enlarged brains) and disturbing patterning of brain structures due to increased cell proliferation and decreased cell death. Follow up ex vivo experiments showed that PTEN loss dramatically increased total number of neurons in foetal brain and, more importantly, an increase in the number of neuronal stem cells capable of growth.

Zhang, et al., found that deletion of PTEN in the murine hematopoietic system resulted in the depletion of current hematopoietic stem cells (HSCs) and increased the proliferation of leukemogenic stem cells (Zhang et al., 2006). The result of this was the mice developed myeloproliferative disorders which eventually led to leukaemia. Yilmaz, et al., in turn demonstrated that treating murine PTEN null cells with rapamycin, an mTOR inhibitor, blocked the growth of the leukemogenic stem cells and increased the proliferation of normal HSCs (Yilmaz et al., 2006). This strengthens the theory that PTEN's effect on stem cells arises through its regulation of the PIP3/AKT pathway.

2.3.6 Senescence/Apoptosis

As PTEN has been shown to increase antioxidant activity, it seems counterintuitive that PTEN could promote senescence or apoptosis, especially when one considers that complete acute loss of PTEN promoted a strong senescence response that opposes tumour progression (Z. Chen et al., 2005). However, complete loss of PTEN has been found in many cancers, leading to speculation that loss of PTEN leads to the formation of tumours.

Be this as it may, Gil et al., showed that apoptotic stimuli promote the nuclear import of PTEN, implying the nuclear functions of PTEN include apoptosis (Gil et al., 2006). While the mechanisms of PTEN's pro-apoptotic functions are still unclear, PTEN has been found to augment doxorubicin-induced apoptosis in PTEN-null Ishikawa cells (cells that express truncated PTEN proteins) (Wan, Li, Xie, & Lu, 2007). Of note in this study was that the doxorubicin reduced the levels of phospho-AKT/PKB suggesting PTEN's role in apoptosis is through its regulation of the PIP3/AKT pathway. This hypothesis is supported by Vasudevan, et al.'s, study that showed that NFκB, up-regulated by AKT, suppresses PTEN activation which reduces apoptosis (Vasudevan et al., 2004).

PTEN has also been found to induce apoptosis in a PI3K/AKT independent manner, through the association of p53. Much as PTEN and p53 work together to induce cell cycle arrest, it has been found that p53, in association with its family members p63 and p73, can activate apoptotic genes in response to DNA-damage (Flores et al., 2002) and that a p73-PTEN complex enhances this response (Lehman et al., 2011). This at first appears to be in direct contrast to the above stated antioxidant activities of PTEN-p53 in response to DNA damage, but much as Wan, et al. found that the level of doxorubicin affected the activation of PTEN induced apoptosis (Wan et al., 2007), the degree of DNA-damage may indicate whether PTEN-p53 would induce apoptosis or antioxidant release.

As the apoptotic functions of PTEN are controversial, it is difficult to ascertain the result on longevity. Speculation, however, is that apoptosis effectively removes potentially harmful cellular toxins, misfolded proteins and damaged DNA from the system by destroying the cell (J. L. Liu et al., 2005; Ming & He, 2012). This removes the potential for malignant cells or for these toxins to spread. While not a direct influence on longevity as has been seen elsewhere, this cellular housekeeping functions to keep tissues and organs healthy, indirectly increasing longevity.

2.3.7 Caloric Restriction

A major breakthrough in the study of PTEN and how it relates to longevity has been the recent study done by Ortega-Molina. Of special interest was not the link between

tumour suppression and longevity as has been found with other tumour suppressors such as p53, Ink4a, Arf (Matheu et al., 2009; Matheu et al., 2007), but the effect PTEN has on caloric restriction and the implications this has for longevity.

Ortega-Molina, et al.'s study showed both a median and mean increase in survival and longevity (between 9-16%). Testing showed this was due to the PTEN transgene, independent of other variables. It was observed that the PTEN transgene extended longevity independently of its tumour suppressive functions, as cancer-free mice also showed a significant increased longevity. This would leave its effects on the PI3K/AKT pathway, nuclear functions and/or insulin pathways to be the culprit.

The authors found that PTEN transgenic mice, both young and old, had lower fasting levels of glucose and insulin serum levels, and a significantly lower value of the insulin resistance index HOMA-IR compared with the wild type control mice. This observed effect has been a widely reported feature of long lived mice, namely that of a decreased insulin and insulin-like growth factor 1 (IGF1) signalling (IIS) pathway (Bartke, 2008a, 2008b). This comes to pass through negative feedback routes from the IIS pathway itself (Carracedo & Pandolfi, 2008; Kamagate et al., 2010; Puig & Tjian, 2005).

Ortega-Molina, et al., further explored these negative feedback signals, especially in white adipose tissue (WAT) and found the main perpetrator was S6K which, along with mediating the nuclear export of PTEN as mentioned above, acts as a primary negative feedback signaller of the IIS pathway (Um, D'Alessio, & Thomas, 2006; Um et al., 2004). The WAT of transgenic PTEN mice presented reduced levels of AKT (as expected of increased PTEN activation) and also lower levels of phosphorylation in two relevant substrates of S6K, namely S6 and IRS1. S6K-mediated phosphorylation of IRS1 at Ser636/Ser639 is inhibitory for insulin signalling resulting in insulin resistance. Due to increased PTEN activation, the AKT pathway, which includes S6K, would be reduced, leading to less phosphorylation by S6K of IRS1 and as a result, less insulin resistance. The authors further strengthened this hypothesis by feeding the mice, control and transgenic, a high-fat diet for 6 months (a well-established technique for inducing metabolic stress, insulin resistance and liver steatosis) and found that while the transgenic mice increased their body weight at similar levels to control mice, they were responsive to insulin injections while control mice were not. Control mice were also shown to have extensive liver steatosis while transgenic mice showed minimal or no signs.

Caloric restriction (CR) has been tested and observed in many organisms, including nematodes, mice and humans, and has been attributed to the effect of oxidation (Mehta & Roth, 2009) from the IIS axis (Fontana, Partridge, & Longo, 2010; Kenyon, 2010). In *C. elegans* specifically, decreased PI3K (AGE-1) and increased PTEN (DAF-18) result in extended longevity (Dorman, Albinder, Shroyer, & Kenyon, 1995; Masse, Molin, Billaud, & Solari, 2005; Morris, Tissenbaum, & Ruvkun, 1996) and in mice, reduced IIS activity also yields and extends longevity (Bartke, 2008a; Ortega-Molina et al., 2012). Studies show that CR results in decreased levels of IGF1, a potent stimulator of IRS1 and thus the PI3K pathway (Hempenstall, Picchio, Mitchell, Speakman, & Selman, 2010; Jiang et al., 2008; Moore et al., 2008).

A commonly observed feature of long-lived mice, along with decreased IIS axis activity, is improved insulin sensitivity (Bartke, 2008b). This is due to the earlier mentioned reduction in the negative feedback loop stemming from S6K (Carracedo & Pandolfi, 2008; Kamagate et al., 2010; Puig & Tjian, 2005). Supporting this, the transgenic mice in Ortega-Molina's study showed lower fasting levels of glucose and insulin serum levels and significantly lower value of the insulin resistance index HOMA-IR (Ortega-Molina et al., 2012). Serum levels of IGF1 were also significantly, if moderately, lower in transgenic mice than their controls.

Despite being hyperphagic, these transgenic mice had a decreased body weight compared to control mice (27-28% decrease in young mice, (35-44% decrease in old mice) (Ortega-Molina et al., 2012). Transgenic mice had a higher resting metabolic rate relative to lean mass, and this was shown to be an effect of PTEN independent on lean mass. Respiratory quotients and body temperature were ruled out as possible explanatory factors. Epididymal WAT relative to body mass was also significantly lower in transgenic males, as well as serum levels of leptin and cholesterol.

The brown adipose tissue (BAT) of transgenic mice also showed an increased activity. This is significant as BAT is an efficient source to dissipate heat (Kozak & Anunciado-Koza, 2008; Nedergaard & Cannon, 2010), and the significant uptake of glucose in the BAT observed in the study suggests that the reduced weight of the transgenic mice may be due in part to their increased energy expenditure.

The importance of this to humans is twofold. Firstly, PTEN encourages caloric restriction which, through the decreased activity of the IIS axis and PI3K/AKT

pathway, enhances longevity. Secondly, PTEN reduces insulin resistance, assists weight loss and lowers cholesterol, which when taken together means PTEN is crucial in the fight against diabetes, obesity and high blood pressure, again enhancing longevity.

2.4 Cancer Properties

There are two reasons that PTEN's association with cancer is presented below. The first and most obvious reason is that cancer prevention is a straightforward extension of one's longevity by preventing the possible mortality associated with cancer. While it has no effect on the genetic enhancement of longevity, it cannot be understated that prevention of an illness is a key to extended longevity.

The second reason for the inclusion of this part of the review is that PTEN's functions in relation to cancer are closely linked to its nucleic and cellular functions. Its inhibition of the PI3K/AKT pathway and its functions with p53 in DNA-damage are used for normally functional cells as well as often damaged and deteriorated cancer cells. It is by the inhibition of normal PTEN functions that malignant tumours may form.

2.4.1 Mutations

2.4.1.1 Complete Loss/Haploinsufficiency

As with mutations that have a regulatory effect on the function of PTEN, mutations of PTEN is also a leading cause of cancer, with an estimated 50%–80% of sporadic tumours (includes endometrial carcinoma, glioblastoma and prostate cancer) and at 30%–50% in breast, colon, and lung tumours having monoallelic mutations of PTEN in common (J. Li et al., 1997; Risinger, Hayes, Berchuck, & Barrett, 1997; Tashiro et al., 1997; Whang et al., 1998). Complete loss of PTEN is associated with advanced cancers, metastases, and more recently been observed to be common in breast cancers caused by BRCA1 deficiency (Saal et al., 2008), and have been found mostly in endometrial cancer and glioblastoma (Ali et al., 1999).

Complete loss of PTEN is not certain to cause cancer, as it needs specific circumstances. Studies done on mice have shown that complete loss of PTEN is lethal in early development, yet heterozygous mice were viable only to develop a variety of tumours in later life (Cantley & Neel, 1999). More recently, however, a study showed that complete acute loss of PTEN did not induce hyperproliferation as expected, but promoted a strong p53-senescence response (Z. Chen et al., 2005). This suggests that complete loss of PTEN, in the absence of other mutations, may be detrimental to tumour growth, in contrast to the above finding of PTEN loss in various cancers. It does provide evidence though for why PTEN haploinsufficiency is more often presented in cancer than complete loss of allelic function.

The notion that complete loss of PTEN does not have a direct effect on cells, rather an indirect effect through downstream substrates is strengthened by several studies that have conditionally mutated both PTEN alleles using lox recombination to promote Cre recombinase specific tissues, both germ cells and somatic cells. The result was mutational inactivation of PTEN. This loss did not result in oncogenesis, rather subsequent generations of these PTEN null cells transformed into malignancies (Backman et al., 2001; Crackower et al., 2002; Kimura et al., 2003; C. H. Kwon et al., 2001; A. Suzuki et al., 2001). This suggests that, like the senescence response via p53, this oncogenic response is through another downstream effector of PTEN, possibly PIP3/AKT.

As reviewed in Salmena, et al., various mechanisms can create a gradual loss of PTEN and thus a gradation of tumour suppression, ranging from 0% loss to 50% heterozygous to 100% (homozygous) loss (Salmena et al., 2008). These mechanisms can include mutations, transcriptional repression, post-translation modification, epigenetic silencing and aberrant localisation, many of which have been reviewed earlier in this thesis. Many of these, in combination can create a continuum of PTEN functionality. For example, in mice PTEN is haploinsufficient for tumour suppression and thus 50% of total PTEN is insufficient for tumour suppression.

Other studies using PTEN haploinsufficient mice have shown favourable tumour conditions where mice develop colonic adenomas, lymph node hyperplasia and prostate tumours at greater rates than controls due to deregulation of the PI3K/AKT pathway (Kwabi-Addo et al., 2001; Podsypanina et al., 1999). Other studies with mice have shown that loss of one PTEN allele promotes development of lethal polyclonal

autoimmune disorders (Di Cristofano et al., 1999) and that PTEN heterozygosity is a driving force for epithelial cancers like prostate cancer (Di Cristofano, De Acetis, Koff, Cordon-Cardo, & Pandolfi, 2001), suggestive of PTEN haploinsufficiency.

Despite this evidence, the theory that PTEN is haploinsufficient is still undetermined, although there are reports that substantiate this. However, PTEN is haploinsufficient for PHTS due to PTEN heterozygosity results in distinctive phenotypes such as developmental disorders and benign polyps, PHTS also severely increases the risk of developing malignant tumours (Dahia, 2000). Tumours that have been derived from individuals with Cowden syndrome have a tendency not to present detectable biallelic mutations of the PTEN gene (Marsh et al., 1999), suggesting only one allele remains, strengthening the haploinsufficiency hypothesis.

2.4.1.2 Germline Mutations

Germline mutations of PTEN have mostly been found in hereditary, autosomal dominant cancer syndromes with shared characteristics such as developmental defects and disorders, neurological deficits, multiple benign hamartomas and an increased risk of cancers. The aberrant growth and hamartomas have been shown to penetrate all three germ layers (Rustad et al., 2006), evident of a mutation in the germline.

These syndromes are Cowden syndrome, Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba syndrome, and Proteus syndromes which collectively make up the PTEN hamartoma tumour syndromes (PHTS) (Liaw et al., 1997; Marsh et al., 1997; A. Suzuki et al., 1998; B. P. Zhou et al., 2001). These have been associated with the deregulation of the PI3K/AKT pathway and PI3K inhibitors have been suggested as potential treatments (Blumenthal & Dennis, 2008). Mice with PTEN mutations have been shown to be susceptible to tumours in various organs such as skin, prostate and mammary glands (Backman et al., 2004; G. Li et al., 2002; Ming et al., 2011; G. Suzuki, 2003; Yanagi et al., 2007).

More recently, studies have reported PTEN germline mutations in patients with macrocephaly, mental retardation and autism spectrum disorders, while showing little to none of other PHTS symptoms (Busa, Chabrol, Perret, Longy, & Philip, 2013). These studies have shown that as many as 10% of cases involving autism spectrum disorders and macrocephaly had PTEN germline mutations (McBride et al., 2010). While the

connection between PTEN mutations and these neurological disorders remain unclear, there have been associations made with the PI3K/AKT pathway, much like with PHTS.

2.4.1.3 Somatic Mutations

Somatic mutations of PTEN have been documented in a variety of cancers and tumours, both at early and advance stages. These mutations can lead to several different results: either inactivation of PTEN's phosphatase activity through mutation on the C-terminal or partial or total loss of either mRNA and/or protein expression (Simpson & Parsons, 2001). While typically a mutation is accompanied by loss of heterozygosity, resulting in the above mentioned possible haploinsufficiency, some tumours have appeared to evolve mechanisms to reduce concentration of PTEN without visible mutations of the gene (Whang et al., 1998).

Of note is that the frequency of PTEN mutation is nearly as great as that of p53 (Cantley & Neel, 1999) and that both have been associated with total inactivation. Both have been putatively linked to stimulation of the PI3K/AKT pathway. With the closely linked activities of both genes in cancer suppression, cell cycle arrest and DNA-damage repair, it is unsurprising that both would be targeted for mutation.

PTEN mutations have also been studied in model organisms such as Drosophila where it alters cell size, proliferation, apoptosis and cell migration (X. Gao, Neufeld, & Pan, 2000; Oldham et al., 2002; Scanga et al., 2000), much like in humans. However, tissue-specific PTEN mutation in mice revealed that, among several phenotypes, PTEN inactivation did not result in tumours but created an environment that selects for tumour growth. This is potentially linked with the above mentioned Cre recombinase experiments.

2.4.2 Regulatory Effects Causing Cancer

Unsurprisingly, the regulatory effects of and on PTEN implicated in cancer development will have close ties with the previous section on regulation of PTEN. This is because, as PTEN is a tumour suppressor, its effects on tumours are only observed once it becomes inactive or down-regulated. While the earlier section on PTEN

regulations focused on the mechanisms of regulation, this section will focus on the effects.

Reports have indicated that a large part of PTEN's tumour suppression is due to its nuclear functions. It was found that the absence of nuclear PTEN is commonly associated with the more aggressive diseases in patients with cutaneous melanoma (Whiteman et al., 2002; Zhou et al., 2000), large B cell lymphoma (Fridberg et al., 2007), colorectal cancer (Zhou et al., 2000, oesophageal squamous cell carcinoma (Tachibana et al., 2002) and pancreatic islet cell tumours (Perren et al., 2000). In these studies, the absence of nuclear PTEN is more markedly observed in undifferentiated and metastatic tumours (Gimm et al., 2000). With this, Liu, et al. found that forced nuclear expression of PTEN antagonizes anchorage-independent growth (Liu et al., 2005).

A more general study regarding the tumour suppressive functions of PTEN was done by Ortega-Molina, et al. In this study the authors found that mice with an extra transgenic PTEN gene had significantly less incidence of fibrosarcomas than wild type mice. Histological exploration revealed a significantly reduced number of malignant tumours, most notably lymphomas and histiocytic sarcomas in transgenic mice.

While the exact mechanisms and causes are as of yet unclear, it can be suggested that the nuclear co-operation between PTEN and p53 in regards to DNA-damage may be significant. These two genes can arrest the cell cycle, leading to senescence and can initiated DSB repair of damaged DNA as well as other antioxidant effects through downstream effectors of p53. The antioxidant properties can prevent damage occurring to DNA. DNA repair mechanisms can repair any damage that does occur, and if these fail, the cell cycle can be arrested to induce senescence. These are all working to prevent the formation of tumours.

Another oncogenic protein suppressed by nuclear function is the protein MSP58, which Okumura, et al., discovered transformed PTEN null mouse embryonic fibroblasts, yet was abrogated in the presence of introduced PTEN (Okumura, Zhao, Depinho, Furnari, & Cavenee, 2005a; Okumura et al., 2005b). MSP58 was also abrogated by a phosphatase inactive PTEN mutant suggesting that, as with the other nuclear PTEN functions described earlier, this is done potentially through physical interaction of the C-terminal domain.

One of the most commonly observed methods by which PTEN has been suppressed has been during transcription. Methylation of the PTEN promoter region has been associated with reduced PTEN expression in various cancers (Soria et al., 2002). The Ras-Raf-MEK-ERK pathway has been demonstrated to have a link with aberrant down-regulation of PTEN transcription in both epithelial and fibroblast cell types in human cancer cells through the factor c-Jun (Hettinger et al., 2007; Vasudevan, Burikhanov, Goswami, & Rangnekar, 2007) and through transforming growth factor beta (TGFβ) dependent mechanism in pancreatic adenocarcinoma. MiRNAs, and miR-21 in particular, disrupts expression of PTEN through binding to transcripted sequences (Meng et al., 2006; Meng et al., 2007).

As well as the nuclear functions regarding p53 described above, the suppression of PTEN would lead noticeably to the unregulated behaviour of the PI3K/AKT pathway leading to uncontrolled proliferation and cell growth. This would result in the proliferation of cells containing, potentially, deleterious segments of DNA, which may result in tumours.

There is also positive regulation of PTEN involved when regarding cancer. Tuberous sclerosis 1 and 2 (TSC1 and TSC2) forms a complex that inhibits mTOR and so inhibits S6K (Chung et al., 2006). Along with reducing insulin resistance, this also reduces the nuclear export of PTEN. The TSC1 and 2 complex, however, are down regulated by AKTA via phosphorylation of TSC1. This phosphorylation leads to the degradation of the TSC1 and 2 complex, disallowing it from inhibiting mTOR. Both TSC1 and TSC2 are tumour suppressors and are mutated in tuberous sclerosis (TSC). TSC is associated with benign hamartomas and brain abnormalities (Gil et al., 2006), much like PHTS, although both are required for efficient TSC suppression. As the TSC1 and 2 complex helps reduce incidence of PTEN nuclear export, and PTEN reduces AKT phosphorylation of the TSC1 and 2 complex, one can see that the tumour suppressive functions of PTEN are much broader than only its own syndromes.

3 PTEN Genetic Overexpression and Protein Level Determination

3.1 Introduction

This chapter will cover the transfection of the HUVEC cultures and the ELISA assay that was used to verify the transfection. These were the first necessary steps in the experiment and the importance of these cannot be understated. The processes and protocols listed below follow the preliminary trials that were done to assess the viability of the methodology. The failures of the preliminary trials' 1µg plasmid doses inspired the notion of a dose dependent study. The initial culturing of the cultures will be discussed in Chapter 5, while this chapter will deal solely with the transfection and ELISA.

3.2 Methods and Materials

3.2.1 Procurement of Materials

The PTEN (RefSeq: NM_000314.3) plasmid was obtained from Origene as a complete plasmid, category number: RC202627. The reason for obtaining the complete plasmid rather than only the cDNA was because of time constraints regarding the cloning of plasmids.

The PTEN ELISA kit was acquired from Antibodies-online.com. The reason for choosing an ELISA kit over a Western Blot kit was that ELISA would deliver more

quantifiable results that would be much better suited to be used in mathematical modelling than Western Blot.

3.2.2 Transfection protocol

The day prior to transfection, the cultures were trypsinized, the cells were counted and replated at $8x10^4$ cells per well in 1ml of Complete Medium 200. This was to ensure cell density was at 50-80% confluent at time of transfection.

The first step as shown in Figure 2 was to dilute 10µg of DNA into 100µl of distilled water and 10µl of this was removed for transfection use. The rest were frozen, ready to be used in emergency cases. The 10µl of diluted DNA were then diluted again into 6 aliquots of 0.1µg of DNA, 5 aliquots of 0.01µg of DNA and 1 aliquot of 1µg of DNA. The purpose of this was to determine what dose of DNA was best for transfection. The DNA was then added into 12 vials of 200µl Opti-MEM Reduced Serum Medium.

Into each vial, PLUS reagent was added at a 1:1 ratio to the diluted DNA and the mixtures incubated for 5-15 minutes at room temperature. Lipofectamine LTX was added to each vial. For the 3 columns of the 12 well plate, Lipofectamine LTX was added at a ratio of 2:1, 3:1, and 5:1 to the DNA in each respective column. The vials were left to incubate for 25 minutes at room temperature.

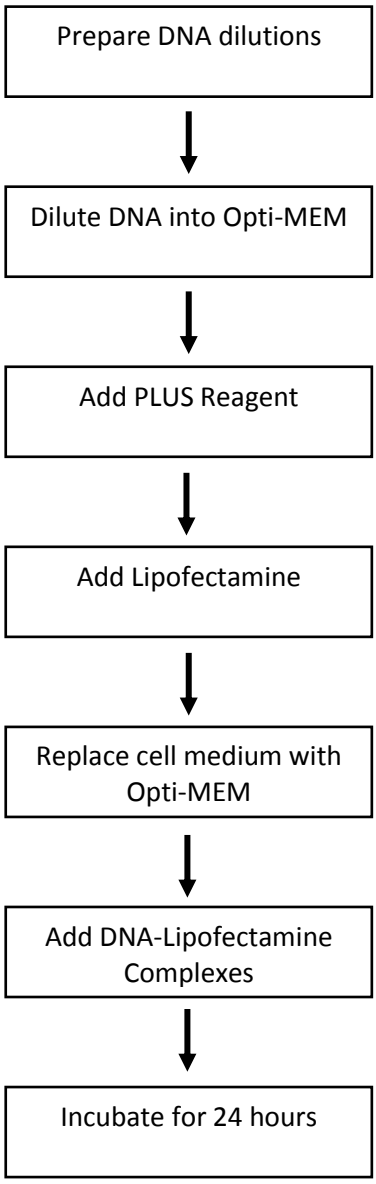

Figure 2: Simplified Protocol for Lipofectamine transfection.

The growth medium was removed from the cells and replaced with Opti-MEM Reduced Serum Medium. 200µl of the DNA-Lipofectamine LTX complexes were added to each cell, making sure to mark the well to indicate the amount of DNA and Lipofectamine LTX in that well. The plate was then incubated at 37°C, 5% CO2 in an incubator for 24 hours before the Opti-MEM Reduced Serum Medium were replaced with Medium 200.

3.2.3 ELISA protocol

Reagent Preparation

Wash Buffer was diluted by a factor of 20 in Milli-Q water.

Sample Preparation

Cells were washed once with Phosphate Buffered Saline (PBS). PBS was removed and Cell Lysis Buffer were added and homogenized. Cells were centrifuged for 10 minutes at 4°C and the supernatant removed for use.

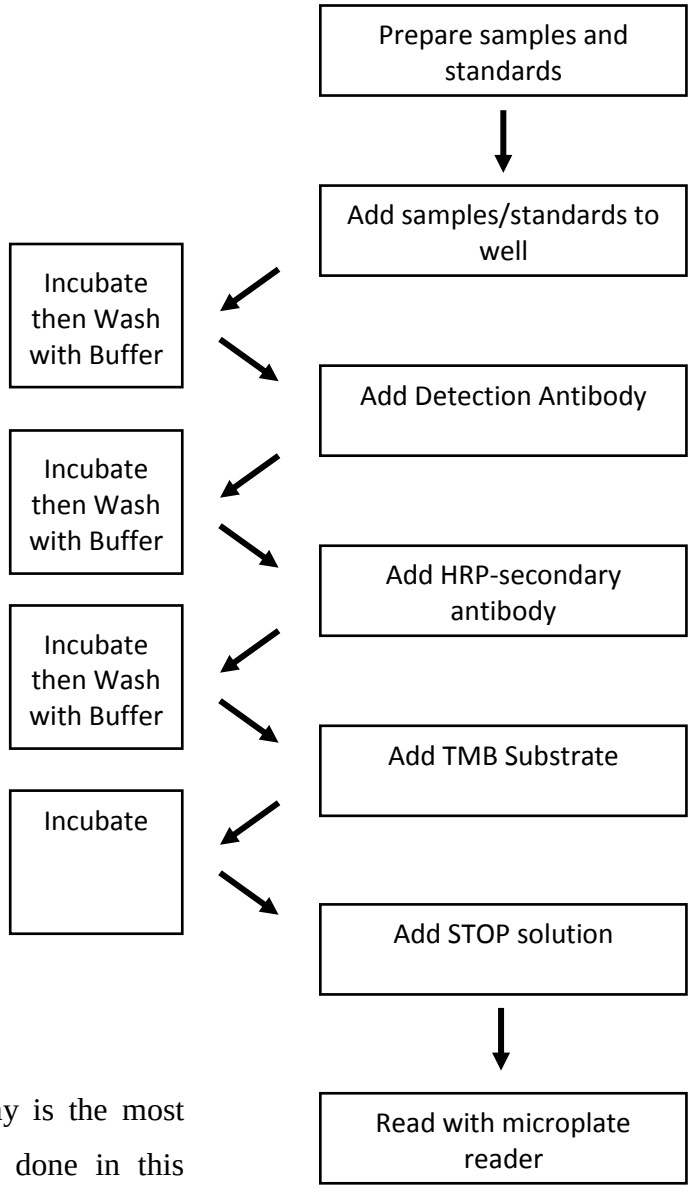

Figure 3: Protocol for PTEN ELISA.

Assay Procedure

As shown in Figure 3, the assay is the most difficult experiment to have been done in this thesis. For each well to be assayed, 100µl of Sample Diluent and 100µl of cell lysate were vortexed before 100µl of this diluted cell lysate were added to each well. The plate was then incubated for 2 hours at 37°C. The plate contents were removed and the wells washed 4 times with 200µl of Wash Buffer. 100µl of Detection Antibody were added to each cell and the plate incubated for 1 hour at 37°C before being washed again. 100µl of HRP-linked secondary antibody were added to each cell and the plate again incubated for 30 minutes at 37°C. The wells were then washed again as previously described. 100µl of TMB Substrate were added to each well and the plate incubated for 10 minutes

at 37°C. 100µl of STOP Solution were added to each cell and the plate agitated. The plate was then read by a microplate reader at 450nm to ascertain results.

3.3 Statistical Analysis

To ascertain the levels of PTEN in the various samples, the cells in each well to be used were counted prior to performing the experiment. The purpose of this was to display the data per $10x10^4$ cells; otherwise the results would be skewed towards those wells with higher cell numbers. The normalised results were then compared against the control samples as well as each other using linear models and *t*-tests for tests of significance.

3.4 Results

The first major milestone in this thesis was the transfection of exogenous PTEN plasmids into the HUVEC cultures. To test the success or failure of this transfection, an ELISA was performed.

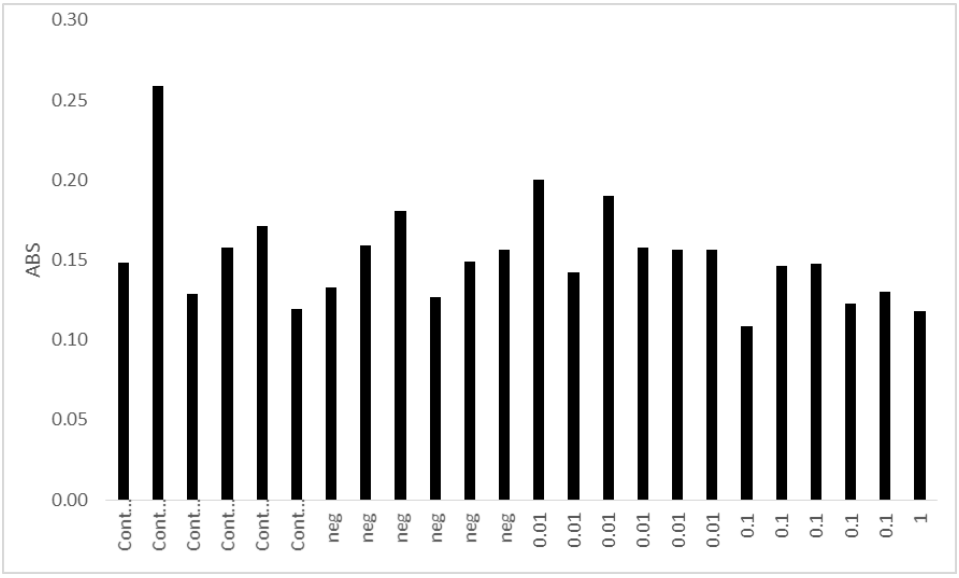

Figure 4: Raw data extracted from ELISA assay, measured in ABS, 24 hours after transfection. The categories are the positive and negative controls, and the three doses of PTEN: 1µg, 0.1µg and 0.01µg.

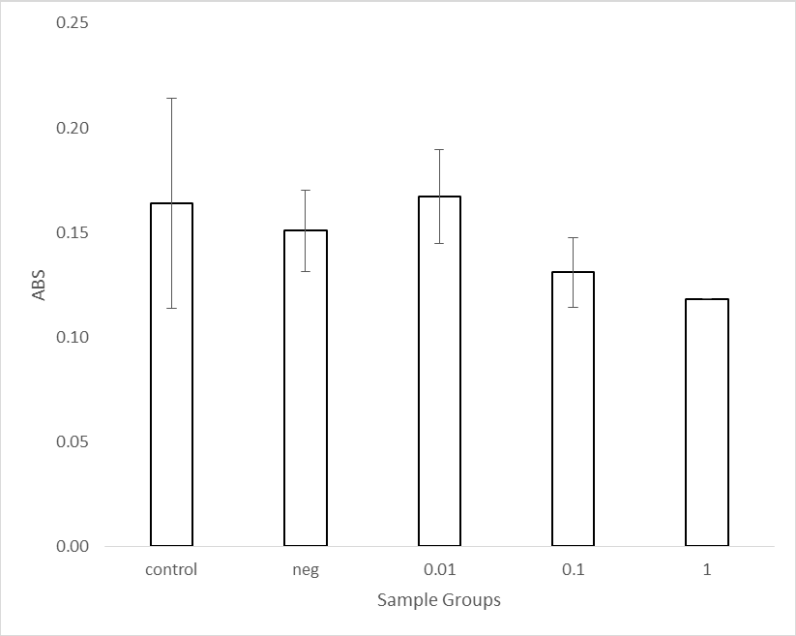

Figure 5: Average of raw data extracted from ELISA assay, measured in ABS, 24 hours after transfection, with standard deviations above and below the mean. The categories are the positive and negative controls, and the three doses of PTEN: 1µg, 0.1µg and 0.01µg.

As shown in Figure 4, the raw data obtained from the result of the ELISA shows, at first glance, a mixed field of results ranging from its minimum of 0.1083 (0.1µg PTEN) to 2.590 (Control sample). The mean of each category, shown in Figure 5, emphasises this. The Control and 0.01µg mean samples were 0.1641 ±0.05 and 0.1671±0.023 respectively, and lowest mean sample was the 1µg dose at 0.1183±0.

The reason for only one 1µg sample in the ELISA sets, and all following data, was because of the preliminary tests that were run prior to the beginning of the main experiments. In these preliminary tests transfection of 1µg per well resulted in a uniform death of cells before ELISA could be performed. This was believed to have been because the apoptotic effects of PTEN may have been too overabundant at 1µg, and so it was decided that, in the main experiments, only one well of 1µg would be run to test if this was the case.

As can be seen in Figure 4, the second data point, the Control sample at 2.590 is significantly higher than any of the other data points. This can also be seen in the standard deviation of the Control mean samples in Figure 5. A possible reason for this may be that, because of the photometric method used in ELISA tests, the well was dirty and so gave a false, or inflated, value. As shown when the values are normalised per

10x104 cells in Figure 6, this is even more pronounced as the value is significantly higher than the other Control sample values.

Figures 6 and 7 also show that when the samples are normalised per the cell numbers, the data appears more even, and that there is a distinct grouping between those cells that have not been transfected and those that have. Amongst the individual data points, the lowest value is now 0.0604 (Negative Control) while the highest value is 0.2093 (0.1µg PTEN). This is in stark contrast to the previous minimum and maximum values.

When comparing the data of Figures 6 and 7, one can see that where before there were no significant differences between the five categories of samples, there is now a clear difference between the categories, especially between the 0.1µg and 0.01µg PTEN sets of samples and the other three sets.

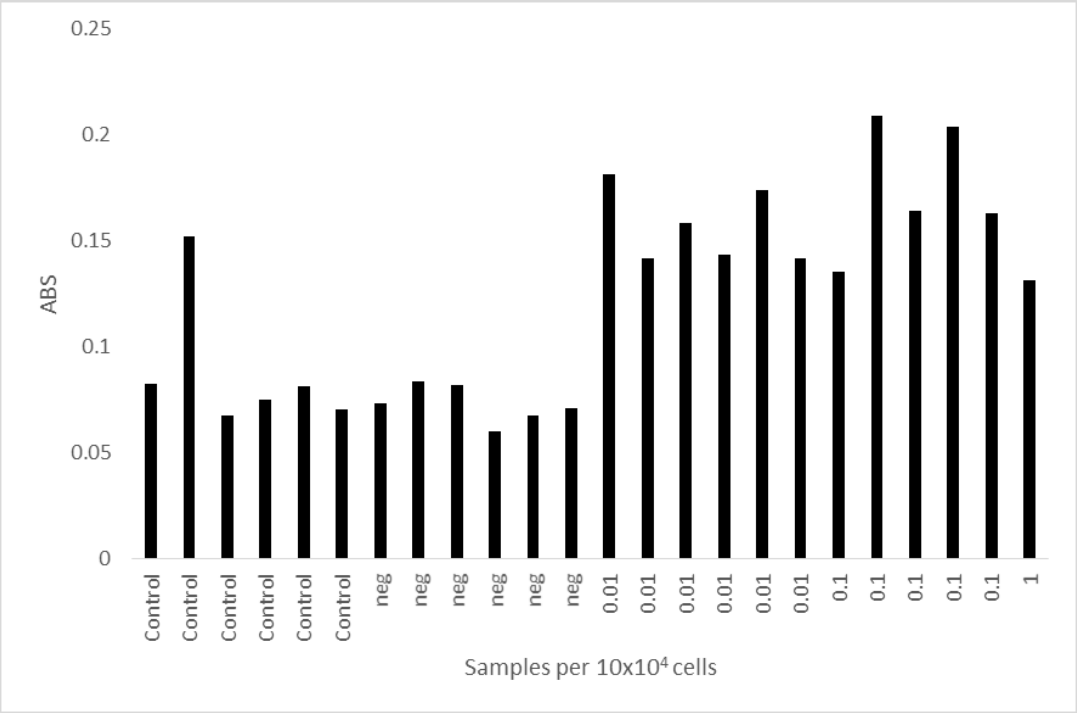

Figure 6: Values of ELISA normalised per $10x10^4$ cells. Categories remain as previously described.

Using *t*-tests, both 0.1µg and 0.01µg samples show a statistically significant increase over the control samples: P-value for 0.1µg is 0.0015; P-value for 0.01µg is 0.0019. This shows that there is a clear increase in the production of PTEN protein in the transfected cultures. This means that the transfection was a success.

T-tests showed that there is no statistically significant difference between the 0.1µg and 0.01µg sets: P-value of 0.2877. If one combines these two sets and then test this new set against the control, it is revealed that there is, unsurprisingly, a statistically significant difference between them: P-value = 0.0008.

There is no statistically significant difference between the negative control and the positive control sets: P-value = 0.3085. This suggests that the empty vectors that were transfected into these cultures had no overt effect on the cells.

As there is no significant difference between the transfected samples and between non-transfected samples, it is possible to test these two groups as a whole against one another for significance. This revealed that there was a great difference between transfected and non-transfected samples: P-value = 5.283×10^{-8}.

As the 1µg set only contains one sample, it was not possible to do a *t*-test between it and the Control set.

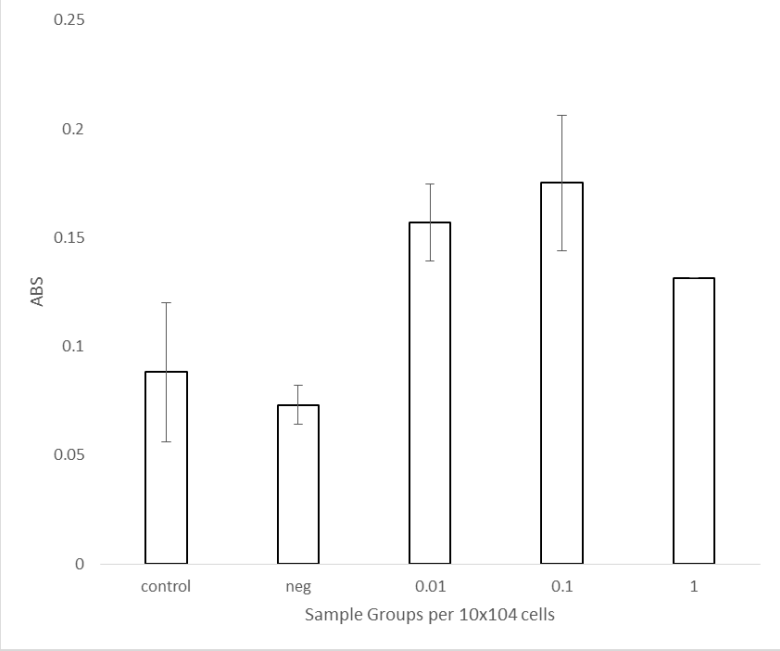

Figure 7: Mean values, with standard deviations above and below the mean, of ELISA values normalised per 10×10^4 cells. Categories remain as previously described.

Figure 8 shows the mean number of cell divisions, per culture group, for the period between the seeding of the plates for transfection and the counting of the cells for the ELISA test compared with the overall mean number of cell divisions underwent per passage by each culture group.

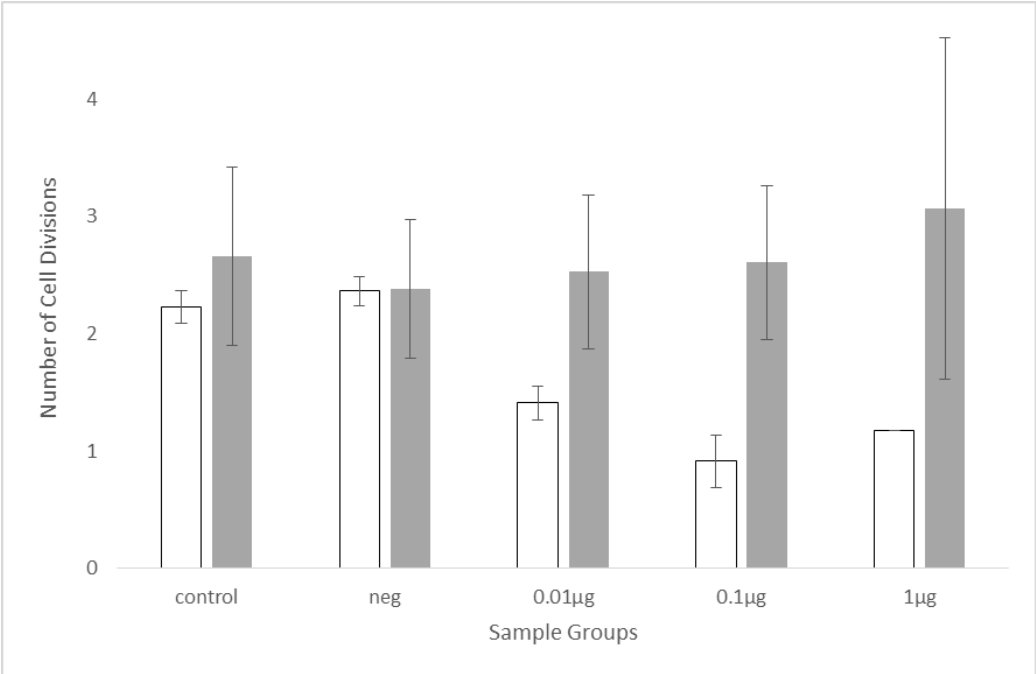

Figure 8: Mean number of cell divisions: for the period between seeding for transfection and ELISA in white; overall per passage level in grey. Separated per culture group, with standard deviations above and below the mean.

As seen, there is a distinct difference in the values between the non-transfected cultures and the transfected cultures. There is no statistically significant difference for the non-transfected cultures between the overall mean number of cell divisions and mean number of cell divisions for the period of the transfection (P-value = >0.05). However, there are large statistically significant differences in the transfected cultures between the sets of data, as shown in Table 3.

Table 3: P-values of *t*-tests done between the mean number of cell divisions for the period of the transfection and the mean number of cell divisions per passage level.

	Control	Negative Control	0.01µg	0.1µg	1µg
P-value	0.5764	0.5425	0.001037	0.00003139	NA

The P-values for the transfected cultures are all <0.05. The exception is the 1µg set due to it only containing one sample and therefore a *t*-test cannot be done using it. However, the 1µg set has the highest difference, of 1.899, between the two data sets, compared with the 1.695 difference between the 0.1µg sets: this implies a high degree of significance.

3.5 Discussion

It goes without saying that without a successful transfection, the rest of this thesis' experiments could not have taken place. Thus, the transfection and the subsequent ELISA was the first major milestone to be achieved in this thesis.

After the ELISA was done and the results normalised, the data showed a clear distinction between cells that have been transfected and those that have not been. As had been expected, the transfected cells showed a significant (P-value = 5.283×10^{-8}) increase in the levels of PTEN above that of the non-transfected cells. Of these transfected cells, the group that showed the greatest levels of PTEN was the 0.1µg group at 0.175±0.031 compared with the lowest levels of PTEN, the Negative Control group at 0.073±0.009. However, this was not significantly higher than the 0.01µg group at 0.157±0.018 (P-value= 0.2877). This early data would suggest that the transfection success was independent of its dose.

The decision to include three doses of the PTEN plasmids during the transfection was as much for practicality's sake as for scientific curiosity's sake. During the preliminary trial of the transfection, only one dose (at 1µg was used) was used. Although manufacturer's protocol was followed, this resulted in total cell death of all transfected culture wells.

Thinking that it might be an overdose of the plasmid that caused this, due to PTEN's known ability to cause senescence and apoptosis (Gil et al., 2006; Wan et al., 2007), it was decided that in the subsequent transfection, 0.01µg and 0.01µg doses of the PTEN plasmids would be used with only one well transfected with a 1µg dose to determine if the cell death would occur again. As can be seen in Figure 4 it clearly did not. This, however, lead to the issue that because the 1µg group only contained one sample, it was not possible to do a *t*-test comparison between it and any other individual group.

In the same vein, to determine the toxicity of the PTEN plasmids, the number of cell divisions between the period of seeding the plates for transfection and the ELISA test were compared against the mean number of cell divisions each culture group underwent each passage. As was shown in Figure 8 the mean number of cell divisions underwent,

for transfected cultures, during the transfection process was remarkably low compared to what was expected. This was first presumed to be due to the known toxic effects of the Lipofectamine reagent, although the Negative Control showed no significant decrease in cell divisions.

As the PTEN plasmids are the only point of difference between the Negative Control and transfected cultures, one can assume PTEN was to blame. As with the preliminary trials, the most likely reason for the decrease in cell division numbers is the apoptotic functions of PTEN. If more than one copy was transfected into the cells' DNA, it is possible that, rather than producing the sought for longevity extension effects, the excess of PTEN may have caused the cells to enter apoptosis. Comparing the 0.01µg and 0.1µg sets, this seems likely, although the 1µg set do not contain enough samples in order to determine if this trend continues.

The result of the Negative Control group is as important as those of the transfected cultures. As the Negative Control group was only transfected with an empty vector, the results indicate that this vector had no influence on PTEN. The mean cell count for the Negative Control group was 20.7±1.6 while the Control group was 18.8±1.7, an insignificant difference (P-value = 0.107). This is in stark contrast to the PTEN transfected cells whose mean cell count was 9.25±1.7. This, supported by the other results, show that the empty vector had no discernable effect on the cells, and that it was by the PTEN plasmid alone that any effect was observed. This is important as it allows the further results to be interpreted in the secure knowledge that it is only PTEN's effects that is being witnessed.

Apart from the importance that a successful transfection has to this study alone, the successful transfection of PTEN into a HUVEC culture is significant for further studies as it was the first to be done that this author could find in current literature. HUVEC has been used as a cellular model in the past, mainly for vascular studies (Rhim et al., 1998), and it has been described as "difficult to transfect using standard non-viral transfection methods" (Jenson, 2012). Due to the possible applications that PTEN could have as a drug, this successful transfection then represents a major step forward for future studies involving human transfection of PTEN.

3.6 Conclusion

The transfection of PTEN plasmids into HUVEC cultures was done as the first step to determine whether increased levels of PTEN had a positive effect on cellular longevity. An ELISA assay was done to verify this transfection and to record the results for further use. This transfection was found to be a success with transfected cultures showing a significantly greater level of PTEN than non-transfected cultures (P-value = $5.283x10^{-8}$). The greatest levels of PTEN was found in the 0.1µg set although it was not a significant departure from the 0.01µg (P-value= 0.2877). The data obtained represented a good result in itself as it could be applied to the rest of the thesis in modelling and correlations.

4 Determination of Total Antioxidant Potential

4.1 Introduction

The importance for an antioxidant assay lies with the published literature surrounding PTEN's capacity to increase antioxidant levels. The two main pathways for this to occur are through the p53 (Sahin & DePinho, 2012) and PI3K/AKT (Salminen, Hyttinen, Kauppinen, & Kaarniranta, 2012) pathways. The role of antioxidants and autophagy to remove oxidants such as ROS, thereby preventing DNA and telomere damage is well documented (Blagosklonny et al., 2010; Kenyon, 2010; Rubinsztein et al., 2011; von Zglinicki, 2002) and it is thought that the main method by which PTEN can improve cellular longevity is by increasing antioxidant levels. This assay was chosen in order to test that hypothesis.

4.2 Methods and Materials

4.2.1. Procurement of Materials

The AOP-450 assay kit was obtained from OxisResearch, a division of Oxis Health Products, for the purpose of determining the difference in total antioxidant potential between transgenic cells and control cells. The assay accomplishes this through the reduction of Copper+2 (Cu^{+2}) to Copper+1 (Cu^{+}). Cu^{+2} has the ability to form ROS molecules, increasing oxidative damage. By reducing Cu^{+2} to Cu^{+}, the potential oxidative stress is reduced and so the total antioxidant potential of the culture is increased.

4.2.2 AOP-450 protocol

Sample Preparation

Cells were washed 3 times with PBS prior to lysis. Cells were lysed by homogenization and then were centrifuged at 3000 x g for 12 minutes. The supernatant was removed and used for the assay.

Assay Procedure

Both the samples and provided Standards were diluted in the Dilution Buffer at a ratio of 1:40 to create a 600µl solution. 200µl of diluted samples or standards were pipetted into each well with pure Dilution Buffer used as Reagent Blanks. The plate was then read by a microplate reader at 450nm for a reference measurement. 50µL of Copper Solution was added to each well. As shown in Figure 9, the only incubatory period for this experiment was this time for 3 minutes at room temperature. 50µL of Stop Solution was then added and the plate read for a second time at 450nm.

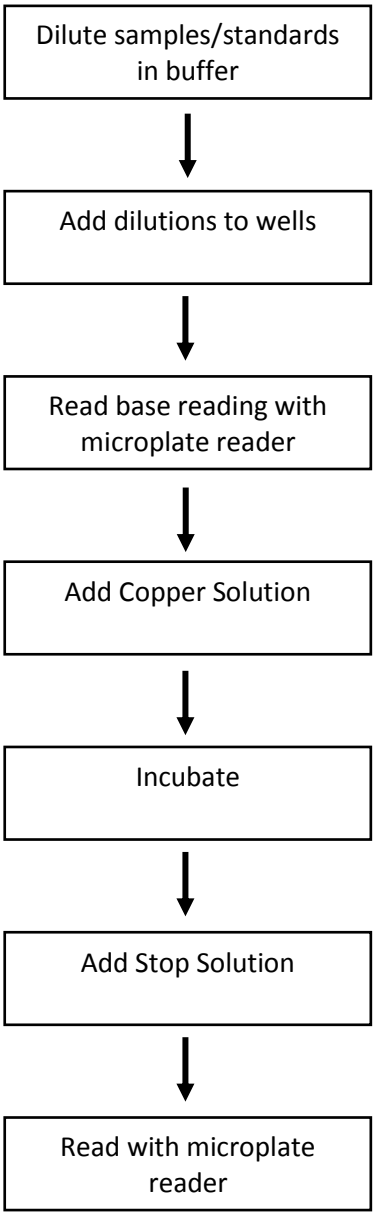

Figure 9: Protocol for AOP-490

4.3 Statistical Analysis

To calculate the total antioxidant power of the cells, the readings taken before the Copper and Stop Solutions were added was subtracted from the readings taken after the Solutions were added. This equated to the net absorbance. Next, a standard curve was created using the Trolox standards. As per the protocol, to display the data in terms of µM Trolox equivalents and µM copper reducing equivalents (CREs), the samples were divided by the slope, calculated from the standard curve. Or expressed as:

Solve for x:

$$y=mx$$

Where: y= assay readings

m= slope

x= trolox concentration

While this would give a reliable result, a more proper investigation of the data as per (P. J. Brown, 1982) would provide more accurate results, but due to time constraints this would be better dealt with in further studies (Hankin, 2014).

As with the ELISA test, the samples were then normalised per the cell numbers and compared, firstly, to the control samples and then each other by using *t*-tests.

4.4 Results

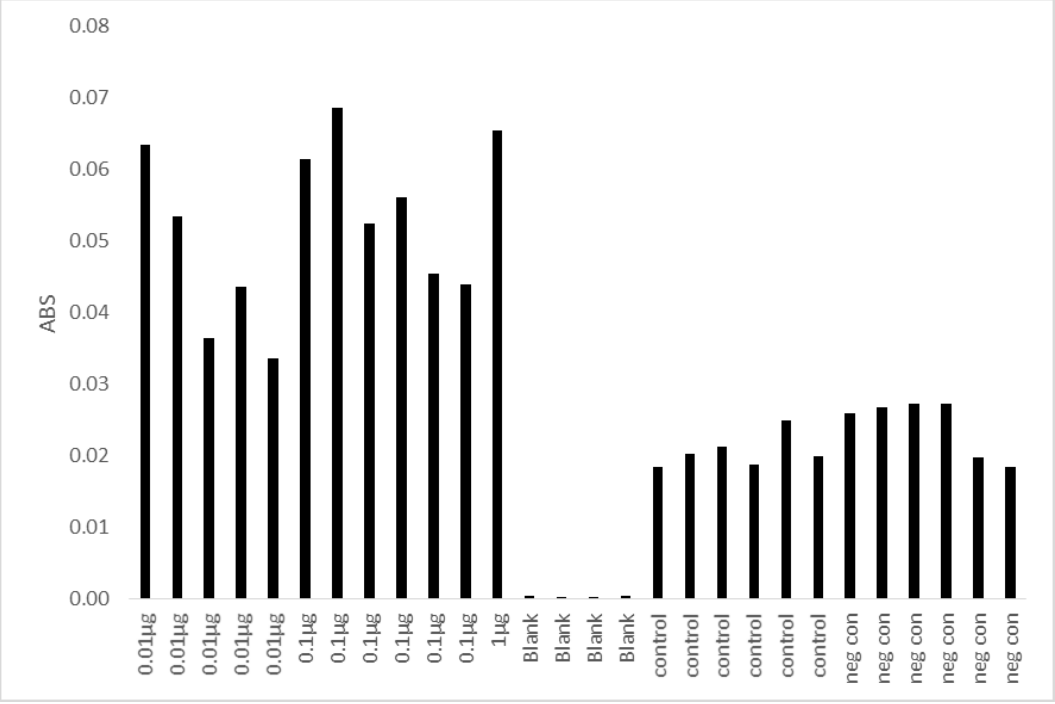

Figure 10: Net absorbance, expressed in ABS, of the AOP-450 assay. The six categories of samples are Blank [Wells], Control, Negative Control, 0.01µg, 0.1µg and 1µg.

The purpose of the AOP-450 assay was to determine the total antioxidant potential in the cultures with the intent on discovering if the cultures with transfected PTEN would produce a higher total antioxidant potential than those without. To that end, the assay works by taking two measurements, one before the STOP solution (explained previously) is added and one afterwards, whereupon one subtracts the former from the latter to achieve a net absorbance rate as shown in Figure 10 above.

As with the ELISA, the data was normalised by taking into account the cell numbers from each culture. The data was then expressed as Net Absorbance per 10x104 cells, as shown in Figure 12. At first glance there is not an immediate difference between the two sets of data; there is still a distinct difference in the ABS between the control sets and the transfected sets (albeit a lesser difference in Figure 12). The maximum value amongst both graphs belongs to the 0. 1µg sets (0.0686 before normalisation, 0.0510 for after), while the minimum value belongs to the Control set (0.0185) before normalisation and to the Negative Control set (0.0132) after.

The reason for this similarity is that the standard curve for the assay has not yet been created and thus the data has not yet been expressed in terms of antioxidant equivalents.

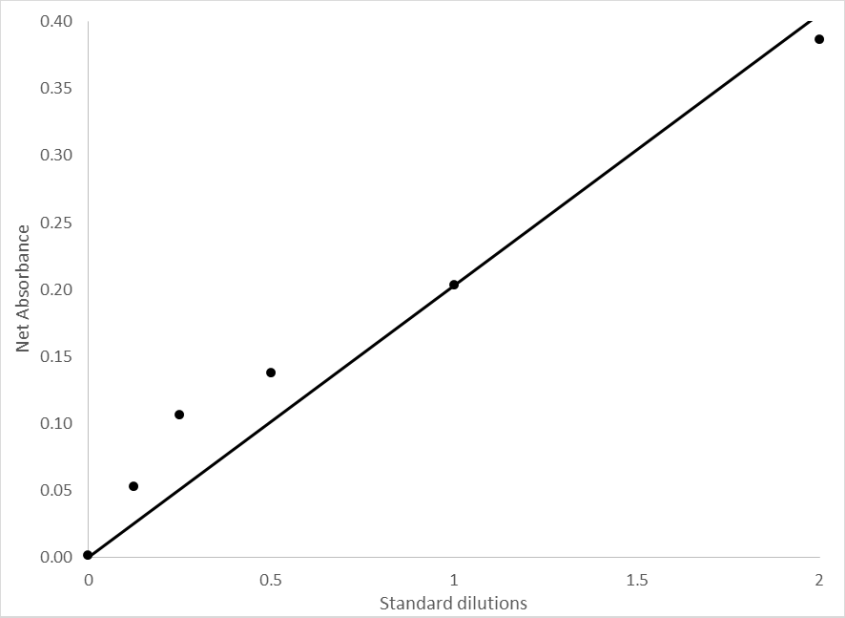

Figure 11: Standard curve produced from the net absorbance values taken from the AOP-450 assay with the slope forced through an intercept of 0.

The standard curve was comprised of six dilutions of the Trolox standard: 0mM, 0.125mM, 0.25mM, 0.5mM, 1mM and 2mM. The standard curve that resulted from the assay lead to a slope with an intercept of 0.03287. This was because the 0% concentration standard's net absorbance used in the assay was 0.0006 rather than a true 0. A *t*-test on whether this closeness to 0 was significant resulted in P-value of 0.0002. With no evidence that the intercept was not 0, the intercept was forced to zero which led to a slope of 0.20286.

The result of the data expressed, after being adjusted via the standard curve's slope, can be seen in Figures 13 and 14. As expression in µM copper reducing equivalents (CRE's) is merely the µM Trolox equivalent figure multiplied by 2189, there are no discernable differences between the two graphs beyond the measurements that are used. As such, they are shown here in the interest of completeness but priority will be given to CRE for the purposes of examination.

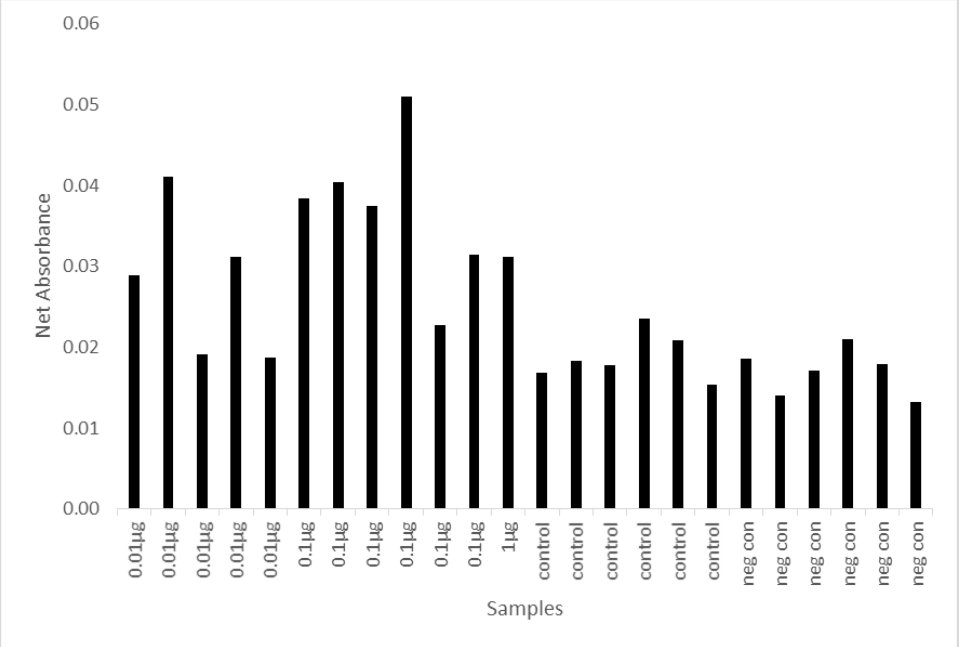

Figure 12: Net absorbance, expressed in ABS per $10x10^4$ cells, of the AOP-450 assay. The five categories of samples are Control, Negative Control, 0.01µg, 0.1µg and 1µg.

Firstly, as with the previous and ELISA results, the minimum value is a part of the non-transfected cultures, while the maximum value is of the transfected cultures. The minimum is the Negative Control (183.0997±31.4) and the maximum is the 0.1µg (398.1716±101.6). With a Control value of 202.4562±31.9, it is unsurprising that the *t*-test between the 0.1µg set and Control set resulted in a P-value of 0.0041, showing a great statistical significance.

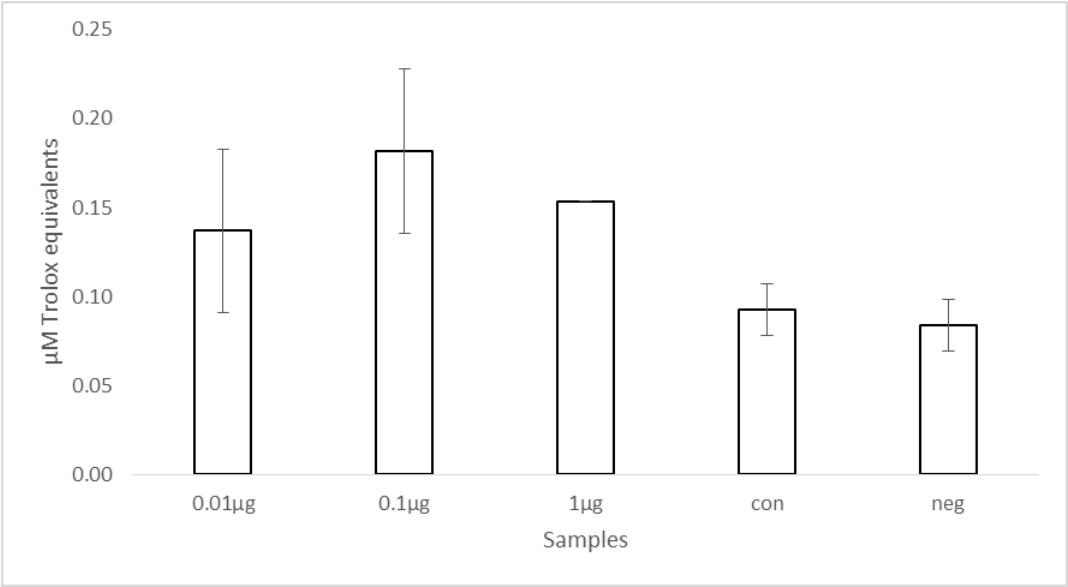

Figure 13: Mean µM Trolox equivalents with standard deviations above and below the mean of the five sets of samples, normalised to$10x10^4$ cells.

Dissimilar to previous results, a *t*-test performed between the 0.01µg set and Control set resulted in a P-value of 0.0957, showing that the difference between these two sets are not statistically significant. This could potentially be due to the high degree of variance in the 0.01µg set. Similarly, the difference between the Control and Negative Control sets was also deemed not significant: P-value = 0.3142.

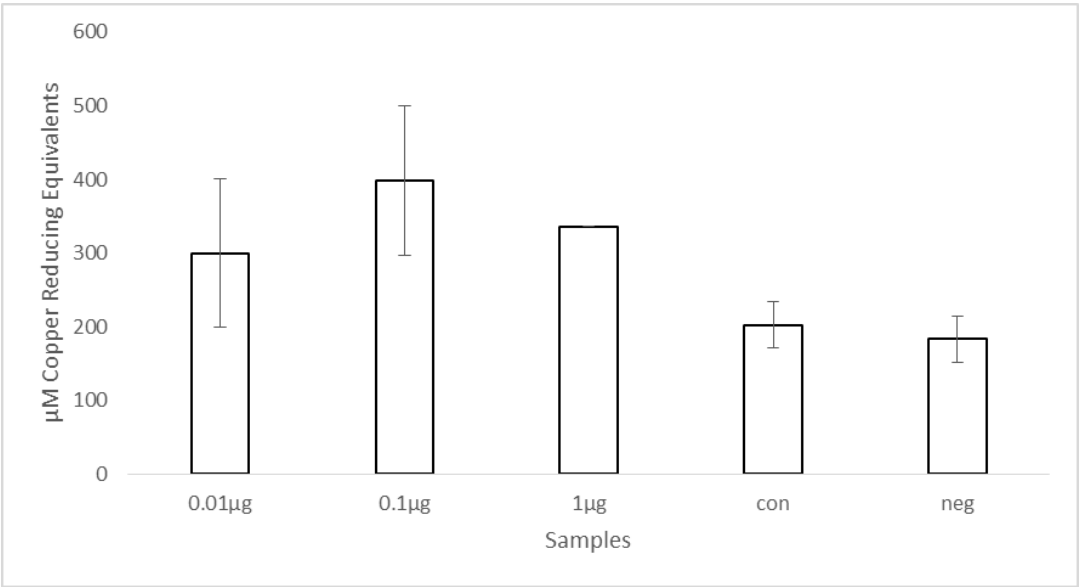

Figure 14: Mean µM Copper reducing equivalents with standard deviations above and below the mean of the five sets of samples, normalised to$10x10^4$ cells.

The *t*-test done to test the significance between the 0.01µg and 0.1µg sets gave a P-value of 0.144. The same done between the Control and Negative Control sets gave a P-

value of 0.3142. Because both sets did not have a significant difference between them, one can combine them to test if there is a significant difference between transfected cells and non-transfected cells, the result of which was: P-value = 0.0002089.

In respect to the variance of each set, the 0.01µg and 0.1µg sets showed a variance of 8084.8 and 8607.4 respectively. The Control and Negative Control sets were at an order of magnitude less at 846.8 and 821.1 respectively. This large difference between transfected and non-transfected cells was shown again when these two sets were taken as a whole with a variance of 9888.9 for transfected cells and 927.6 for non-transfected cells.

With the data collected from both assays, it is possible to compare the two. Figure 15 shows the correlation scatter plot between the mean normalised results of the ELISA and AOP-450 assays. A test of whether the slope had any significance to being 0 had a P-value of 0.51 and as such was kept at the intercept of 41.17. A visual positive correlation between the two sets of data was supported by a correlation coefficient of 0.936 suggesting an extremely close correlation between an increase in PTEN and an increase in total antioxidant potential. However, when each data set was taken by their individual data points as shown in Figure 16, the Pearson's product-moment correlation showed the correlation was only 0.568.

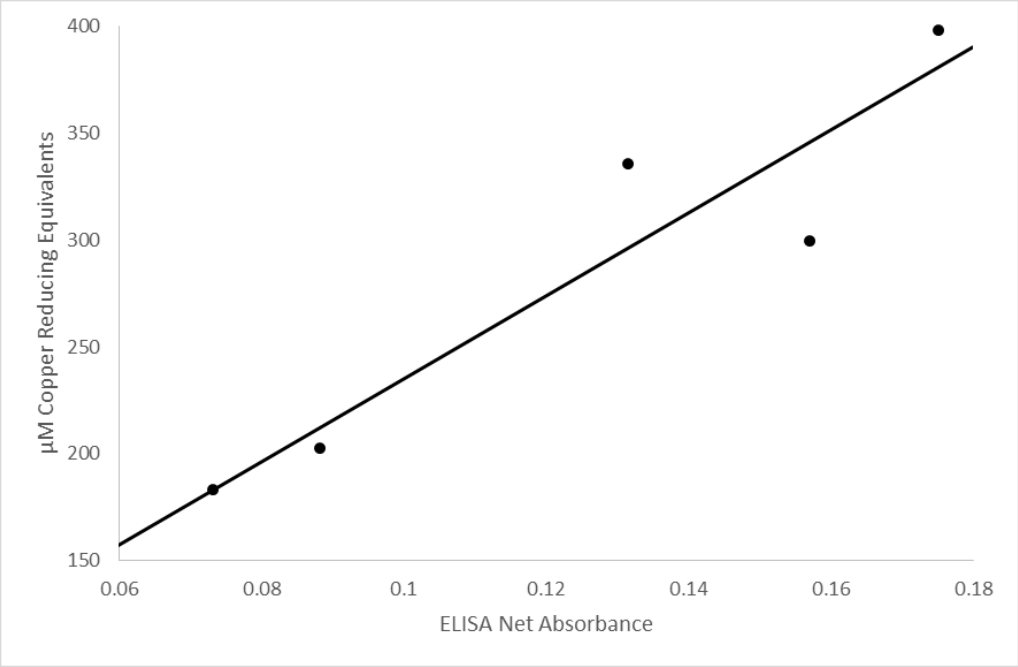

Figure 15: Scatter plot comparing the mean results of the ELISA and AOP-450 assays. All values are normalised and displayed per $10x10^4$ cells.

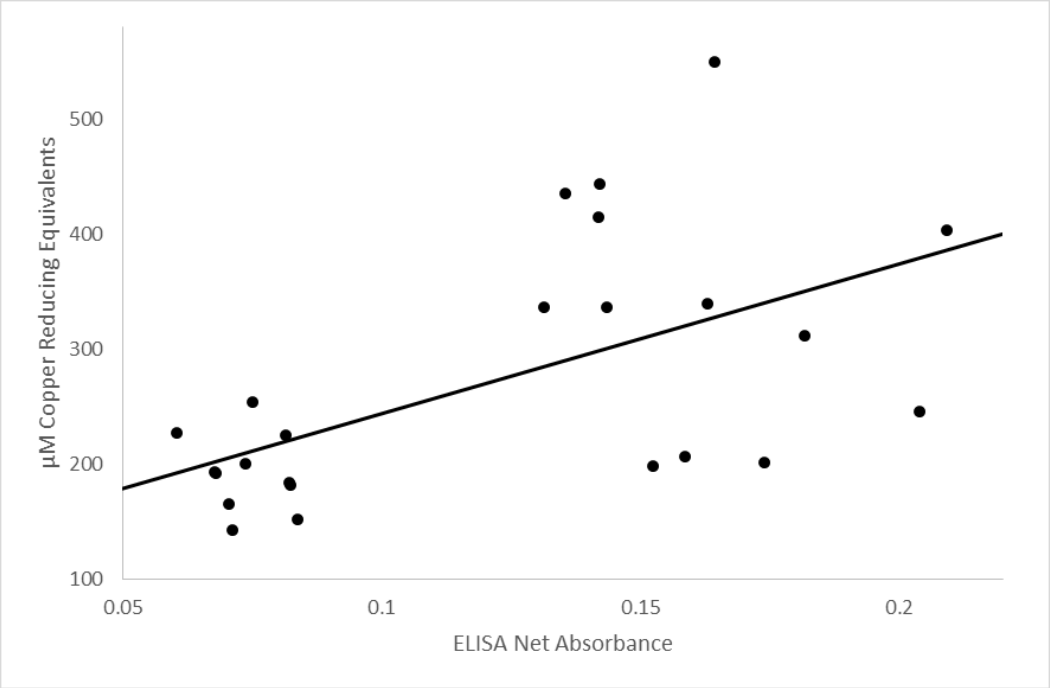

Figure 16: Scatter plot comparing the individual results of the ELISA and AOP-450 assays. All values are normalised and displayed per $10x10^4$ cells.

4.5 Discussion

In regards to ageing and longevity, PTEN's most prominent effect to potentially extending both is in its ability to positively regulate autophagy through its effects on the PI3K/AKT pathway (M. Ashcroft et al., 2002) and caloric restriction (Ortega-Molina et al., 2012). It was for this reason that an antioxidant assay was used to determine whether an increase in PTEN levels would lead to an increase in antioxidant, and thus autophagic, levels.

The principle behind the AOP-450 assay is the reduction of levels of Copper+2 (Cu^{2+}) to Copper+1 (Cu^{+}). Copper, in its forms of Cu^{2+} and Cu^{+}, is involved in many metabolic processes (L. Garcia, Welchen, & Gonzalez, 2014), but its most pertinent ability, to this thesis, is Cu^{2+}'s capacity to form ROS molecules and increase oxidative damage (Gaetke & Chow, 2003). By reducing Cu^{2+} to Cu^{+}, it is effectively reducing the potential oxidative stress, and, logically, the higher the antioxidant potential, the more Cu^{2+} will be reduced to Cu^{+}.

Figure 14 shows the hoped for and expected results from the AOP-450 assay, after normalisation, that an increase in PTEN levels does in fact increase the total antioxidant potential. Of note, the figures representing the data before and after normalisation do not show as extreme a change as the ELISA results did. This is because, unlike the ELISA, the cells had an opportunity to be passaged and thus be roughly of equal numbers. So, one can see a clearer increase in the transfected sets than in the non-transfected sets in either graph.

However, as explained in previous sections, the difference between the 0.01µg set and Control set is not statistically significant (P-value = 0.0957). This can be relegated to the large variance in the 0.01µg and 0.1µg sets (and one can presume the 1µg, had it had more samples), compared with the non-transfected sets. This sheds some doubt that PTEN can reliably increase a cell's antioxidant rate and may indicate the processes involved in these pathways may not be uniformly affected by PTEN, thus showing greatly varying antioxidant levels.

In saying that, there was no significant difference between the two sets of transfected cultures (excluding the 1µg set), once again owing to the large degree of variance, and when the transfected groups as whole were taken against the non-transfected groups, the resulting P-value was merely 0.0002089. This shows that despite the large degree of variance, PTEN does have an effect on antioxidant levels.

A way in which one can be more certain of this is by looking at the results of the Negative Control set and its associated P-value when compared the Control set, namely 0.3142, which was highly insignificant. With the only differences between the Control set and transfected sets being differing levels of PTEN and a vector, once the vector is ruled out, one can say with a confident degree of certainty that this is due to the levels of PTEN.

The significance of this, beyond a statistical sense, is the effect of antioxidants on the longevity of cells. Oxidative damage can reduce a cell's longevity by damaging key parts of the DNA required for proper operation of the cell (Friedberg, 2003). By damaging these key areas, the cell stops proper operation and enter senescence and apoptosis. Another area prone to DNA-damage are the telomeres, which, when reaching a critical stage will force the cell to enter senescence (Blasco, 2007). By the reduction of

oxidants such as ROS and thus DNA-damage, it is possible to increase cellular longevity.

Considering PTEN's noted capacity to positively regulate genes and proteins involved in autophagy, such as FoxO (Calnan & Brunet, 2008), and its negative regulation of pathways resulting in oxidative damage (Carracedo & Pandolfi, 2008; Salminen & Kaarniranta, 2009), if an increased level of PTEN leads to an increased antioxidant potential (which the AOP-450's results suggest) then it is quite possible to speculate that a possible route to enhancing longevity is by increasing the PTEN levels.

The graphs in Figures 15 and 16 cast doubt upon this. Figure 15 shows a very tight grouping of data points along the slope and a high correlation coefficient of 0.936. At first glance, this would support greatly the above hypothesis that an increase in PTEN leads to an increase in total antioxidant potential and thus an increase in cellular longevity. However, in Figure 16 one can see a very different narrative. With only a correlation coefficient of 0.568, one cannot say the above is as definitive as it was before.

While correlation of 0.568 still represents a positive correlation, as one can visually verify from Figure 16, it is not, however, a correlation in which one can have a high degree of confidence predicting future results. With an ELISA value of 0.3, one can determine with some confidence that, following the correlation of Figure 15, the µMCRE of the same sample would be in the 600s. Following Figure 16, while one can say it will be higher than an ELISA value of 0.1, one would be able only to determine a very vague range for its µMCRE value.

A possible statistical reason for such as disparity between the two figures may lay in the large variance observed in Figure 14 among the transfected culture groups but not the non-transfected groups. These two groupings are easy to identify in Figure 16, as there are two distinct groupings, with the left most group the most spread out showing this high degree of variance.

A possible biological explanation for this could lie in the method by which PTEN influences autophagy and antioxidant levels. As mentioned above, PTEN does not directly influence autophagy or antioxidant levels, rather affects it indirectly through various pathways. These pathways can be categorised broadly into two groups: those that PTEN affects in the cytoplasm and those PTEN affects in the nucleus.

The main impact PTEN has on antioxidant levels while in the cytoplasm is by down regulating the PI3K/AKT pathway (Chalhoub & Baker, 2009; Stambolic et al., 1998). By down regulating PIP3, downstream effectors of the PI3K/AKT pathway such as NF-κB have a reduced capacity to down regulate FoxO (Adler et al., 2007; Salminen & Kaarniranta, 2009; Salminen et al., 2008). This in turn allows FoxO to induce greatly the expression of several antioxidant enzymes (Calnan & Brunet, 2008). Conversely, PTEN's impact on antioxidants in the nucleus derives from its interaction with the p53 protein. By binding with p53, PTEN is able to enhance p53-mediated functions (Z. Chen et al., 2005), among which is the increased production of the p53 downstream antioxidant gene Sestrin.(Wempe et al., 2010).

While there are many mechanisms by which PTEN may travel between the cytoplasm and the nucleus, such as by diffusion, active shuttling and pathway depended exportation (Liu et al., 2007; Planchon et al., 2008); one mechanism of note is the promotion of nuclear of PTEN as a result of apoptotic stimuli (Gil et al., 2006) where it enhances these apoptotic stimuli. This can be seen as another process of PTEN where it is in direct confrontation with the PI3K/AKT pathway, which opposes apoptosis (Datta et al., 1997) as one of its mechanisms to promote survival and proliferation. A speculation on the purpose behind PTEN's enhancement of apoptotic stimuli is the destruction of cells containing potential toxic material, thus keeping the overall health of tissues at an optimal level.

The link between this and the variance seen in the 0.01µg and 0.1µg results Figure 14 and the low correlation coefficient in Figure 16 is one of timing. The AOP-450 assay was performed during the ninth passage of the cultures. This is near the end of the passaging lifespan of HUVEC cultures, and as such the proportion of cells entering apoptosis would have been higher than in earlier cultures. The apoptotic stimuli in these cells would have resulted in a greater nuclear import of PTEN than normal, leading to a higher proportion of p53 mediated antioxidant than FoxO mediated antioxidants.

As these two pathways operate by different means, their differing proportions may have been responsible for the great variance. With two independent pathways, the mechanisms by which they affect antioxidant levels also would be different. As such this could lead to different cultures having greatly differing results, depending on the proportion of PTEN nuclear import in each culture. The antioxidative properties of the

FoxO and p53 in relation to PTEN import in response to apoptotic stimuli would be a fascinating avenue for future research.

4.6 Conclusion

The AOP-540 assay revealed that, within the dosage range used, increased levels of PTEN results in increased antioxidant potential (a correlation between 0.568 and 0.936). As with the ELISA assay, the highest value belonged to the 0.1µg set but, once again the difference between it and the 0.01µg set was not statistically significant (P-value = 0.144). The 0.01µg set also did not have a statistically significant difference in regards to the Control set (P-value = 0.0957), most likely due to the fact that the transfected sets had a variance ten times greater than the variance of the non-transfected sets. It is unknown what caused this great degree of variance, but in regards to literature, it may well be due to the different antioxidant pathways PTEN influences.

5 Cell Culture, Maintenance and Evaluation of Cell Growth

5.1 Introduction

The bulk of the experiment consisted of growing, maintaining and passaging the cultures. This was divided into three clear categories of when they occurred. The first bulk of culturing occurred before the experiment. This was the initial culturing where the cultures were prepared for transfection, and excess cultures were frozen as stock for emergency purposes. Due to unforeseen circumstances in the preliminary trials, this was a wise choice. The second bulk of culturing was the Triplicate Culturing, this occurred directly before the transfection in order to create the three culture groups: the Transfected Group, the Control, and the Negative Control. The last bulk of culturing was the maintenance cultures. This was done after transfection in order to grow and maintain the cultures until they all achieved senescence and apoptosis to determine if there was a significant difference between the culture groups.

The purpose for investigating cell growth of the HUVEC cultures was to use this as a measure for longevity. As noted in Chapter 4, literature shows that the most likely method by which PTEN would increase longevity is by reducing the rate at which telomeres shorten. If this is the case, it would logically mean that the cells would undergo more cell divisions than normal before the reduction in telomeric length would initiate senescence.

5.2 Methods and Materials

5.2.1 Procurement of Materials

The HUVEC and the media required for it (Medium 200 and Low Serum Growth Supplement), were acquired from Life Technologies. The HUVEC were all from a single donor so as to ensure similarity of cellular physiology.

5.2.2 Initial culturing

5.2.2.1 Thawing

As seen in Figure 17, the process of thawing HUVEC cells began with removing them from liquid nitrogen storage and thawing in a 37ºC water bath. When the vial contents had thawed, the vial was moved to a Class II, type A laminar flow culture hood and the contents were agitated to disperse the cells. 20µl were removed and suspended in 20µl of trypan blue solution for cell counting. The vial contents were diluted, with Medium 200 with added LSGS, to a concentration of 1.24 x 10e4 before 15ml of cell suspension was added to four 75cm2 culture flasks. The flasks were then incubated at 37ºC, with 5% CO2.

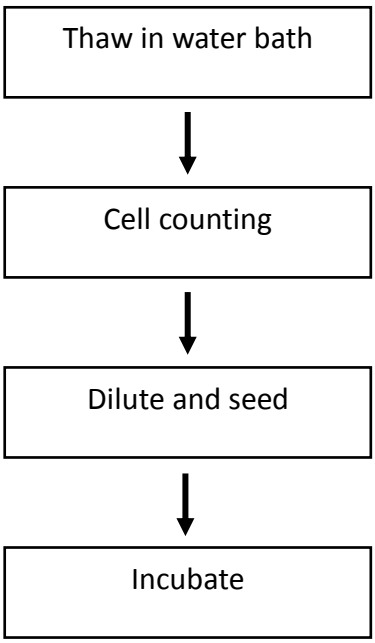

Figure 17: Protocol for thawing and starting HUVEC cultures

5.2.2.2 Culture maintenance

The culture medium was replaced with fresh Complete Medium 200, 24 hours after establishing the culture from cryopreservation. For subsequent subcultures, the medium was replaced on a 48 hourly basis until the cultures were 80% confluent. If sub-culturing did not occur yet, the culture medium was replaced daily until then.

5.2.2.3 Subculture of HUVEC

Before any subculturing occurred, the flasks were viewed under a microscope to ascertain their condition, i.e.: confluency, mitotic activity, and signs of contamination. If the flasks were at, or more than, 80% confluent, the flasks would be subcultured.

To subculture the 75cm2 flasks, the culture medium as removed and 6ml of TrypLE Express was added to each flask and incubated for 1-3 minutes. The flasks were viewed under a microscope to ensure the cells had been dislodged from the surface, and force was gently applied to the flask to encourage this. 10ml of Complete Medium 200 was added to neutralize the effects of the TrypLE Express. This solution was agitated to remove any further cells and to break up cell clumps in the solution. The cells were then pipetted into centrifuge tubes and centrifuged at 180 x g for 7 minutes. The supernatant was removed from the tube, with care being taken not to dislodge the cell pellet. The cell pellets were re-suspended in Medium 200 and distributed to a further four flasks, creating a total of 8 75cm2 flasks. These were finally incubated at 37ºC, with 5% CO2.

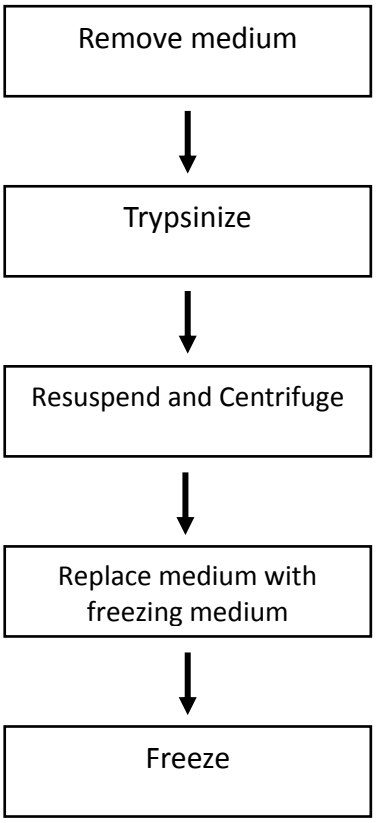

Figure 18: Protocol for freeing HUVEC cultures

5.2.2.4 Freezing

Figure 18 shows the protocol for freezing cultures. To initiate the cryopreservation, the culture medium was removed from the flasks and TrypLE Express added to trypsinize the cells. The cells were re-suspended in Medium 200 and these suspensions were pooled together. 20µl were removed and suspended in 20µl of trypan blue solution for cell counting. The cell suspension was pipetted into centrifuge tubes and centrifuged at 180 x g for 7 minutes. The supernatant was removed and the cells suspended in freezing medium at a density of 1-2 x 10e6. 1ml aliquots were dispensed into sterile cryotubes. The cryotubes were placed in a styrofoam container and placed into a -20°C freezer for 1 hour before being transferred to a -70°C freezer overnight. The cryotubes were placed into liquid nitrogen the next day for long term storage.

5.2.3 Triplicate culturing

Following the culturing procedure outlined above and Figure 1, three 12-well plates were seeded. They were marked Control, Negative Control and Test respectively. The Control cultures were not modified in any way to serve as a baseline. The Negative Control cultures had an empty plasmid vector added to ensure it was the PTEN gene that affected the cells' longevity and not the plasmid vector. The Test cultures had the complete PTEN plasmid added and were the focus of this thesis.

5.2.4 Maintenance Cultures

The maintenance of the cultures after the transfection occurred, followed the same protocols that have been listed above. This culturing process was continued until such a time that all three of the culture groups had undergone senescence and apoptosis in order to determine their longevity.

5.3 Statistical Analysis

Throughout the culturing process, the cell numbers were counted at each passage level and recorded. The purpose of this was to determine the number of cell divisions each culture underwent as a measure of their longevity. The number of cell division was ascertained by dividing the duration of the culture by the doubling time of the cells.

The doubling time was established by using the following formulae:

$$N1 = N0*2t/T$$

$$T=t*ln(2)/(ln(N1)-ln(N0))$$

Where: N0 = the initial concentration of cells

N1 = the final concentration of cells

t = the duration of culture

T = the doubling time

ln = natural logarithm

The total cell divisions of each well in each culture could then be determined. These were then compared between the Test, Negative Control and Control samples using a T-test to determine whether there was a statistically significant difference in the samples.

To determine whether the total antioxidant potential and/or the PTEN levels were responsible for any change in longevity, correlatory plots were done using longevity against antioxidant potential and against PTEN levels, testing the significance of the slope.

5.4 Results

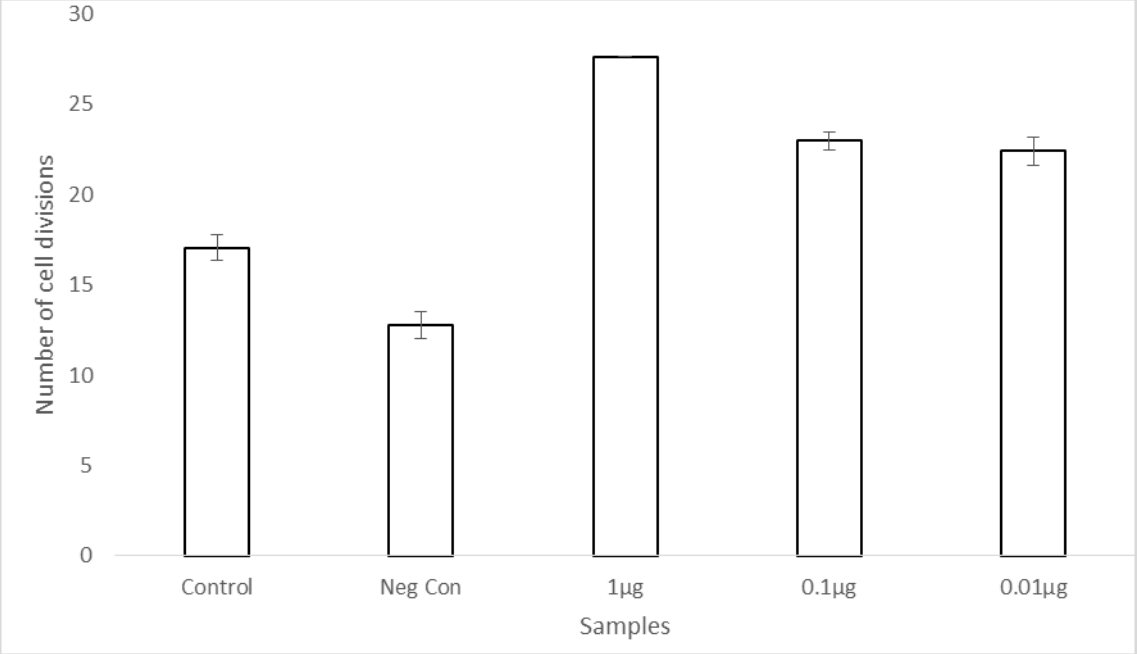

Figure 19: Mean number of cell divisions underwent by each sample culture with standard deviations above and below the mean.

The central purpose of this study was to determine the effect on longevity of HUVEC cultures when transfected with PTEN. Thus, the final milestone of the thesis was to grow and passage the cultures until all cells died of senescence and apoptosis. Figure 19 shows the mean number of cell divisions each sample culture underwent. As

with the previous results, one can immediately see a distinct difference between the transfected cultures and non-transfected cultures.

Unlike previous results, however, there is no visual similarity between the Control and Negative Control cultures. This is supported by a *t*-test for significance which found a P-value of 9.04×10^{-13}. The difference in values between the Control culture and the 0.1µg (P-value = 1.25×10^{-11}) and 0.01µg (P-value = 3.72×10^{-6}) was significant. The difference between the 0.1µg and 0.01µg was found to be statistically insignificant: P-value = 0.2035. Contrasting the transfected cells against the Control gave a P-value of 2.48×10^{-9}.

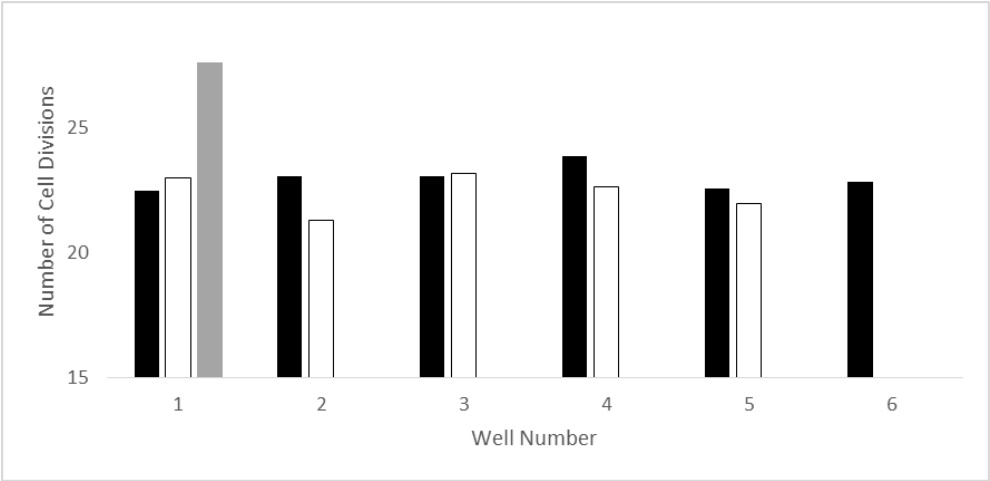

Figure 20: Total number of cell divisions underwent by each well in the transfected culture group. Black wells are the 0.1µg set, the white wells are the 0.01µg set and the grey well is the 1µg set.

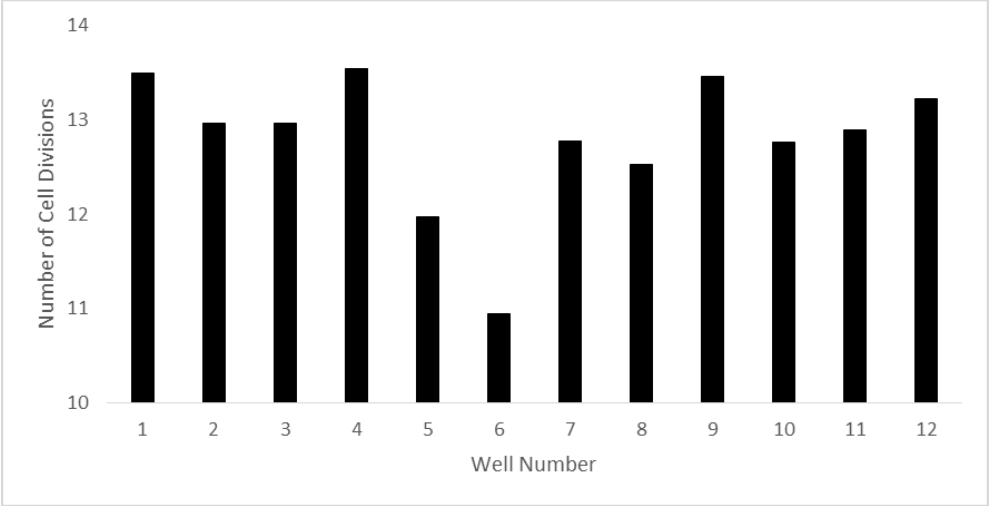

Figure 21: Total number of cell divisions underwent by each well in the Negative Control culture group

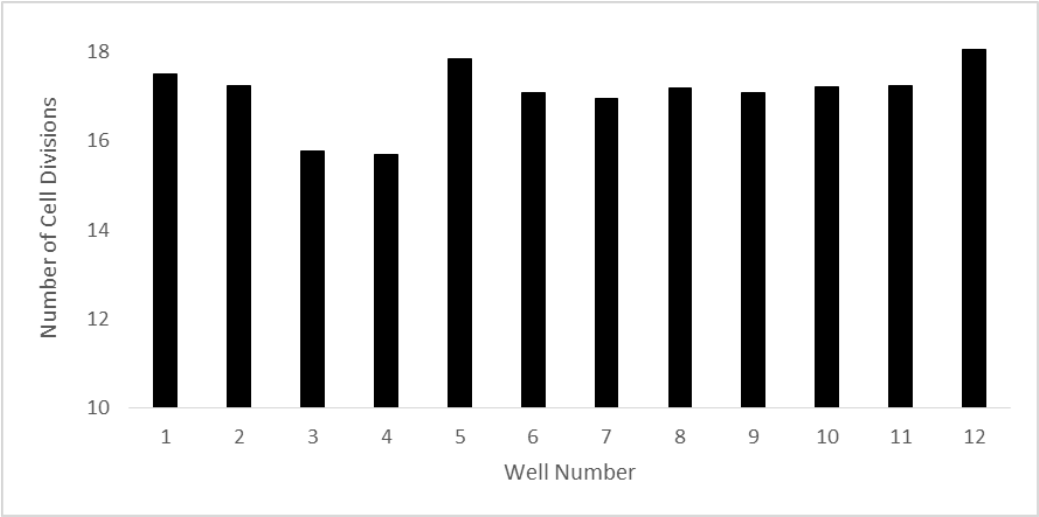

Figure 22: Total number of cell divisions underwent by each well in the Control culture group

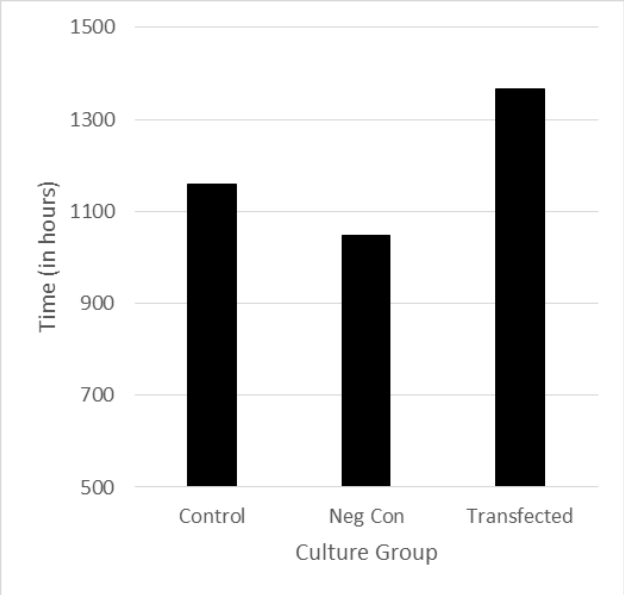

Figure 23: Total length of time, expressed in hours, that each culture group underwent division.

Figures 20 - 22 show the variance in each of the culture sets. The least amount of variance was shown in the 0.1µg culture set (0.21) while the highest was the Negative Control culture set (0.495). Figures 20 - 22 also shows that the lowest total cell division was Well 6 of the Negative Control culture set while the highest (if one ignores the 1µg well) was Well 4 of the 0.1µg culture set.

Figure 23 shows the length of culture each group underwent as another measure of longevity. Because all transfected culture sets were on the same plate, they were passaged at the same time, meaning they have the same time elapsed. However, it is still clear to see that the transfected culture group continued for a substantially longer time than the Control (208 hours) and the Negative Control groups (318 hours).

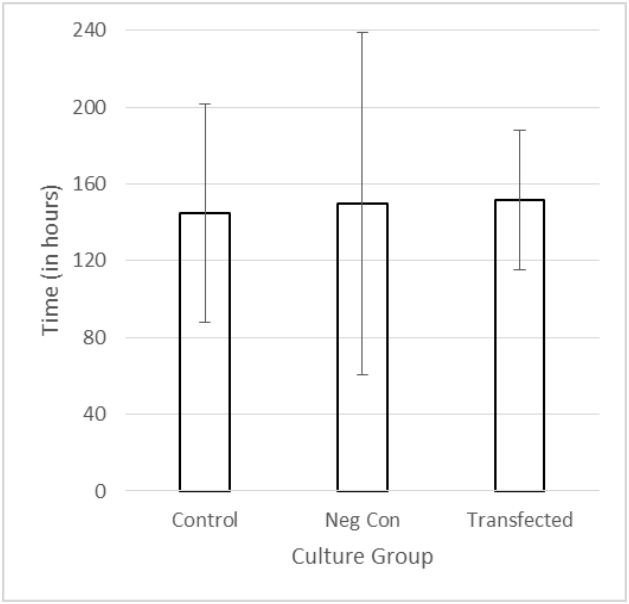

Figure 24: Mean length of time, in hours, between passages per culture group, with standard deviations.

As with the number of cell divisions, the Negative Control group did poorly (110 hours less) in comparison with the Control group.

Figure 24 shows the average length of time between passages for the three culture groups. While the transfected group had the highest value (151.78) there is no statistical difference between any of the three groups, as shown in Table 4. The Negative Control group had the highest variance (7932.2) while the Transfected group had the least (1332.6).

Table 4: P-values from *t*-tests of significance on the average length of time between passages for each culture group

	Control	Negative Control	Transfected
Control	1	0.8459	0.6182
Negative Control	0.8459	1	0.6039
Transfected	0.6182	0.6039	1

Figure 25 shows the correlation between the mean values given by the ELISA and the mean total cell divisions.

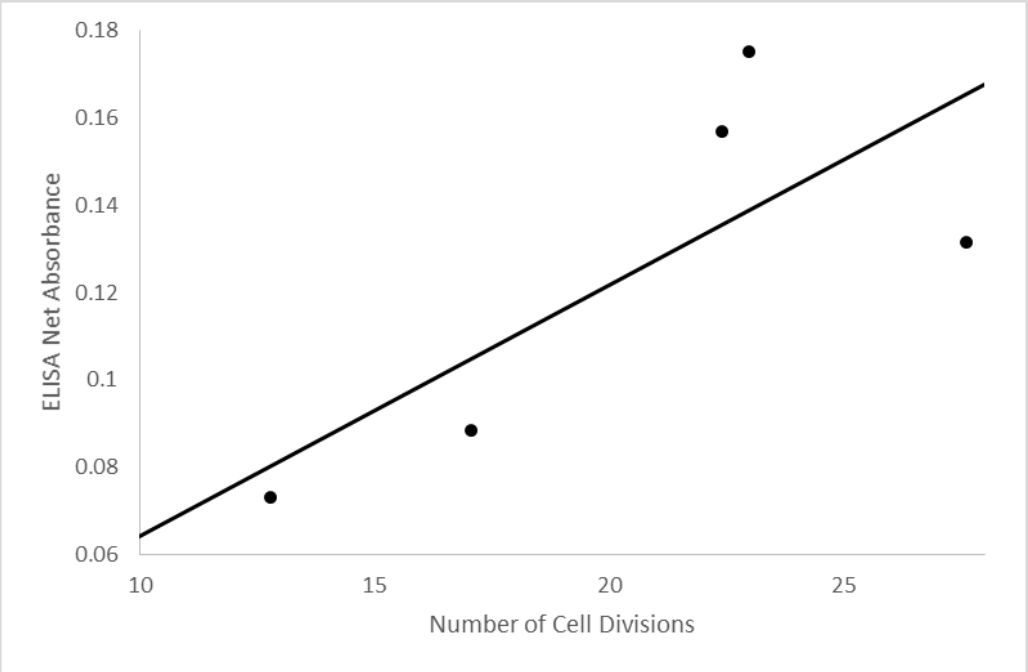

Figure 25: Scatter plot comparing the mean values of the ELISA and number of cell divisions. ELISA values are normalised and displayed per $10x10^4$ cells.

There was no evidence (P-value = 0.1396) of the slope's intercept being 0, and as such was left at 0.006668. A Pearson's product-moment correlation test returned a correlation coefficient of 0.7557 suggesting a significant similarity between the two data sets. The ELISA did not account for each of the Control and Negative Control's individual well, rather only six of each. Thus, to look at an individual comparison between cell division and PTEN levels, one must equally reduce the cell division numbers to six. This was done by grouping the wells into sets of two and finding the average. The result is shown in Figure 26.

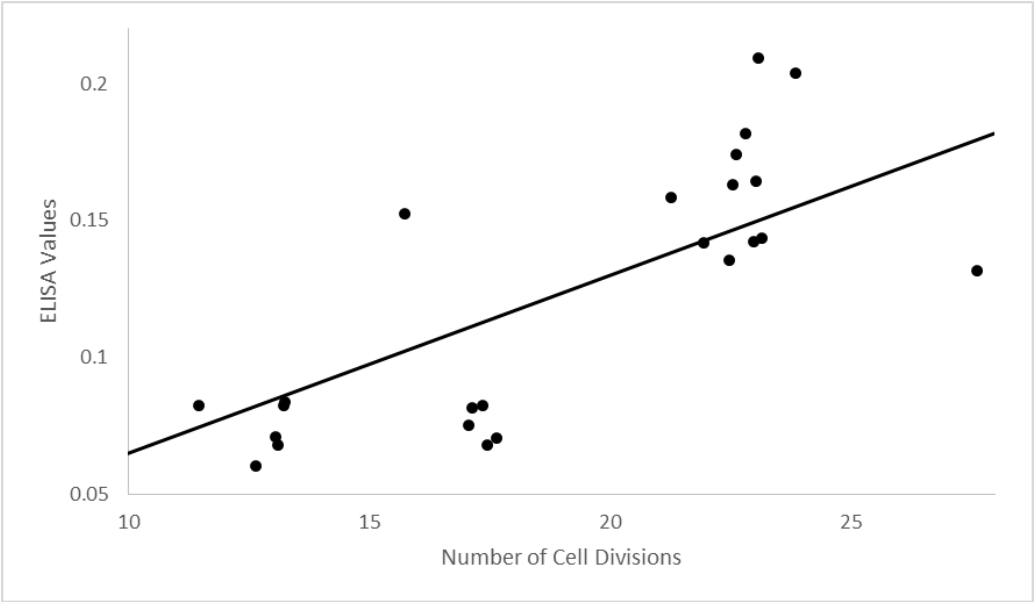

Figure 26: Scatter plot comparing the individual values of the ELISA and number of cell divisions. ELISA values are normalised and displayed per $10x10^4$ cells.

Unlike the previous graph, there was strong evidence that the slope's intercept must be zero (P-value = $3.52x10^{-5}$) and thus the intercept was forced to 0. Initial observations clearly show two groupings of values, those from 11 to18 on the horizontal axis, and those from 21 to 28 on the same. The former represent the non-transfected cells while the latter the transfected cells. A correlation coefficient for this data results in a value of 0.7824.

There are two observable outliers, one at 15.7356, 0.1524 and the second at 27.6174, 0.1314. If one takes these two data points out of the set, one attains a correlation coefficient of 0.8727.

A similar correlation graph between mean CRE values and mean number of cell divisions is shown in Figure 27. No evidence was found that the slope's intercept must be 0 and was left at 0.92 (P-value = 0.92). A correlation coefficient of the data was given as 0.8376 showing an even closer correlation than ELISA vs. Cell division.

As with Figure 26, the data was similarly transformed in order to view the individual data retrieved from the AOP-450 assay. This is displayed in Figure 28.

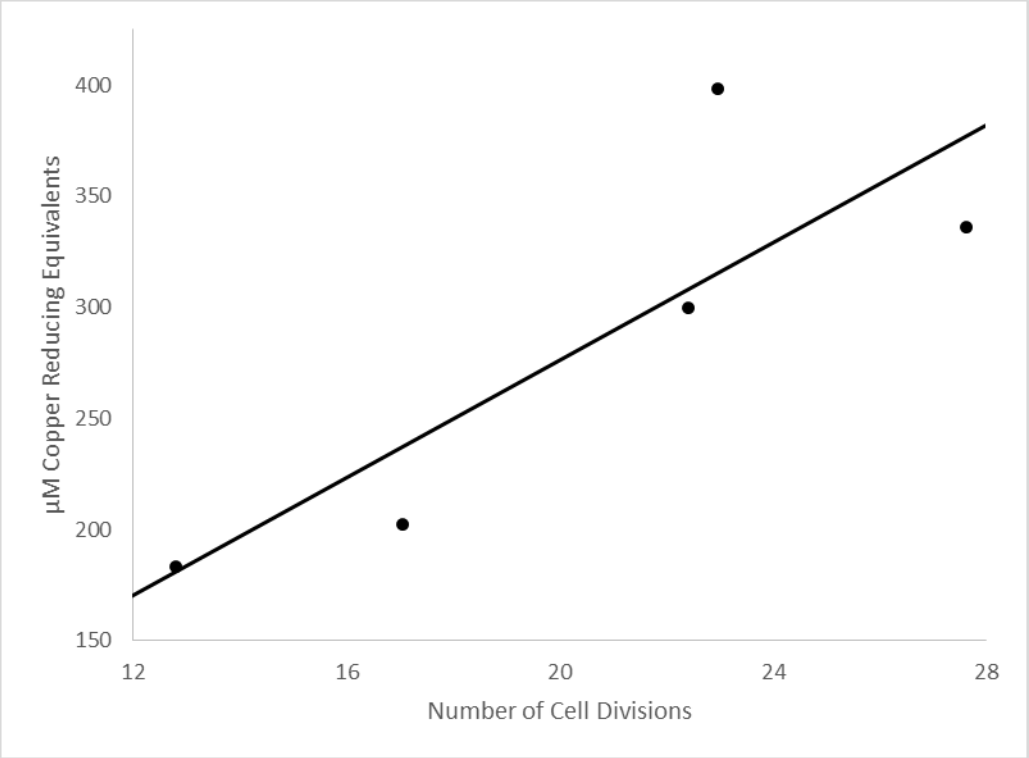

Figure 27: Scatter plot comparing the mean values of the AOP-450 and number of cell divisions. AOP-450 values are normalised and displayed per µM Copper reducing equivalents per $10x10^4$ cells.

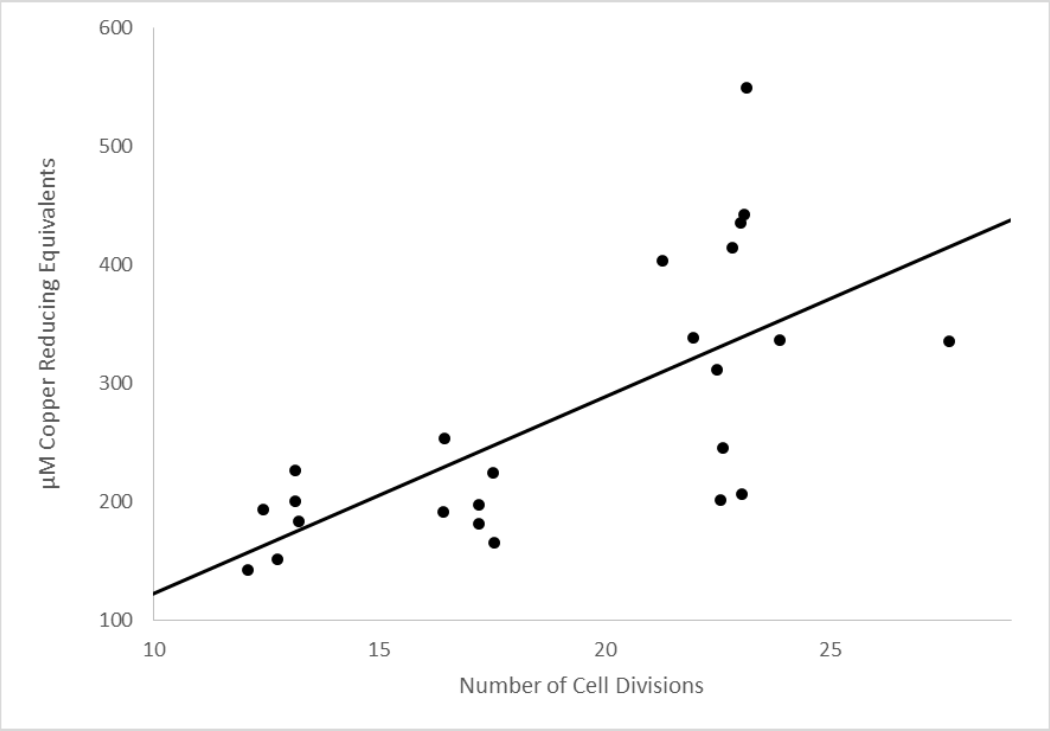

Figure 28: Scatter plot comparing the individual values of the AOP-450 and number of cell divisions. AOP-450 values are normalised and displayed per µM Copper reducing equivalents per $10x10^4$ cells.

There was strong evidence to suggest an intercept of 0 (P-value = 0.0005) and it was thus set at 0. Visually, there are three distinct groupings: from 11 to 14, from 15 to 18

and from 21 to 24 on the horizontal axis. The first grouping is the Negative Control group, the second is the Control group while the third grouping is the Transfected group. As with the results shown in Figures 13 and 14, one can see the large degree of variance in the Transfected group. This is supported by a correlation coefficient for this data being at 0.6564, suggesting only a moderate correlation between µM CRE and number of cell divisions.

5.5 Discussion

The ultimate goal of this thesis was to test the hypothesis that an increased level of PTEN, achieved via transfection, would increase the cellular longevity of HUVEC cultures. The purpose for this was to generate a proof of concept that could be applied in future studies, namely that PTEN could be beneficially used to increase human longevity. The literature review discussed the many and varied studies that have been done in the past related to this, but the literature was severely lacking in human studies of PTEN when focused on longevity.

As PTEN was first discovered as a cancer prevention gene, the majority of research has been spent in uncovering this aspect of PTEN. Recent work, however, has seen researchers studying the effects of PTEN on longevity, revealing functions relating to longevity such as protection from DNA damage (Ming et al., 2011); caloric restriction and protection from insulin resistance (Ortega-Molina et al., 2012); and an increase in antioxidant levels (Calnan & Brunet, 2008; Wempe et al., 2010). It was on the basis of this research that this thesis attempted to apply these effects to human cells. The scope of a Master's thesis meant that not all effects could be tested and thus antioxidant and total longevity was chosen.

Previous results have shown the first successful transfection of PTEN plasmids in human cells in regards to longevity. It showed that an increase in PTEN levels resulted in an increased total antioxidant potential. This supported prior literature that PTEN does have a positive effect on antioxidant levels, however these results alone would not show in any conclusive manner that an increase in PTEN would lead to an increase in cellular longevity.

As such, the final milestone of the thesis was to grow and passage the cultures until completion to discover if the hypothesis was indeed correct.

Initial results shown in Figure 19 would seem to support the hypothesis. There is a clear difference in the mean number of cell divisions between the transfected culture groups and the non-transfected culture groups. This was reinforced by *t*-tests that delivered P-values of <0.01 for the 0.1µg vs Control, and for 0.01µg vs Control groups. The transfected group as a whole also delivered a P-value of <0.01. Such statistically significant results greatly support the hypothesis that increased levels of PTEN do indeed increase HUVEC longevity.

Of note is the Negative Control culture. As discussed previously, the results have shown that the empty vector transfected into this culture has had no significant effect on the culture beyond what was seen in the Control culture group. However, as seen in Figure 19 and Figures 20 - 22, the Negative Control cultures consistently underwent fewer cell divisions than the Control cultures. In respect to the number of passages, the Negative Control cultures underwent 10 passages while the Control cultures underwent 11. As the empty vector can be discounted as a culprit in this, the most likely cause would be the natural variance in longevity experienced by HUVEC cultures as described in literature where HUVEC cultures normally experience only 9-11 passages.

One of the cellular functions of PTEN is to arrest the cell cycle during the G1 phase via p53 (Chang et al., 2008). Because of the other functions previously discussed concerning the connection between PTEN and p53, this cell cycle arrest is thought to facilitate DNA repair and antioxidant production, in effect a pause in the cellular cycle in which to do maintenance. PTEN was also found to arrest the cell cycle, also during the G1 phase, by the suppression of cyclin D1 transcription and prevention of its nuclear import (Radu et al., 2003).

As the ELISA results showed a significant increase in PTEN level, logic dictated that there would be a higher degree of cell cycle arrest in the transfected cultures. This would lead to a slower rate of cell division and thus the culture would persist for a longer period of time. To determine if such a hypothesis is correct, the length of time of each passage was recorded and displayed in Figures 23 and 24. Figure 23 seems to show support for this hypothesis, but the difference in the total length of time for each

passage can easily be explained by the fact that the Control cultures underwent 11 passages; the Negative Control cultures 10; and the Transfected cultures 13.

A more appropriate look at the mean length of time for each passage does not show any significant differences between culture groups. In Figure 24, the Transfected cultures had the highest value at 151.78 while the Control cultures had the lowest value at 144.75. While promising, this is offset by the high degree of variance in each set. This resulted in P-values of >0.6 for each *t*-test done on the sets. While a cell cycle arrest may increase the length of a culture, there is no evidence to support it in this thesis.

As mentioned previously, the only additions made to the Transfected cultures were the PTEN plasmids (containing the PTEN cDNA and a vector); and only an empty vector was added to the Negative Control cultures. Throughout the culturing process, all cultures were provided equal amounts of medium and LSGS. As the Transfected cultures alone showed a significant increase in longevity, one can reasonably rule out the effect of the medium, growth serum or empty vector as having played a significant part in this.

Figures 25 and 26 support the hypothesis that PTEN alone was responsible for the increased longevity observed in the cultures. Unlike the correlation done between ELISA values and µM CRE, when both mean and individual data points were taken into account, the correlation coefficients were remarkably similar: 0.7557 for mean values and 0.7824 for individual values. The latter correlation could be higher if the two outliers (a Negative Control point and the 1µg set) were removed, resulting in a correlation of 0.8727.

Of interesting note is the correlation coefficients when each culture group is taken separately. As seen in Figure 26, there are two distinct groupings representing the Non-Transfected and the Transfected culture groups moving left to right on the *x*-axis. The Non-Transfected correlation coefficient is 0.109 while the Transfected is -0.163. If the outliers are removed these are 0.017 and 0.472.

This presents a paradox. If one accepts the outliers, then the individual groups' coefficients suggest that, at best, there is no correlation between PTEN levels and cellular longevity and at worse, as the levels of PTEN increase, the longevity of the cell decreases. However, as can be seen in Figures 25 and 26 this is patently not true. The

transfected group have significantly higher values than the non-transfected groups with a strong correlation of between 0.7824 and 0.8727.

A possible explanation that could reconcile these results lies in the biological pathways surrounding PTEN. These were examined extensively in the literature review and one can see many pathways that both positively and negatively regulate PTEN, and pathways that are regulated, positively and negatively, by PTEN. With all the pathways and their associated feedback loops that influence PTEN, it is highly possible that the homeostasis involved in these prevent the positive effects of PTEN on longevity. This is logical as it is only by transfection that an increase in longevity was seen. Therefore, it is not too surprising to see no correlation between PTEN and longevity in non-transfected cells.

The correlation between mean µM Copper reducing equivalents and the mean number of cell divisions was even closer than the ELISA values, with a coefficient of 0.8376. The link between antioxidants and cellular longevity has been well reported (Hung et al., 2010; Matheu et al., 2007; von Zglinicki, 2002), so this correlation was not surprising. It was surprising to see, however, the correlation in Figure 28, it being quite lower than expected, at 0.6564: only a moderate correlation. This is a much greater difference between individual and mean results than the correlations than the ELISA/Cell Division correlations.

An explanation for this, as with the ELISA/µM CRE correlations, lay in the large variance observed in the results obtained from the AOP-450 assay. As this variance affected the ELISA/µM CRE correlations, so it affected this correlation. It must be said that there is a slightly higher correlation between the µM CRE and longevity than µM CRE and ELISA, 0.6465 compared with 0.568. It is not a considerable difference but it does suggest there is, as literature shows, a closer relationship between antioxidants and longevity than with PTEN.

It has been demonstrated that there is a significant correlation between PTEN levels and cellular longevity suggestive of causation. The correlation between µM CRE and longevity of 0.6465 also suggests that antioxidants did indeed have a positive effect on the longevity of the cultures, and the potential rationale behind the variance seen in the AOP-450 results of differing ratios of activated antioxidant pathways may be

responsible for the low correlation. However, such a moderate correlation may indicate PTEN may be influencing longevity by additional means.

The most likely culprit once one eliminates any possible pathway that could increase antioxidant levels would be DNA-damage repair. In Ortega-Molina's, et al., (2012), examination of PTEN's effect on mice revealed that PTEN transgenic mice showed a significantly reduced level of DNA damage in their livers. Accumulation of DNA damage leading to deterioration in liver tissue is a biomarker for ageing (Matheu et al., 2007; C. Wang et al., 2009). While HUVEC are not liver cells, the principle of DNA-damage repair by PTEN, especially via DSBR is through PTEN's up-regulation of Rad51 (Shen et al., 2007). The Rad51 pathway has been show to promote telomere maintenance (Yu, Kojic, Holloman, & Lue, 2013), even in the absence of telomerase (Le, Moore, Haber, & Greider, 1999).

PTEN may have positively influenced the longevity of the transfected HUVEC cultures through the indirect maintenance of DNA-damage and telomeres via Rad51 as well as through the increase in antioxidant levels.

5.6 Conclusion

As with previous results, the culture group that had the highest mean number of cell divisions was the Transfected culture group. Discounting the single 1µg sample set, the 0.1µg set had the highest value as per previous results, but as with previous results this was not a statistically significant departure from the 0.01µg set (P-value = 0.2035). The transfected culture groups as a whole had a very significant increase compared with the control (P-value = 2.48×10^{-9}). Both the results from the ELISA and AOP-540 assays correlated well with the mean total number of cell divisions indicating that, within the dosage of PTEN used, an increase of PTEN leads to longer cellular longevity via an increased antioxidant levels. The moderate correlation of the µMCRE and the mean total cell numbers may indicate that PTEN may be influencing other pathways that increase cellular longevity, other than antioxidants.

6 Overall Discussion

6.1 Summary of Research

The central purpose of this thesis was to determine whether an increased level of PTEN, acquired via transfection, would increase significantly the longevity of HUVEC cultures. Recent papers have linked PTEN to longevity independently of its anti-cancer properties (Masse et al., 2005; Ming & He, 2012; Ortega-Molina et al., 2012) although no conclusive studies have been done to test this on humans. The importance of a human trial is to demonstrate a proof of concept upon which further gerontological studies can be based.

The importance of longevity research is not solely for the extension of human lifespan, rather also for the extension of the human "health span". The most common symptoms of ageing are sarcopenia (Thomas, 2010); a decreased strength and mobility (Leveille, 2004); osteoporosis (Tung & Iqbal, 2007); wrinkles caused by degradation of the skin; reduced immune response (Goronzy & Weyand, 2012); anemia and fatigue (Balducci, 2010); reduced stress response and wound repair (Emmerson & Hardman, 2012); and the most important: deterioration of the brain's cognitive abilities. To increase the human lifespan while keeping these conditions intact is not only greatly unethical and immoral, but also highly impractical and uneconomical.

Using the USA as a model for OECD nations, in 2009 it was speculated that the cost of age related health care was 6% of GDP (Hosseini, 2011), this accounted for 40% of all healthcare costs. This number is only expected to rise in coming years with an exponentially increasing ageing population (Christensen, Doblhammer, Rau, & Vaupel, 2009). Concerning another of PTEN's features, that of caloric restriction, it is intriguing to note that the costs of obesity account for over US$90 billion annually (Daviglus, 2005). These figures exclude the well documented worldwide costs of the treatment of cancer. The potential usage of PTEN in humans as a preventative health measure then

goes beyond the health of the individual and potentially can improve the health of society as a whole.

This thesis looked to provide a basis for this ambitious goal. In Chapter 3 it was shown this was achieved through the transfection of HUVEC cultures with PTEN plasmids. This was then verified by using an ELISA assay to present quantifiable results to be used in other chapters. These results demonstrated that PTEN plasmid transfection is possible in HUVEC cell lines, with transfected culture groups showing a statistically significant increase in PTEN levels higher than the control culture groups. This was the first step in showing that PTEN could be applied to humans, and showed the first caveat to this: that the dosage of the transfected PTEN plasmids can have a significant effect of the cultures.

Chapter 4 presented the antioxidant assay to test the most likely means by which PTEN could enhance cellular longevity. By using the AOP-450 assay, the total antioxidant potential of the cultures was displayed in Copper Reducing Equivalents. These results were not as uniform as they were optimistically expected to be, showing a large degree of variance in the transfected culture group. However, the transfected culture group had a significantly greater µMCREs than the non-transfected culture group, establishing that PTEN can increase the antioxidant levels of HUVEC cultures. The correlation between this and Chapter 3's results proved to be little more than nominal, suggesting that there may be underlying regulatory pathways of PTEN that may be influencing the antioxidant levels in unforeseen ways.

As with any cellular study, the bulk of the work done revolved around the maintenance of the cultures themselves and Chapter 5 outlined the methods used for this. This chapter also presented the findings of the total number of cell divisions the cultures underwent. This was the yardstick chosen to measure the cultures' longevity because literature has shown that, through antioxidants and DNA-damage repair pathways, PTEN's effect on longevity would be through the maintenance of the telomeres. By reducing the rate at which telomeres shorten, cells would be able to divide more. The results from Chapter 5 showed that the transfected cultures underwent a significantly higher amount of cell divisions than the non-transfected cultures.

Chapter 5 also presented the correlations between the results given in Chapters 3 – 5. These data showed that there was a clear correlation between PTEN and the mean total

number of cell divisions. As the PTEN plasmids were the only difference in treatment between the transfected cultures and non-transfected cultures, this strongly implies that PTEN was the cause of this. The correlation between µMCREs and mean total number of cell divisions was more moderate but still a strong indication that antioxidant levels are closely linked with cell division.

With the correlations taken from Chapter 4 and 5, one can conclude that the most likely cause for the significantly higher number of cell divisions in the transfected culture groups was the increased levels of PTEN, achieved via the increased total antioxidant potential.

6.2 Caveats

The most importance caveat to this study's results is the dosage of PTEN plasmids that were used to transfect the HUVEC cultures. While the results from the thesis are optimistic, it must be remembered that they are only valid within the ranges of the doses used, namely 0.01µg, 0.1µg and 1µg per $8x10^4$ cells per 1ml well plate. In all of the Chapters' results, there has not been a statistically significant difference between the 0.01µg and 0.1µg sets. This implies that any dosage between 0.01µg and 0.1µg used would also not have had a statistically significant difference to those above mentioned sets. However, in all cases there was a distinct difference between the 0.01µg and the 0.1µg sets and the 1µg sample. This shows that the effects of PTEN may be radically different at higher doses. For example, the 1µg set had the highest value in Chapter 5 for the mean total number of cell divisions. However, amongst the ELISA results in Chapter 3, the 1µg set had the lowest value for the transfected cultures.

This was further complicated by the preliminary transfection trial in which the dosage used for all transfected wells was 1µg per $8x10^4$ cells, which resulted in all cells uniformly dead. This was the reason for including one well of 1µg dose, to determine if the same effect would reoccur. It clearly did not, which raises the issue of why this did not occur. It is more troubling because the 1µg sample used in the experiments did surprisingly well in all areas.

The second great caveat to consider is the large variance that was observed in the results from Chapter 4. The variance amongst the transfected cultures were ten times

that of the non-transfected cultures. As mentioned in Chapter 4, this could indicate that the pathways used by PTEN during a cell's lifecycle may have a different impact upon the total antioxidant potential. The results, however, did correlate well with the mean total number of cell divisions in Chapter 5 showing that there was a link between antioxidants, PTEN and cell divisions.

6.3 Avenues for Future Research

As mentioned in the previous section, the results of the study only apply to the doses that were used. The correlations, however, do allow one to predict to a certain extent, how different doses would affect the longevity of the cultures. It would be of great research interest to investigate further how different doses of PTEN can affect cell cultures. This would be highly interesting when dealing with the two extremes of doses: to determine what the highest dose of PTEN could be used without destroying the cultures and what the lowest dose could be while still showing a statistically significant effect.

In a similar vein, the use of other cell lines in respect to PTEN transfection would also prove interesting. HUVECs have been used as a cell model in many studies previously, although this has been mostly for endothelial or vascular studies. Using other cell lines would prove beneficial towards determining if PTEN's observed effects in this study are replicated in other cells. The importance of this clearly would be to provide further groundwork for any future human trials, especially if deleterious effects were to be found.

The variance observed in Chapter 4, of the AOP-450 results, and the cause of the moderate correlations in Chapters 4 and 5 could do well to be further elucidated upon. A replication of this study would be an obvious method to determine if, like the outliers mentioned in Chapter 3's ELISA results, the microplate reader used in the assay may have read dirty wells as false positives. A different antioxidant assay may also provide a different measure of total antioxidant potential. This could be done to discount the possibility of a faulty product.

Although, as mentioned in Chapter 4, the different pathways by which PTEN indirectly affects antioxidant levels may have been the culprit. A study done measuring the antioxidant potential of both the p53 and FoxO pathways in relation to PTEN

overexpression would be of substantial research interest, not only as a verification of this Chapter 4's results, but also to ascertain if overexpression of these pathways in concert with PTEN could produce more favourable results in regards to longevity.

Another avenue for future research would be to look at using PTEN proteins instead of PTEN plasmid transfections to increase cellular longevity. Using PTEN proteins as an administered drug would allow a degree of precision over the effect produced more than transfection would. This also would allow a more rapid testing to deduce what the most favourable concentration of PTEN is to produce the most significant cellular longevity.

In regards to PTEN transfection, a future study that would be of great interest would be the combined transfection of PTEN and Telomerase reverse transcriptase (hTERT) in mice to determine whether the combined effect of these two genes are more than the sum of their parts. PTEN transfection and overexpression in mice have been shown to increase longevity and metabolism, and protect the organism from both insulin resistance and cancer (Ortega-Molina et al., 2012). Overexpression hTERT leads to telomerase reactivation, which was found to reverse tissue degeneration in artificially aged mice (Jaskelioff et al., 2011). There is a potential that PTEN may offset telomerase's capacity to induce the formation of tumours.

7 References

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