2017

Employing the enzyme cofactor function of ascorbic acid to affect oncogenic pathways in human melanoma: modulating Hypoxia Inducible Factor-1α and DNA demethylation to reduce malignant potential

Adam Patrick Fischer
fischer35@marshall.edu

EMPLOYING THE ENZYME COFACTOR FUNCTION OF ASCORBIC ACID TO AFFECT ONCOGENIC PATHWAYS IN HUMAN MELANOMA: MODULATING HYPOXIA INDUCIBLE FACTOR-1α AND DNA DEMETHYLATION TO REDUCE MALIGNANT POTENTIAL

A dissertation submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
In
Biomedical Sciences
by
Adam Patrick Fischer
Approved by
Dr. W. Elaine Hardman, Committee Chairperson
Dr. Sarah L. Miles
Dr. Pier Paolo Claudio
Dr. Beverly Delidow
Dr. Todd L. Green

Marshall University
August 2017

APPROVAL OF THESIS

We, the faculty supervising the work of Adam P. Fischer affirm that the dissertation, *Employing the Enzyme Cofactor Function of Ascorbic Acid to Affect Oncogenic Pathways in Human Melanoma: Modulating Hypoxia Inducible Factor-1 Alpha and DNA Demethylation to Reduce Malignant Potential*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

W. Elaine Hardman — 7/12/2017

Dr. W. Elaine Hardman, Committee Chairperson — Date
Department of Biomedical Sciences

Sarah L. Miles — 7/12/2017

Dr. Sarah L. Miles, Committee Member — Date
Department of Biomedical Sciences

Beverly C. Delidow — 7/21/2017

Dr. Beverly C. Delidow, Committee Member — Date
Department of Biomedical Sciences

Todd L. Green — 7/21/17

Dr. Todd L. Green, Committee Member — Date
Department of Biomedical Sciences

Pier Paolo Claudio — 7/16/2017

Dr. Pier Paolo Claudio, Committee Member — Date
Departments of BioMolecular Sciences and Radiation Oncology, The Univ. of Mississippi

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents for their unwavering support during challenging times I have encountered over the past five years. I would like to thank Julia Schrieber and Connie Berk for everything they have done to keep our lab running, and especially Margaret McFarland for all the help she has provided at the TGRI. My work would not have been possible without encouragement from several department chairs: Dr. Richard M. Niles, Dr. Donald Primerano, and Dr. Gary Rankin. I thank all of you for your past and continuing support. I also wish to acknowledge my undergraduate mentor and friend, Dr. Gary Z. Morris. Without his inspiration and reassurance, I never would have applied to the BMS program at Marshall. Lastly, I want to thank all the members of my committee for their contributions to my life and education over the past five years. I would like to thank Dr. Todd L. Green for helping me enter and remain in the BMS program, Dr. Beverly Delidow for her classroom instruction, especially her efforts to improve my speaking skills, both Drs. Pier Paolo Claudio and W. Elaine Hardman for their continuing mentorial support, and lastly Dr. Sarah L. Miles. Dr. Miles took me into her lab when my confidence was at an all-time low. With her encouragement, I was able to restore normalcy to my life in-and-out of the lab. Simply stated, I would not have been able to complete my degree at Marshall without her support, and for that I will be forever grateful.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Dioxygenase enzymes such as the HIF hydroxylases (PHD1-3, FIH) and the Ten-eleven translocation (TET1-3) enzymes regulate the activity of the hypoxia inducible factor-1α (HIF-1α) transcription factor and the DNA methylation status of cells, respectively. Aberrant accumulation and activation of HIF-1α can allow malignant cells to acquire attributes that promote progression, chemotherapy resistance, and survival, while aberrant hypermethylation of gene promoters can silence the expression of tumor suppressor genes essential to preventing tumorigenesis. Inadequate levels of intracellular ascorbic acid (AA), a necessary cofactor for optimal dioxygenase enzyme function, could potentiate these tumorigenic conditions. In fact, plasma levels of AA have been found to be below normal physiological levels in individuals with melanoma, as well as other cancer types. Interestingly, melanoma tumors frequently demonstrate both high expression of HIF-1α and increased promoter methylation. AA therefore may present a valuable adjuvant therapy option for melanoma patients by regulating the HIF-1 and DNA demethylation pathways. In these studies, we investigated the ability of AA supplementation to decrease aberrant HIF-1α accumulation and activity in melanoma cell lines and to increase global 5-hydroxymethylcytosine (5hmC) content of DNA in metastatic melanoma to ultimately reduce the malignant potential. Our studies showed that treatment with AA (reduced vitamin C) significantly increased intracellular vitamin C content of cultured melanoma cells and significantly decreased the stability, accumulation, and transcriptional activity of HIF-1α. Supplementation with AA also significantly increased global 5hmC content and ultimately resulted in the significant decrease of malignant potential of metastatic melanoma cells. Additionally, we discovered novel evidence that HIF-1α may regulate the DNA methylation status of melanoma cells by influencing the expression of TET2. Taken together,

these results suggest that deficiencies in AA levels, commonly observed in malignant tissue and blood plasma of cancer patients, may be a contributing factor to the deregulation of the HIF-1α and DNA demethylation pathways. Deregulation of these pathways is likely due to the loss of its cofactor function for the dioxygenase enzymes that tightly regulate various oncogenic factors. Furthermore, AA has the potential to be a powerful adjuvant therapy for metastatic melanoma, and further investigations in multiple cancer types are warranted to advance the use of AA to the clinical setting.

CHAPTER 1
INTRODUCTION

Ascorbic acid (AA; reduced vitamin C) is a well-known water soluble anti-oxidant and a key nutrient that is necessary for most life on the planet. AA is a simple six carbon molecule closely related to glucose. AA was first isolated in the late 1920's and early 1930's by a Hungarian scientist named Albert von Szent-Györgyi, who later won the Nobel prize in physiology or medicine in 1937, in part because of his discovery. A high physiological concentration of ascorbic acid has long been considered an indicator of good health. Currently, there is mounting evidence that ascorbic acid may be useful for treating or preventing multiple medical conditions including cardiovascular disease, diabetes, Alzheimer's disease, and especially cancer (Bowman, 2012; Gokce *et al.*, 1999; Harding *et al.*, 2008). While AA therapy may likely be useful in treating a variety of different malignancies, it may well be most advantageous for treating melanoma.

Melanoma is a malignancy derived from melanocytes in the epidermis and is responsible for 75 % of skin cancer fatalities (Siegel, Miller, and Jemal, 2016). Once identified, primary melanomas can be easily cured by surgical excision; however, patients with metastatic disease have few treatment options, generally resulting in a poor prognosis. Thus, evaluation of the anti-malignant properties of AA as an adjuvant therapy against melanoma is of timely importance.

In this work, we explore and investigate the effect of AA on human melanoma cells via its often-overlooked enzymatic cofactor function. The results of these studies provide evidence that supplementation with fully reduced vitamin C (AA) can decrease the malignant potential of metastatic melanoma cells, likely by inhibiting the aberrant protein accumulation of Hypoxia inducible factor-1α (HIF-1α) and increasing 5-hydroxymethylcytosine (5hmC) content of the DNA.

Mammalian synthesis of ascorbic acid

All known plant species synthesize and accumulate ascorbic acid, albeit some species use different mechanisms to do so. Plant species that are able to accumulate high concentrations of AA are consumed as a dietary source of vitamin C by numerous animal species; however, most vertebrates also have the ability to endogenously synthesize their own AA. AA is synthesized in a multi-step enzymatic process from glucose, where the terminal reaction is the conversion of L-gulono-1,4-lactone to L-ascorbic acid by the enzyme L-gulonolactone oxidase (GULO) (Fig. 1) (Linster and Van Schaftingen, 2007).

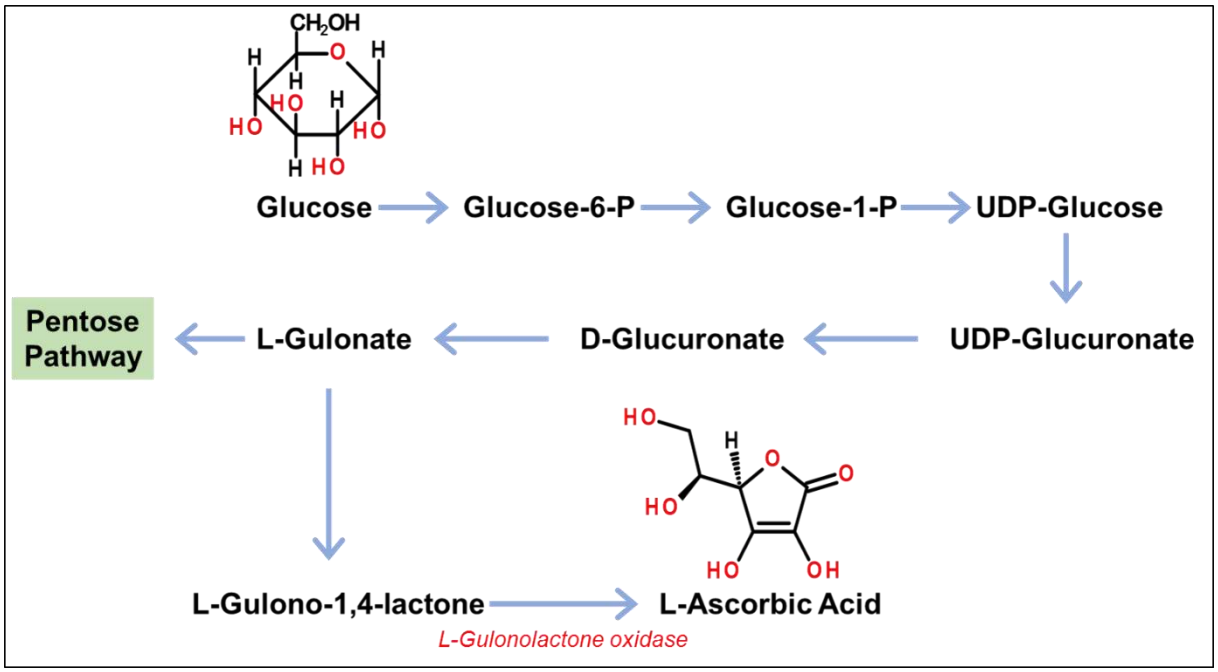

Figure 1. Mammalian synthesis of ascorbic acid.
In mammals, ascorbic acid is synthesized in a multiple step enzymatic process within the liver. In the final reaction, the enzyme L-gulonolactone oxidase catalyzes the conversion of L-gulono-1,4-lactone to L-ascorbic acid. In addition, the intermediate L-gulonate can be shunted into the pentose pathway.

Mammals synthesize AA in the liver, whereas lower vertebrates such as fish, amphibians, and reptiles do so in the kidneys (Linster and Van Schaftingen, 2007). While the ability to

synthesize AA has been highly conserved throughout the animal kingdom, several bat species, guinea pigs, and anthropoid primates (including humans) are unable to endogenously synthesize AA (Drouin, Godin, and Pagé, 2011; Linster and Van Schaftingen, 2007). The inability to synthesize AA is caused by severe mutations to the GULO gene. In humans, seven out of twelve exons are deleted, resulting in the transcription and translation of a non-functional protein (Drouin *et al.*, 2011). Although bats, guinea pigs, and anthropoid primates have this trait in common, evidence suggests there is not a single common ancestor where loss of GULO evolved linking these mammalian species together. Using a combination of phylogenetics and evolutionary biology, researchers have determined that the loss of AA synthesis has occurred at least three distinct times during mammalian evolution, with primates and guinea pigs developing this trait approximately 61 million and 14 million years ago, respectively, while bats are yet to be determined (Fig. 2) (Drouin *et al.*, 2011).

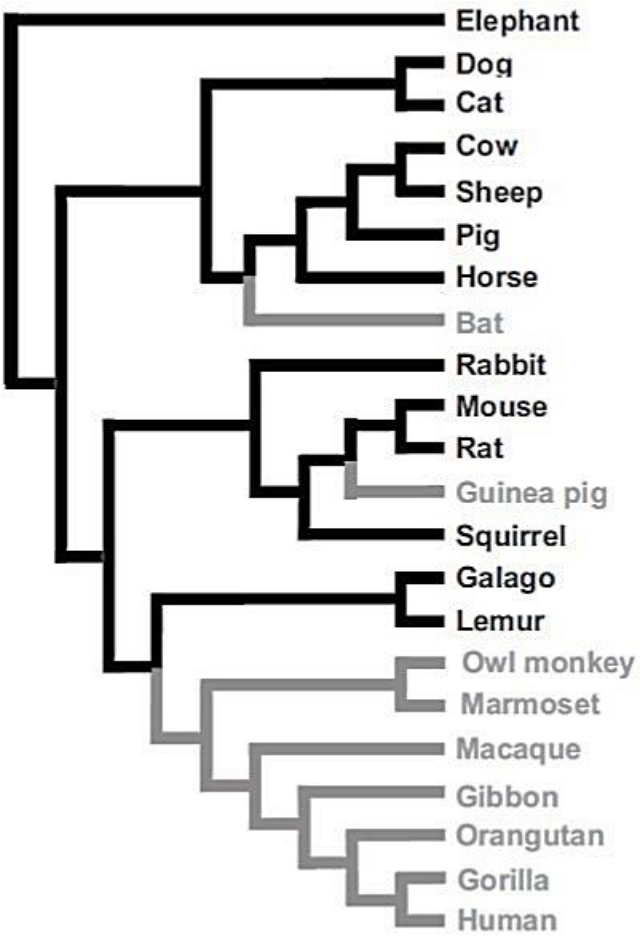

Figure 2. Phylogenetic distribution of the ability to synthesize vitamin C in mammals. Lineages able to synthesize vitamin C are in black; those incapable are in gray. The phylogenetic relationships are based on those in reference (Prasad, Allard, and Green, 2008).

Loss of ascorbic acid synthesis may have provided a selective survival advantage for these species, possibly by inhibiting the production of H_2O_2, a known byproduct of GULO activity (Linster and Van Schaftingen, 2007). This may have been beneficial by limiting free radical induced DNA damage. Another plausible benefit from the loss of AA synthesis may have been the conservation of glucose or the accumulation of AA intermediates that could be shunted into other metabolic pathways such as the pentose synthesis pathway. These benefits may have been particularly advantageous given that all species that lost GULO function had diets that were

naturally rich in vitamin C, thus losing AA synthesis was not detrimental to survival. However at least two bat species within two different families of Chiroptera have been found to reactivate GULO activity, although it was several fold lower than that found in mice (Drouin *et al.*, 2011). This finding has prompted some researchers to hypothesize loss of GULO did not encourage survival but instead was a physiologically insignificant mutation that has been preserved over time.

Transport of vitamin C in humans

Non-AA synthesizing animals have developed intricate systems for maintaining consistent steady-state AA levels through intestinal absorption, dissemination, recycling, and excretion. Dietary vitamin C[1] exists in two forms, as ascorbic acid (AA; reduced vitamin C) or a fully oxidized form called dehydroascorbic acid (DHA). Both forms of vitamin C are absorbed by the brush border membrane within the lumen of the small intestine, albeit AA absorption in humans is greatest in the distal ileum, while maximum DHA absorption occurs in the jejunal segments (Malo and Wilson, 2000). Differences in regional vitamin C absorption are likely due to the distribution of vitamin C transporters within the intestine. AA is specifically transported across the plasma membrane by sodium dependent vitamin C cotransporters (SVCT1 and 2), while DHA competes with glucose to enter cells through glucose transporters (GLUTs) (Du, Cullen, and Buettner, 2012b).

The SVCT1 isoform is primarily responsible for AA absorption and reabsorption from the intestinal lumen and kidneys, and is confined to the apical surface of enterocytes and renal tubule cells (Du *et al.*, 2012b; Lindblad, Tveden-Nyborg, and Lykkesfeldt, 2013). Transport of AA is dependent on a sodium gradient where two moles of sodium enter the cell per every mole of AA (Wohlrab, Phillips, and Dachs, 2017). Alternatively, DHA enters the apical side of

enterocytes via GLUT1 and 3. Intestinal uptake of DHA via GLUT transport has a lower efficiency (Km = 0.8 mM) compared to AA uptake by intestinal SVCT1 transporters (Km = 0.2 mM), however maximal uptake rates between the two systems are similar in the absence of glucose (Du *et al.*, 2012b; Wilson, 2002).

Once DHA enters the enterocytes, it is enzymatically reduced to AA in the cytosol either by glutathione-dependent or nicotinamide adenine dinucleotide (NADPH)-dependent DHA reductases (Lindblad *et al.*, 2013; Linster and Van Schaftingen, 2007). Additionally, cytosolic glutathione can spontaneously reduce DHA. However, this reaction is not thermodynamically favored, and thus is thought to contribute little to DHA reduction (Linster and Van Schaftingen, 2007). Accumulated AA in the intestinal epithelium is released into the plasma by diffusion or hypothesized volume sensitive anion channels, allowing systemic dissemination of vitamin C in the form of AA (Lindblad *et al.*, 2013; Wilson, 2002). Circulating levels of DHA are very low (2 – 5 μM) compared to levels of AA (40 – 80 μM) (Du *et al.*, 2012b; Liang, Johnson, and Jarvis, 2001). Low intra-enterocyte and plasma concentrations of DHA help promote continual DHA uptake from the intestinal lumen down its concentration gradient.

The expression of the SVCT2 isoform is wide-spread throughout most tissues, allowing this transporter to maintain systemic organ and tissue levels of AA (Fig. 3) (Corti, Casini, and Pompella, 2010; Lindblad *et al.*, 2013). While SVCT1 and 2 share approximately 65 % amino acid identity (Lindblad *et al.*, 2013), SVCT2 (Km ≈ 8 – 62 μM) has a higher efficiency for AA uptake than SVCT1 (Km ≈ 65 – 240 μM) allowing tissues to accumulate a higher level of intracellular AA than is found in the plasma (Du *et al.*, 2012b). Some tissues, such as muscle and cardiac tissue, accumulate relatively low concentrations of AA (0.2 mM), while the brain accumulates the highest concentration of AA at approximately 2 – 10 mM (Lindblad *et al.*,

2013). These levels are achieved even though SVCT2 is absent in the blood brain barrier. Instead, AA enters the brain by SVCT2 mediated transport into the cerebrospinal fluid at the choroid plexus (Lindblad *et al.*, 2013).

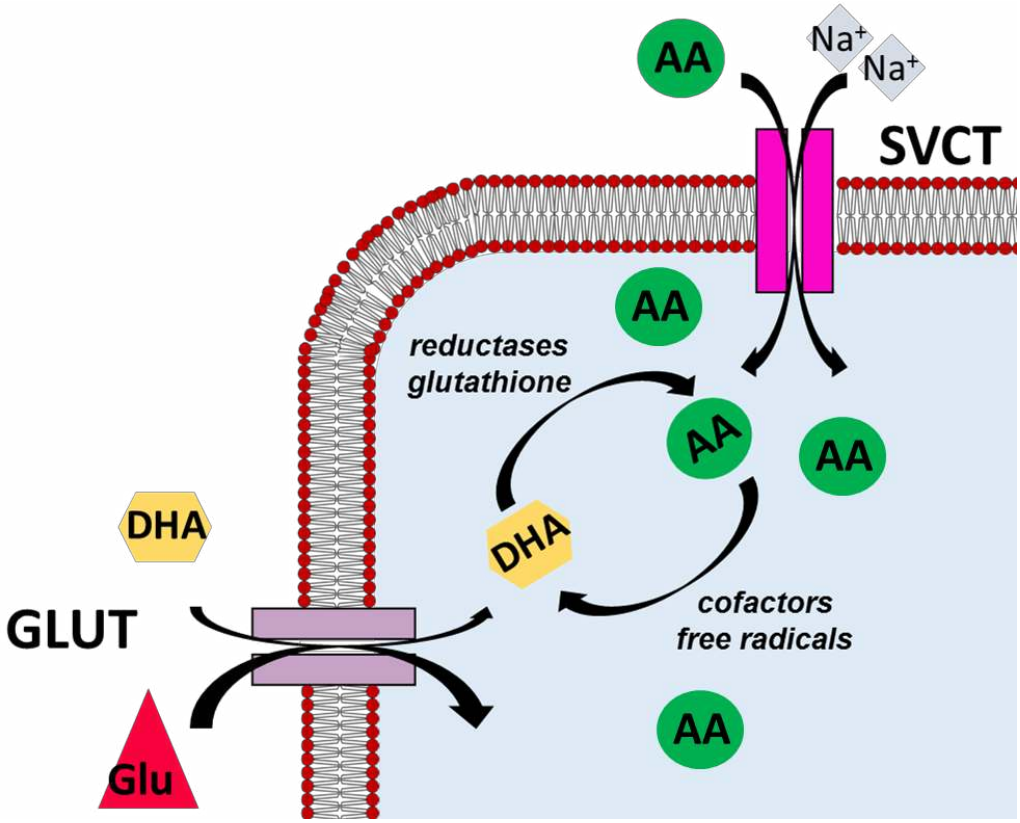

Figure 3. A schematic diagram of the entry and recycling of ascorbic acid and DHA in the cell.
Ascorbic acid enters the cell through sodium-dependent vitamin C co-transporters (SVCTs). Alternately, dehydroascorbate (DHA) competes with glucose (Glu) to enter the cell via the glucose transporters (GLUTs). Once inside the cell, DHA can be enzymatically or non-enzymatically reduced in the cytosol to AA, promoting the accumulation of intracellular AA.

Metabolism and maintenance of vitamin C

Once AA accumulates in the cytosol it can be used either as an anti-oxidant or an enzyme cofactor by donating an electron to a free radical or enzyme. In the process, AA becomes oxidized to form DHA. DHA itself has no biological activity because it is already fully oxidized. To preserve vitamin C activity, tissues can reduce DHA back to AA, a process called vitamin C

recycling, using the same enzymatic and non-enzymatic mechanisms present in the enterocytes. Vitamin C recycling also contributes to high AA accumulation within the cytosol; however, in some cases recycling of intracellular DHA may not be possible. Low activity of DHA reductases and/or depletion of glutathione and NADPH can inhibit the reduction of DHA to AA. Moreover, DHA is relatively unstable at physiological pH, resulting in its spontaneous and irreversible degradation to 2,3-diketogulonic acid (2,3-DKG), which can be sequentially metabolized into terminal end products of L-erythrulose and oxalic acid (Linster and Van Schaftingen, 2007). Metabolites of DHA are exported into the plasma where they are filtered and excreted by the kidneys. Thus, intracellular reduction of DHA to AA serves to not only provide functional AA, but also to prevent DHA degradation and loss of total vitamin C.

As previously stated (Du *et al.*, 2012b), normal physiological levels of plasma AA levels range from approximately 40 – 80 μM, but this range is highly variable depending on several factors including age, sex, medical conditions, and the socioeconomic status of an individual. Nonetheless, the body takes great measures to maintain a constant steady-state of AA. Dietary vitamin C absorption becomes saturated as plasma AA levels approach the upper physiological limits (Lindblad *et al.*, 2013). Excessive circulating levels of AA are filtered by the kidneys and excreted in the urine, as AA is highly water soluble. Conversely, during periods of mild to moderate deficiency, AA is reabsorbed in the tubular system (Corti *et al.*, 2010). Plasma contains low circulating levels of DHA, primarily because of its minimal release from intestinal epithelium and its instability (Lindblad *et al.*, 2013; Linster and Van Schaftingen, 2007; Wilson, 2002). In addition, intracellular loss of DHA from somatic cells through GLUTs and oxidant dependent oxidation of AA in the plasma can elevate circulating DHA levels causing the risk of vitamin C loss. However, vitamin C loss is minimized via DHA uptake by erythrocytes.

Erythrocytes lack both SVCT1 and 2 transporters, but are adept at rapid DHA uptake from GLUT1 (Du *et al.*, 2012b; Lindblad *et al.*, 2013). Once recycled and accumulated as AA, the AA in erythrocytes is slowly released, presumably by diffusion, back into the plasma (Mendiratta, Qu, and May, 1998), preserving functional AA.

Vitamin C deficiency and disease

Although non-AA synthesizing animals are proficient at maintaining vitamin C homeostasis, lack of vitamin C consumption for prolonged periods of time results in a medical condition known as scurvy. Scurvy is a potentially fatal condition characterized by chronic fatigue, joint pain, gum bleeding, poor wound healing, and internal hemorrhaging. Scurvy was commonly associated with transoceanic European sailors from the 15th through 18th centuries and is estimated to have killed nearly two million men during that time (Carpenter, 1986; Hemilä, 2006). Today, a diagnosis of scurvy is rare in industrialized countries; however, it is more prevalent in individuals living below the poverty line and in third world countries (Institute of Medicine, 2000). Death from scurvy is typically due to exsanguination from massive internal bleeding occurring after weeks of AA depletion, yet scurvy can be reversed, with a full recovery, by vitamin C consumption, even when death is imminent. The severity of the symptoms of scurvy, culminating in death, highlights the critical importance of vitamin C in human physiology. In fact, the term "vitamin C" was given to AA to indicate it is essential for human survival, while the same molecule technically is not a vitamin for AA-synthesizing animals.

Unfortunately, some chronic medical diseases, such as cancer, are now thought to play a role in nutrient depletion such as vitamin C. In 1952 Oscar Bodansky M.D. performed one of the first studies documenting significantly lower plasma AA levels in cancer patients compared to healthy individuals (Bodansky, Wroblewski, and Markardt, 1952). Evaluation of AA levels in

cancer patients continued into the next decade (Fraenkel-Conrat, Stoy, and Tsai, 1967), prompting some of the first clinical trials of AA supplementation therapy for patients with advanced cancer by Cameron and Pauling (Cameron and Campbell, 1974; Cameron and Pauling, 1976). In these trials, Cameron and Pauling reported that high dose infusion of I.V. AA extended the life of patients by approximately 160 days compared to those without treatment. Almost immediately contradictory results were published (Creagan *et al.*, 1979), embroiling vitamin C cancer therapy into controversy that still exists today. Modern clinical trials still investigate the use of high dose AA therapy on cancer patients with mostly mixed or inconclusive results. The rationale for such a treatment is that intravenous administration of high dose AA can bypass the strict regulation of the gut and increase peak plasma AA levels to approximately 500 times typical physiological levels (Stephenson, Levin, Spector, and Lis, 2013). At these concentrations AA acts as a pro-oxidant facilitating the formation of H_2O_2. Excessive H_2O_2 has been observed to be selectively cytotoxic to malignant cells *in vitro* and *in vivo* models (Chen *et al.*, 2005; Chen *et al.*, 2007; Ohno, Ohno, Suzuki, Soma, and Inoue, 2009), presumably because glutathione depleted malignant cells, likely from an elevated endogenous oxidative burden or decreased synthesis of endogenous antioxidant enzymes, cannot tolerate the added oxidative stress of H_2O_2. While the efficacy of high dose AA therapy continues to be debated, it is generally accepted that it is not uncommon for individuals with cancer to have below normal levels of plasma AA. In some instances, oncology patients can become vitamin C deficient to the point of developing scurvy. Case reports from the Jean Verdier Hospital in France during the mid-1990's indicated that 3 % of their cancer patients were concurrently diagnosed with scurvy, approximately 15 times higher than the scurvy diagnosis of non-cancer patients (0.2 %) (Fain, Mathieu, and Thomas, 1998). Conclusive evidence on the mechanism of AA depletion in cancer patients is yet

to be elucidated. Some have postulated that chemotherapy and radiation treatments as well as the associated emesis contribute to or causes the observed AA deficiency in these patients. However a study by Campbell et al. found that GULO -/- knockout mice inoculated with B16 murine melanoma and Lewis Lung Carcinoma had significantly lower plasma and liver tissue AA levels (determined after tumor volume reached 1000 mm^3) compared to non-inoculated mice, even though they were supplemented with an identical concentration of AA (Campbell *et al.*, 2014). This evidence suggests an active malignancy, and not chemotherapy induced malnutrition, may be responsible for AA depletion observed in cancer patients. Regardless of the mechanism, even moderate AA deficiency may significantly increase the risk of cancer mortality. In a study, individuals with a plasma AA concentration < 28 μM had a 62 % increased likelihood of dying from cancer compared to those with AA levels ≥ 74 μM (Loria, Klag, Caulfield, and Whelton, 2000). Moreover, that risk decreased dose-dependently with increasing plasma AA (Goyal, Terry, and Siegel, 2013).

The onset of scurvy following the depletion of vitamin C in non-AA synthesizing animals is not caused by the loss of an anti-oxidant, as there are many anti-oxidant enzymes, vitamins, and minerals found within the body. Instead these effects are caused by the inhibition of Fe II/2-oxoglutarate (2-OG) dependent dioxygenase enzymes that requires AA as a cofactor (Kuiper and Vissers, 2014c). 2-OG dioxygenases regulate a vast number of cellular processes ranging from neurotransmitter synthesis to DNA modifications via the hydroxylation of different substrates. Individual subfamilies of enzymes are categorized either by the substrates they modify or the functions they regulate. Interestingly, aberrant function of several enzyme subfamilies may be related to malignant transformation or progression (Kuiper and Vissers, 2014c). 2-OG dioxygenases regulate processes significant to oncogenesis such as the activation of oncogenic

transcription factors, epigenetic modifications to DNA and histones, and changes in metabolism (Kuiper and Vissers, 2014c). Given the need for adequate AA to function as a cofactor and the observed AA deficiency of cancer patients, it is highly plausible that low levels of AA can contribute to aberrant regulation of cancer promoting pathways by the inhibition of 2-OG dioxygenases.

Ascorbic acid is a cofactor for Fe II/2-oxoglutarate dioxygenases

2-OG dioxygenases likely contributed to the evolutionary history of life as we know it today. Many examples of 2-OG dioxygenases can be found in both bacteria and eukaryotes including fungi, plants, and vertebrates (Farrow and Facchini, 2014). In humans, 2-OG dioxygenases modulate the structure, function and/or activity of cellular substrates via hydroxylation. All 2-OG dioxygenases contain a conserved catalytic core that is comprised of a distorted double-stranded β-helix fold (DSBH) (Hewitson, Granatino, Welford, Mcdonough, and Schofield, 2005; Mcdonough, Loenarz, Chowdhury, Clifton, and Schofield, 2010). The DSBH core consists of 8 anti-parallel β-strands that form a major and minor β-sheet (Clifton *et al.*, 2006; Mcdonough *et al.*, 2010; Ozer and Bruick, 2007), the latter being supported by at least 2 N-terminal helices (Aik *et al.*, 2015). While the enzyme core is highly conserved, different structures surrounding the DSBH are thought to influence substrate recognition, lending to the high degree of substrate variability observed between subfamilies (Aik *et al.*, 2015; Clifton *et al.*, 2006; Flashman and Schofield, 2007; Hausinger, 2004). Before substrate binding and hydroxylation can occur non-heme ferrous iron (Fe II) coordinates with a conserved HXD/E…H motif, often described as a facial triad, within the DSBH core (Aik, Mcdonough, Thalhammer, Chowdhury, and Schofield, 2012; Hegg and Que, 1997; Valegard *et al.*, 1998; Zhang *et al.*, 2000). Adjacent to Fe II binding is the binding site for the molecule 2-oxoglutarate (2-OG), the

enzyme family namesake (Aik *et al.*, 2012; Clifton *et al.*, 2006; Hausinger, 2004; Mcdonough *et al.*, 2010). Binding of 2-OG is necessary to allow the entry of the substrate into the catalytic core (Kuiper and Vissers, 2014c). Once inside the core, the substrate, more specifically the C—H bond to be oxidized, is held in close proximity to Fe II by hydrogen, hydrophobic, and electrostatic interactions (Aik *et al.*, 2012). Molecular oxygen (O_2) can then enter the core and coordinate with Fe II to induce oxidative decarboxylation of 2-OG producing succinate, CO_2, and a highly reactive Fe IV oxo intermediate. Fe IV rapidly extracts hydrogen from the substrate, replaces it with a hydroxyl group, while concurrently being reduced to Fe II in the process, in conjunction with the release of products and a hydroxylated substrate (Aik *et al.*, 2015). However during the process of oxidative decarboxylation electron transfer to Fe IV can become uncoupled, resulting in the formation of Fe III (Kuiper and Vissers, 2014c). Fe III antagonizes enzyme function prompting the loss of catalytic activity and an inability to modify substrates. To reestablish enzyme activity, 2-OG dioxygenases utilize intracellular AA to further reduce Fe III to Fe II, thus restoring catalytic activity (Kuiper and Vissers, 2014c). Interestingly, the interacting site for AA within the DSBH core is extraordinarily specific for the ene-diol structure of AA. Therefore, other antioxidant molecules are unable to position in proximity to efficiently donate an electron to Fe III (Flashman, Davies, Yeoh, and Schofield, 2010), making AA an essential co-factor for optimal 2-OG dioxygenase activity (Fig. 4).

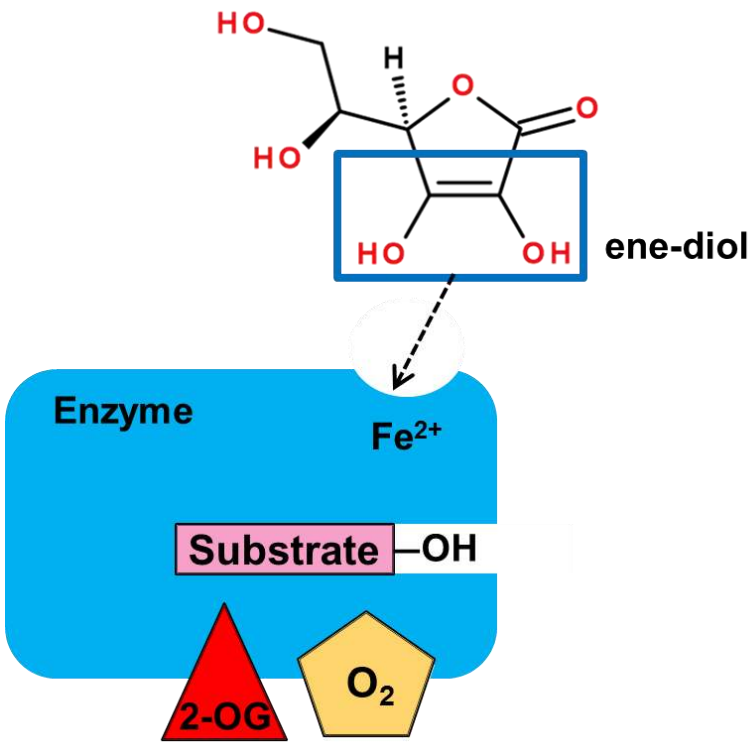

Figure 4. A schematic diagram of Fe II/2-oxoglutarate dioxygenases and the structural significance of AA.
Fe II/2-oxoglutarate dioxygenases are able to hydroxylate a substrate using a common set of cofactors including 2-oxoglutarate (2-OG), molecular oxygen, and enzyme bound non-heme ferrous iron. During the process of hydroxylation, electron transfer to iron can become uncoupled resulting in the formation of Fe^{3+} and loss of enzymatic activity. AA enters into a binding cleft that is highly specific for the ene-diol structure of AA, and donates an electron to reduce Fe^{3+} to Fe^{2+} restoring enzymatic activity.

Regulation of the HIF transcription factors by ascorbic acid

Solid tumors are comprised of multiple malignant and non-malignant cell populations. As the expansive mass continues to grow, oxygen deprivation in localized areas becomes common and allows for regions of intratumoral hypoxia. Oxygen deprivation applies selective pressure that promotes the clonal selection and expansion of malignant populations that are best suited for adaptation, survival, and replication. Surviving cells frequently have a combination of activated oncogenes and impaired tumor suppressors that increase the overall malignancy of the cancer. Hypoxia contributes to adaptation and survival, and thus can augment malignancy by inducing

changes in gene expression. Hypoxic gene expression is mediated by the predominate accumulation of the hypoxia inducible factor-1 transcription factor (HIF-1) in epithelial cells and HIF-2 in endothelial cells. HIF-1α and 2α are heterodimeric transcription factors, where HIF-1α and 2α are the oxygen responsive subunits that mediate transcription factor stability and activity. Elevated expression of either HIF-1α or 2α has been extensively documented in numerous solid malignancies (Semenza, 2010). HIF is known to promote the acquisition of multiple cancer hallmarks, contributing to wide-spread chemotherapy resistance and ultimately decreased patient survival (Semenza, 2010).

Under physiological tissue oxygenation (non-hypoxic), HIF-1α is post-translationally modified on proline 402 and/or 564 within the oxygen dependent death domains (ODD) by HIF-prolyl hydroxylases (PHD1-3), belonging to the 2-OG dioxygenase family. The hydroxylation of proline residues promotes HIF-1α to interact with the von Hippel-Lindau tumor suppressor protein (pVHL), facilitating polyubiquination and subsequent degradation by the 26S proteasome, thereby inhibiting hypoxia gene expression. Alternatively, another member of the 2-OG dioxygenase family, Factor Inhibiting HIF (FIH), hydroxylates asparagine 803 within the C-terminal transactivating domain (C-TAD), inhibiting the interaction of the transcription factor with CBP/p300 co-activator, blocking gene transcription (Schofield and Ratcliffe, 2005) (Fig. 5).

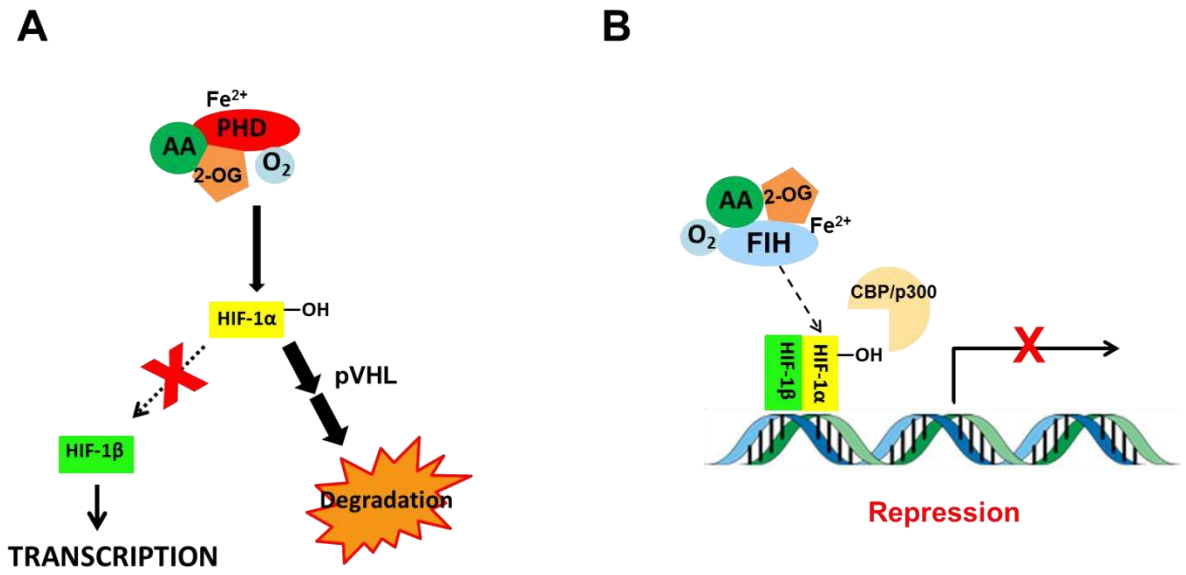

Figure 5. Canonical regulation of the HIF-1α transcription factor under normoxic conditions.
A) The protein stability of HIF-1α is regulated by PHD1-3. During normoxic conditions, PHD enzymes require 2-oxoglutarate (2-OG), O_2, Fe^{2+}, and AA for optimal activity. At optimal activity, PHDs hydroxylate HIF-1α protein, allowing it to interact with pVHL. pVHL polyubiquinates the subunit, targeting it for proteasomal degradation and inhibiting dimerization with HIF-1β. B) Additionally, HIF-1 transcriptional activity can be regulated by FIH. Using the same cofactors, FIH hydroxylates the transactivating domain of HIF-1α, inhibiting interaction with CBP/p300 transcriptional co-activator and blocking HIF regulated gene expression.

When O_2 becomes limiting, as during tissue hypoxia, the catalytic activity of HIF hydroxylases, particularly PHDs, is lost allowing for the accumulation and activation of the complete transcription factor to elicit the cellular hypoxic response.

In addition to the canonical HIF pathway, HIF-1α can also aberrantly accumulate in malignant cells in an oxygen independent manner, which usually involves some mechanism of HIF hydroxylase dysfunction. Inactivating mutations of the HIF hydroxylases are rare; however, a PHD2 gene mutation has been observed in endometrial cancer (Jokilehto and Jaakkola, 2010). Other mutations of consequence are the oncogenic mutations of succinate dehydrogenase (SDH) and fumarate hydratase (FH). These enzymes are integral components of the TCA cycle; however, inhibiting mutations to either enzyme allows for the elevated cellular content of

succinate or fumarate, respectively. These metabolic intermediates competitively bind the 2-OG site on HIF hydroxylases, inhibiting their functionality similar to O_2 deprivation. Individual SDH and FH mutations have been found to be sufficient to induce HIF-1α activation adequate to confer a clear survival advantage to malignant cells (Masson and Ratcliffe, 2014; Raimundo, Baysal, and Shadel, 2011). In fact, additional TCA cycle metabolites including oxaloacetate, malate, isocitrate, and citrate have been observed to differently inhibit PHD and FIH isoforms under various assay conditions (Koivunen *et al.*, 2007; Masson and Ratcliffe, 2014); however, the relevance of this finding in a physiological condition is unclear.

Similar to HIF hydroxylase inhibition by TCA cycle intermediates, inadequate intracellular AA may also promote aberrant normoxic accumulation and activation of HIF-1α in malignant cells. Accumulation of HIF-1α protein, as well as tumor stage, has been found to correlate with low tissue levels of AA in endometrial and colorectal cancers (Kuiper *et al.*, 2014b; Kuiper, Molenaar, Currie, Robinson, Pearson, and Vissers, 2010), suggesting inadequate intracellular AA may contribute not only to aberrant HIF-1α activation but also to malignant progression. *In vivo* studies using GULO -/- mice (a model of human AA dependency) inoculated with murine melanoma cells demonstrate that AA supplementation can decrease tumor accumulation of HIF-1α (Campbell *et al.*, 2014) and decrease tumor volume (Campbell *et al.*, 2014; Cha *et al.*, 2011; Cha *et al.*, 2013). Furthermore, tumor ascorbate levels inversely correlated to the expression of HIF-1 target genes (Campbell *et al.*, 2014), providing further support for the use of AA as an adjuvant cancer therapy.

Ascorbic acid influences DNA methylation patterns via TET enzymes

DNA methylation is the most prevalent form of epigenetic modification and is responsible for regulating many cellular processes, including gene expression, X chromosome

inactivation, genomic imprinting, and the pluripotency of stem cells (Monfort and Wutz, 2013; Suzuki and Bird, 2008). Normally, DNA methylation is generated by the addition of a methyl group to the 5 position of cytosine by DNA methyltransferases (DNMTs), yielding 5-methylcytosine (5mC) (Ooi, O'donnell, and Bestor, 2009). Methylation of cytosine within a gene promoter silences gene expression by inhibiting transcription. Typically, 5mC is present at the same position on both the sense and anti-sense DNA strands, preserving epigenetic regulation following DNA replication and mitosis (Ficz and Gribben, 2014; Rasmussen and Helin, 2016). The status of DNA methylation within the cell can be altered by the three isoforms of the ten-eleven translocation (TET) enzyme. TET1-3 are members of the 2-OG dioxygenase family that catalyze the formation of 5-hydroxymethylcytosine (5hmC) from 5mC (Huang and Rao, 2014). 5hmC is relatively stable and abundant within the genome and is known to interfere with the role of DNMT1/UHRF1 complex in maintaining DNA methylation following DNA replication (Ficz and Gribben, 2014; Huang and Rao, 2014; Rasmussen and Helin, 2016). In addition, 5hmC can be further oxidized by TETs to 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC), which are then excised and replaced with unaltered cytosine by the DNA repair enzyme thymine-DNA glycosylase (TDG) (He *et al.*, 2011; Ito *et al.*, 2011) (Fig. 6).

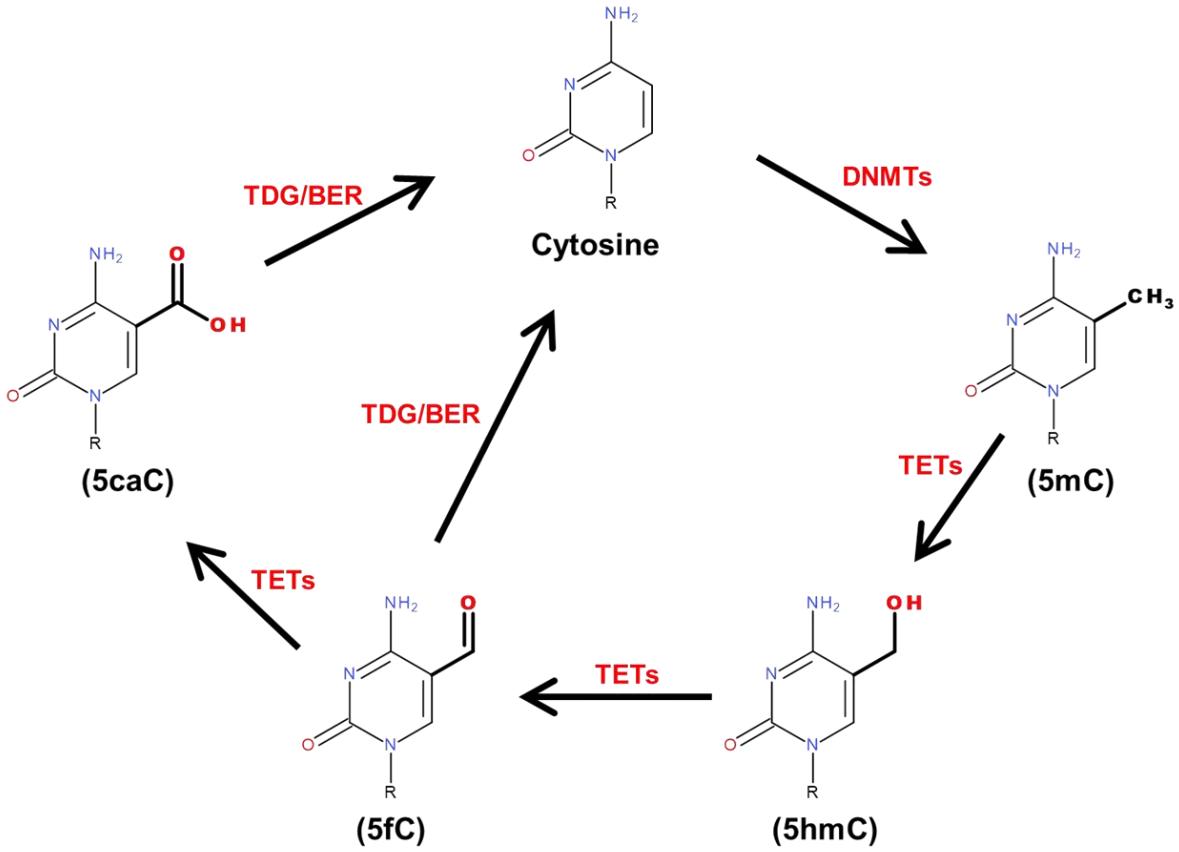

Figure 6. The active methylation and demethylation of cytosine.
Cytosine can be methylated to form 5mC by multiple DNMTs. TET enzymes can facilitate active demethylation by oxidizing 5mC to 5hmC. TET enzymes can then further oxidize 5hmC to 5fC and 5caC, where the latter two intermediates are substrates for the DNA repair enzyme TDG followed by base excision repair (BER), which removes and replaces the modified cytosine with an unmodified cytosine.

Reduced function of TET enzymes either by mutation or deletion has been prominently observed in hematological malignancies. While TET1 and 3 are seldom altered, more than 700 mutations in TET2 have been identified in leukemia patients with a frequency of 50 % and 20 % in chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML), respectively (Huang and Rao, 2014). While the prevalence of TET mutations/deletions in solid malignancies is much lower, reduced protein expression and/or enzyme inhibition has been observed in clear-cell renal cell carcinoma, colorectal, gastric, prostate, liver, lung, and breast cancers, in addition

to glioblastoma and melanoma, suggesting TET enzymes act as tumor suppressors (Huang and Rao, 2014; Rasmussen and Helin, 2016). It has been speculated that TET dysfunction promotes malignant progression by silencing the expression of tumor suppressor genes because of the failure to demethylate gene promoters (Huang and Rao, 2014). Further evidence must be collected before TET mutations are considered a "driver" of oncogenesis; however, there is a strong correlation between the absence of 5hmC in cancer and tumor growth and metastasis (Chen *et al.*, 2017; Hsu *et al.*, 2012; Song *et al.*, 2013). Given that earlier studies determined TET proteins are solely responsible for genome wide hydroxymethylation of cytosine (Ficz and Gribben, 2014; Koh *et al.*, 2011), aberrant TET activity undoubtedly contributes to cancer progression. Like all other 2-OG dioxygenases, TET proteins use AA as a cofactor to maintain enzymatic activity (Monfort and Wutz, 2013). Reliance on AA means that inadequate intracellular ascorbate may hinder the catalytic function of TETs similar to oncogenic mutations, and therefore contribute to cancer progression by silencing tumor suppressors. Multiple *in vitro* studies have demonstrated vitamin C supplementation increases the prevalence of 5hmC while also facilitating DNA demethylation (Blaschke *et al.*, 2013; Dickson, Gustafson, Young, Zuchner, and Wang, 2013; Minor, Court, Young, and Wang, 2013; Monfort and Wutz, 2013; Yin *et al.*, 2013). In these studies, vitamin C activity was dependent on TET expression (Blaschke *et al.*, 2013; Minor *et al.*, 2013; Yin *et al.*, 2013) and the function of SVCT2 (Dickson *et al.*, 2013), an AA specific transporter, confirming the importance of AA in modulating TET activity.

Melanoma as a model for AA therapy

Melanoma is the deadliest form of skin cancer and its incidence has continually risen over the past 30 years. Current projections estimate 1 in 28 men and 1 in 44 women in the United

States will be diagnosed with skin melanoma in their lifetime, resulting in nearly 10,000 fatalities in 2017 (Siegel, Miller, and Jemal, 2017). Aberrant activation of the mitogen-activated protein kinase (MAPK) pathway, caused by NRAS and BRAF mutations, have been described in approximately 90 % of melanomas (Strickland, Pal, Elmets, and Afaq, 2015), contributing to malignant growth, proliferation, and survival (Inamdar, Madhunapantula, and Robertson, 2010). Melanoma growth is commonly classified into one of three stages: radical growth phase (RGP), vertical growth phase (VGP), or metastatic phase (MET) (Table 1). While primary melanomas can be readily cured by surgical excision, once metastasized the disease is highly aggressive and difficult to treat. The use of immunotherapy such as Interleukin-2 (IL-2) and BRAF inhibitors has resulted in more favorable initial responses. Unfortunately, these therapies can be highly toxic and frequently lead to acquired resistance and poor clinical outcomes, highlighting the need for new treatment strategies against metastatic melanoma.

Stage of Malignancy	Characteristics	Cell Lines
Radical Growth Phase (RGP)	Expansive growth confined to epidermis	SbCl2
Vertical Growth Phase (VGP)	Growth has penetrated the dermis and/or subcutaneous tissue; malignant cells may be disseminating into circulation	WM3211, WM1366
Metastatic Phase (MET)	Tumor growth at a secondary site	WM9, WM239A
Unknown	Malignant cells isolated from an unspecified stage	WM3248
Non-malignant	Non-cancerous primary melanocytes	HEMnLP

Table 1. Growth phases of malignant melanoma.
Classification and description of different human melanoma cell lines and primary melanocytes used in this study. Cell lines are classified as designated by Rockland Immunochemicals Inc. at time of publication.

As previously mentioned, it is not uncommon for individuals with cancer to have below normal levels of plasma AA. Melanoma is one of many cancer types that has been documented

to be correlated with AA deficiency (Schleich *et al.*, 2013). Interestingly, advanced melanomas have been shown to contain high aberrant activity of the HIF-1α transcription factor as well as a significant lack of 5hmC content (Kuphal, Winklmeier, Warnecke, and Bosserhoff, 2010; Lian *et al.*, 2012), both of which may be explained, to some extent, by impaired HIF hydroxylase and TET enzyme function due to inadequate levels of AA. In the present work, we investigate the ability of different forms of vitamin C to decrease the malignant properties of human melanoma, in part, by promoting optimal activity of the HIF hydroxylases and TET enzymes. In chapter 2, we examine the ability of AA and an ascorbate analog (ascorbate 2-phosphate; A2P) to decrease the aberrant normoxic and hypoxia-mimetic induced protein accumulation and transcriptional activity of HIF-1α. Here, we demonstrate that not only is A2P more effective than AA in regulating HIF-1α, it was also capable of inhibiting invasion and anchorage independent growth of WM9 metastatic melanoma cells compared to non-treated controls.

A recent hypothesis has suggested that treating malignant cells or patients with DHA would be a more beneficial therapy than AA. The rationale for this idea is that since many aggressive malignancies overexpress GLUT transporters, this would allow for increased uptake of DHA across the plasma membrane of these cells and translate into a higher intracellular AA content following DHA recycling than direct supplementation with AA (Mccarty, 2013). Therefore, in chapter 3 we sought to test this hypothesis by surveying the ability of AA, A2P, and DHA to decrease the hypoxia-mimetic induced activity of HIF-1α in six melanoma cell lines and melanocytes (Table 1). Our studies suggest, AA, particularly in the form of A2P, is significantly more effective than DHA in not only decreasing the activity of HIF-1α but also inhibiting invasive potential as determined using a WM9 tumor spheroid invasion model.

In addition to investigating the impact of AA supplementation on HIF-1α, we also examined if AA could increase the DNA 5hmC content of metastatic melanoma cells by augmenting the activity of TET enzymes. If so, elevated 5hmC content may allow for DNA demethylation and reexpression of tumor suppressor genes. These changes would be particularly relevant and advantageous in melanoma since malignancies retaining high 5hmC levels correlate with lower tumor stage and increased patient survival (Lee, Murphy, and Lian, 2014). In chapter 4 we demonstrate that we are able to significantly increase the 5hmC content of WM9 metastatic melanoma cells following supplementation with A2P, likely by augmenting the activity of TET2. Surprisingly, we found that gene silencing of HIF-1α increased both gene and protein expression of TET2. Furthermore, HIF-1α silencing, in addition to treatment with A2P, dramatically and significantly elevated 5hmC levels compared to cells treated with A2P alone, providing evidence that inhibiting HIF-1α, accompanied by AA therapy, may greatly improve the demethylation of gene promoters and possibly the expression of tumor suppressors in melanoma.

Collectively, the contents of this work provides *in vitro* evidence that the supplementation of melanoma cells with physiological levels of AA may be a beneficial adjuvant anti-cancer therapy as demonstrated by the measured decrease in malignant properties of metastatic melanoma cells. Decreased malignancy is likely attributed, at least in part, to the improved activity of multiple 2-OG dioxygenase enzymes including the HIF hydroxylases and TET enzymes. While additional studies are required before verifying AA supplementation as a bona fide adjuvant therapy for patients with melanoma, these results provide promise by demonstrating success at the cellular level.

Footnotes

1 Typically, it is convention to use the terms vitamin C and ascorbic acid (AA) interchangeably when discussing human physiology because in most cases the two terms

are referring to the same molecule. The term vitamin C is more commonly used to describe nutritional aspects of the molecule, while the term AA is used when describing the biochemistry of the molecule. However, in this work we are describing two distinct forms of vitamin C, reduced vitamin C (i.e. AA) and oxidized vitamin C (dehydroascorbic acid (DHA). In the present study, the term vitamin C is used to describe both forms of ascorbate (AA or DHA), total ascorbate (the summation of AA and DHA), or when the form of vitamin C cannot be distinguished because of assay limitations, thus the terms vitamin C and AA are not necessarily interchangeable.

CHAPTER 2
ASCORBIC ACID AND ASCORBATE-2-PHOSPHATE DECREASE HIF ACTIVITY AND MALIGNANT PROPERTIES OF HUMAN MELANOMA CELLS

The information in this chapter has been published in *BMC Cancer, 15*(1), 867. doi:10.1186/s12885-015-1878-5

Miles, Fischer, Joshi, and Niles. (2015). Ascorbic acid and ascorbate-2-phosphate decrease HIF activity and malignant properties of human melanoma cells. *BMC Cancer, 15*(1), 867. doi:10.1186/s12885-015-1878-5

Sarah L. Miles Ph.D.
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: kittlaus1@marshall.edu
*Corresponding author

Adam P. Fischer
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: fischer35@marshall.edu

Sandeep J. Joshi Ph.D.
Department of Biochemistry and Molecular Biology
University of Maryland School of Medicine
655 W. Baltimore Street
Baltimore, MD 21201
Email: SJoshi@som.umaryland.edu

Richard M. Niles Ph.D.
Department of Biochemistry and Microbiology
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: Niles@marshall.edu

Abstract

Background

Hypoxia inducible factor-1 alpha (HIF-1α) is thought to play a role in melanoma carcinogenesis. Posttranslational regulation of HIF-1α is dependent on Prolyl hydroxylase (PHD 1-3) and Factor Inhibiting HIF (FIH) hydroxylase enzymes, which require ascorbic acid as a co-factor for optimal function. Depleted intra-tumoral ascorbic acid may thus play a role in the loss of HIF-1α regulation in melanoma. These studies assess the ability of ascorbic acid to reduce HIF-1α protein and transcriptional activity in metastatic melanoma and reduce its invasive potential.

Methods

HIF-1α protein was evaluated by western blot, while transcriptional activity was measured by HIF-1 HRE-luciferase reporter gene activity. Melanoma cells were treated with ascorbic acid (AA) and ascorbate 2-phosphate (A2P) to assess their ability to reduce HIF-1α accumulation and activity. siRNA was used to deplete cellular PHD2 in order to evaluate this effect on AA's ability to lower HIF-1α levels. A2P's effect on invasive activity was measured by the Matrigel invasion assay. Data were analyzed by One-way ANOVA with Tukey's multiple comparisons test, or Student-T test as appropriate, with $p<.05$ considered significant.

Results

Supplementation with both AA and A2P antagonized normoxic as well as cobalt chloride- and PHD inhibitor ethyl 3,4-dihydroxybenzoate-induced HIF-1α protein stabilization and transcriptional activity. Knockdown of the PHD2 isoform with siRNA did not impede the ability of AA to reduce normoxic HIF-1α protein. Additionally, reducing HIF-1α levels with

A2P resulted in a significant reduction in the ability of the melanoma cells to invade through Matrigel.

Conclusion

These studies suggest a positive role for AA in regulating HIF-1α in melanoma by demonstrating that supplementation with either AA, or its oxidation-resistant analog A2P, effectively reduces HIF-1α protein and transcriptional activity in metastatic melanoma cells. Our data, while supporting the function of AA as a necessary cofactor for PHD and likely FIH activity, also suggests a potential non-PHD/FIH role for AA in HIF-1α regulation by its continued ability to reduce HIF-1α in the presence of PHD inhibition. The use of the oxidation-resistant AA analog, A2P, to reduce the ability of HIF-1α to promote malignant progression in melanoma cells and enhance their response to therapy warrants further investigation.

Background

Melanoma, a malignancy derived from pigment producing melanocytes found primarily in the epidermis of the skin, continues to be the deadliest form of skin cancer. The global incidence of melanoma is increasing at a faster rate than any other type of cancer. Despite advances in the treatment of metastatic melanoma it remains an incurable disease (Slipicevic and Herlyn, 2012). The only successful cure for melanoma remains early identification of atypical skin lesions and complete surgical excision before invasion of the deeper dermal tissue (Bichakjian *et al.*, 2011; Tsao, Atkins, and Sober, 2004). After dissemination and metastasis of the primary tumor, limited treatment strategies have encompassed the use of conventional chemotherapy such as dacarbazine (Berrocal *et al.*, 2014; Serrone, Zeuli, Sega, and Cognetti, 2000), with only slightly more favorable responses with Interleukin-2 (IL-2), interferon–a2b (IFN-a2b) (Atkins *et al.*, 2002; Tsao *et al.*, 2004) and more recently, mutant BRAF inhibitors and immunostimulants (Culos and Cuellar, 2013; Gyorki, Spillane, Speakman, Shackleton, and Henderson, 2014; Slipicevic and Herlyn, 2012). However, even after initial positive responses, most melanoma tumors become chemoresistant, ultimately leading to treatment failure and refractory disease.

Many factors play a significant role in the initiation and progression of melanoma including genetic alterations, and response to the tumor microenvironment (Bennett, 2008; Postovit, Seftor, Seftor, and Hendrix, 2006; Satyamoorthy and Herlyn, 2002). Within the microenvironment, oxygen availability is thought to play a critical role in melanoma carcinogenesis. Hypoxia inducible transcription factor 1 (HIF-1) is a critical mediator of the cellular response to hypoxia. HIF-1 is a heterodimeric complex of α and β subunits. While both the HIF-1α and β subunit mRNAs are constitutively expressed, the HIF-1α subunit protein is

tightly regulated through post-translational hydroxylation by oxygen dependent Fe II/2-oxoglutarate (2-OG) prolyl 4-hydroxylase (PHD) enzymes (Hirsila, Koivunen, Gunzler, Kivirikko, and Myllyharju, 2003; Kallio, Pongratz, Gradin, Mcguire, and Poellinger, 1997). This hydroxylation targets HIF-1α ubiquitination and degradation by the proteasome. Under conditions of low oxygen tension, these hydroxylase enzymes are disabled, allowing the stabilization and accumulation of HIF-1α in the cell.

Overexpression and stabilization of HIF-1α has been identified in numerous malignancies (Jiang and Feng, 2006; Kimbro and Simons, 2006; Nam *et al.*, 2011; Semenza, 2002), including melanoma, and has been implicated in driving its progression and metastatic potential (Bedogni and Powell, 2009; Giatromanolaki *et al.*, 2003; Goda, Dozier, and Johnson, 2003; Kuphal *et al.*, 2010; Valencak *et al.*, 2009; Zhong *et al.*, 1999). Many tumor types including melanoma stabilize HIF-1α under non-hypoxic conditions (Krieg *et al.*, 2000; Kuphal *et al.*, 2010; Mills, Joshi, and Niles, 2009; Valencak *et al.*, 2009). HIF acts as a positive regulator of proteins known to be important in melanoma cell invasion, spreading and motility (Zbytek, Peacock, Seagroves, and Slominski, 2013). Given the relationship between HIF-1α and melanoma progression, this transcription factor is an attractive target for small molecule inhibitors (Semenza, 2007).

Ascorbic acid (AA) is an essential vitamin in humans due to the evolutionary loss of the gulonolactone oxidase (GULO) enzyme necessary to catalyze the final step in ascorbic acid synthesis. Its antitumor activity has been studied extensively over the past decades. Numerous *in vitro* and *in viv*o studies with both human and animal tumors demonstrated correlations between tumor AA levels, reduced HIF-1 activation, and longer disease free survival (Kuiper *et al.*, 2014b). Additionally, low AA levels are associated with increased HIF-1 activity and more

aggressive tumor phenotypes (Kuiper *et al.*, 2010; Skrzydlewska *et al.*, 2005). Furthermore, cancer patients often have depleted reserves of vitamin C (Bhagat, Ghone, Suryakar, and Hundekar, 2011; Esme *et al.*, 2008; Schleich *et al.*, 2013; Yamamoto, Yamashita, Fujisawa, Kokura, and Yoshikawa, 1998). AA has varying effects on cancer initiation, progression and growth.

The aim of this study was to assess the effect of physiological concentrations of AA on the normoxic expression and activity of HIF-1α in WM9 metastatic melanoma cells and to determine the mechanism for its action. Because of the potential for off target pro-oxidant effects with the use of high concentrations of ascorbic acid (mM concentrations), our studies aimed to determine whether physiologically attainable serum concentrations of AA (Levine *et al.*, 1996; Levine, Padayatty, and Espey, 2011), which would be achievable through the consumption of vitamin C rich foods (yielding up to 100 μM serum AA) or oral dietary supplements (up to 250 μM serum AA), could effectively impact HIF-1α in melanoma cells (Levine *et al.*, 2011). Under normoxic culture conditions, addition of AA to culture media at physiologically relevant concentrations resulted in a rapid reduction of HIF-1α protein, and HIF activity. Interestingly, the transcriptional activity of HIF-1 proved to be more sensitive to AA treatment than the PHD induced degradation of the HIF-1α. We also found that low physiological concentrations of AA were also able to antagonize hypoxia-mimetic (cobalt chloride; $CoCl_2$) induced HIF-1α stabilization and increased HIF transcriptional activity. Ascorbate-2-phosphate (A2P), an oxidation resistant analog of AA, was more potent than its parent compound in reducing HIF-1α levels.

Materials and methods

Cell culture and reagents

WM1366 and WM9 melanoma cell lines were a generous gift from Dr. Meenhard Herlyn at the Wistar Institute (University of Pennsylvania). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified 5% CO_2 / 95% air incubator at 37°C. L-Ascorbic Acid (AA), L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A2P), Cobalt Chloride ($CoCl_2$), and Ethyl 3,4-dihydroxybenzoate (EDHB) were purchased from Sigma Chemical Company.

Western blot analysis and antibodies

Nuclear protein extracts were isolated using the NePER Nuclear and Cytoplasmic Extraction Kit (Pierce), supplemented with Complete Mini Protease Inhibitor Cocktail (Roche) following the manufacturers protocol. Whole cell lysates were extracted using whole cell lysis buffer (50 mM Tris HCl, 150 mM NaCl, 0.25% SDS, 0.25% sodium deoxycholate, 1 mM EDTA; pH 7.4) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche). Equal amounts of protein extracts were separated by SDS-PAGE on 4-20% MP-TGX precast polyacrylamide gels (BioRad), and transferred to nitrocellulose membrane using the BioRad MINIProtean3 system. Membranes were immunoblotted with antibodies that recognized HIF-1α (1 μl/ml; R&D Systems) or EGLN1 (PHD2; 1:1000; Cell Signaling). Anti- β-actin (1:10000; 1 hour at RT; Sigma) was used to assess equal protein loading. Immunoblots were visualized using an enhanced chemiluminescence detection kit (ECL Prime; GE Healthcare) and imaged on a PhotoDyne Imaging system (PhotoDyne Technologies). Densitometry was obtained and quantitated using LabQuant Software.

Small interfering RNA (siRNA) transfection

WM9 cells were transfected at the time of seeding with 10 nM PHD2 siGENOME SMARTpool siRNA (GE Dharmacon; ThermoScientific) or 10 nM non-targeting Control siRNA using Lipofectamine RNAimax (Invitrogen) in standard RPMI culture media following the manufacturers protocol with modifications. Briefly, siRNA transfection complexes were combined in OptiMEM reduced serum media (Invitrogen) following the RNAimax protocol. Cells were removed from the dish via trypsinization, and resuspended in standard RPMI media containing the appropriate siRNA transfection complex. Cells were immediately seeded into 35 mm tissue culture dishes at $1.0x10^5$ cells per dish. Cells were incubated with transfection reagent overnight. Media was replaced the following day with complete RPMI +/- $CoCl_2$, EDHB, AA or A2P at the concentrations and times indicated.

Luciferase reporter assay

$2.0X10^5$ cells were seeded in 60 mm culture dishes 24 hours prior to transfection. Cells were incubated overnight with transfection mixture containing 1.5 μg HIF-1 pTL-Luc (5'-3': GTGACTACGTGCTGCCTAGGTGACTACGTGCTGCCTAGGTGACTACGT GCTGCCTAGGTGACTACGTGCTGCCTAG; Affymetrix, LR0128) and 0.1 μg pSV-β-galactosidase plasmids (Clontech) and eXtreme Gene 9 transfection reagent (Roche) following the manufacturers protocol in OptiMEM Reduced Serum media (Invitrogen). Transfection media was replaced with standard RPMI the following day, and cells treated as described. Luciferase activity was measured using the Luciferase Assay Kit (Promega) and normalized against β-galactosidase activity that was measured using the β-galactosidase Assay Kit (Promega). Luciferase and β-gal were measured separately on a SpectraMax M2e 96-well plate reader.

Matrigel invasion assay

Prior to measuring invasion, WM9 cells were cultured in 10 cm dishes and maintained for 5 days in standard RPMI with or without 100 μM A2P supplementation under standard culture conditions. Invasion was evaluated using BD BioCoat Invasion kits (354481; BD Corning). Cells were dissociated with Accutase (Invitrogen) and $2.5x10^5$ cells were seeded onto the Matrigel coated 6-well inserts and the assay conducted using the manufacturer's protocol with inclusion of 100 μM A2P supplementation in all media chambers. Plates were incubated 24 hours and cells were then fixed with 100% methanol for 5 min and stained with 0.5% crystal violet for 5 min before being washed several times in ddH2O to remove excess stain. Inserts were allowed to dry overnight, then membranes were removed from the inserts, and placed in a microtube with 200 μL of 10% acetic acid for 15 min with vortexing to elute the dye. Sample absorbance was measured in triplicate at 595 nM on a SpectraMax M2e 96 well plate reader. A stained membrane without cells was used as the blank control to account for background staining.

Reverse transcription and PCR

Total RNA was isolated from WM9 cells using an RNeasy Mini Kit (Qiagen) following the manufacturers protocol. RNA quality and quantity was assessed spectrophotometrically using a NanoDrop 2000 UV/Vis spectrophotometer. cDNA was synthesized from total RNA using the Advantage RT-for-PCR kit (Clontech Laboratories, Mount View, CA) following the manufacturers recommended protocol. PCR analysis of target sequences were generated using the Advantage cDNA kit (Clontech Laboratories) with the following PCR primers; EGLN1(PHD2): 5'– GGCAAAGCCCAGTTTGCTGAC-3'(forward), 5' - CCCTCACACCTTTTTCACCTGT-3' (reverse); EGLN2 (PHD1): 5'-

CCAGGCAAGAGAACCAGGAG-3'(forward), 5'-TCAACGTGCCTTACGTACCC-3' (reverse); EGLN3 (PHD3): 5'– GGCTTCTGCTACCTGGACAACT-3'(forward), 5'-AGGATCCCACCATGTAGCTTG-3' (reverse). PCR conditions: 95°C 5 min; 30 cycles of 95°C 1 min, 57°C 1 min, 72°C 1 min; 72° 5 min. PCR products were labeled with Texas Red (Invitrogen), separated on a 1% agarose gel and visualized by UV using a PhotoDyne Imaging system (PhotoDyne Technologies). Target PCR sequences were normalized to β-actin.

Statistical analysis

Data were analyzed using GraphPad Prism 6 software (version 6.0f; GraphPad Software, Inc.). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test, or unpaired Student-t test as appropriate. Each experiment was performed at least three times and represented as mean ± SEM. $p<0.05$ was considered as a significant difference.

Results

Ascorbic acid inhibits the normoxic expression of HIF-1α protein in both invasive and metastatic human melanoma cell lines

We previously reported (Mills *et al.*, 2009) that when grown under normoxic culture conditions, normal human melanocytes and melanoma cell lines isolated from different stages of the disease had either no detectable HIF-1α protein (normal melanocytes) or increased amounts of HIF-1α protein that roughly correlated with their degree of malignancy. In our initial experiments, we tested the ability of physiological concentrations of ascorbic acid (AA) to decrease the amount of HIF-1α protein in a human melanoma cell line (WM1366), isolated from a vertical growth phase melanoma (Fig. 7A-B). The lowest concentration of AA (5 μM)

dramatically decreased the normoxic expression of HIF-1α protein in this human melanoma cell line (Fig. 7B). An increased contrast of the western blot was used to evaluate the signal of the HIF-1α protein by densitometry in the AA treated samples (indicated as HIF-1α^2 in Fig 7).

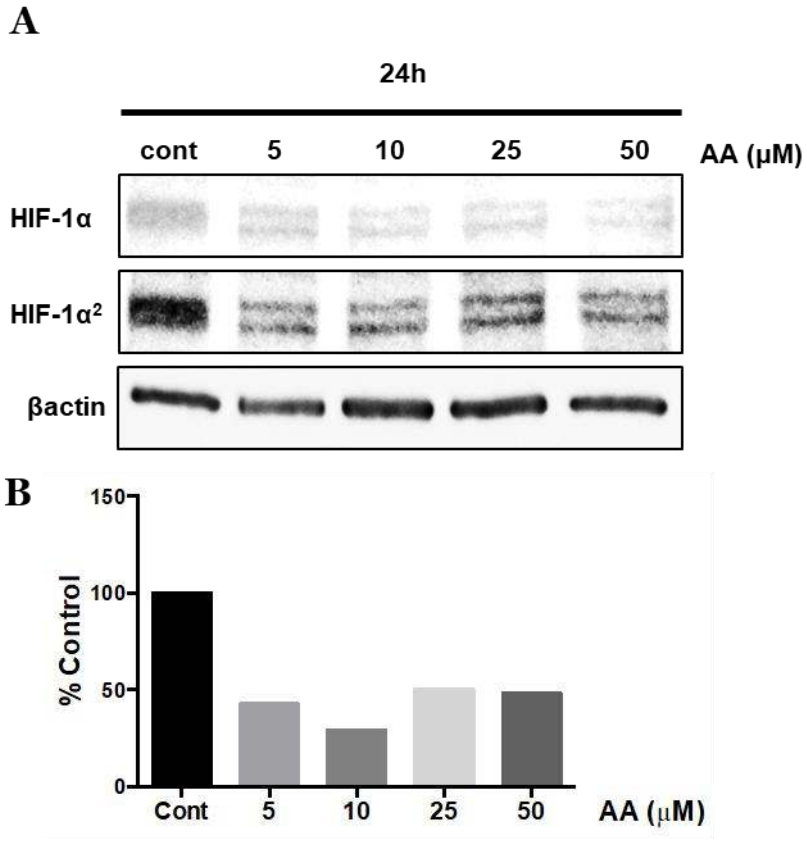

Figure 7. Effect of ascorbic acid on HIF-1α stabilization in WM1366 vertical growth phase melanoma cells.
WM1366 cells were treated for 24 hours with ascorbic acid (AA; 5-50 μM) under standard normoxic culture conditions. A) Western blot analysis of isolated nuclear extracts reveals considerable reduction in the amount of stabilized HIF-1α protein following treatment with AA. B) Densitometry analysis demonstrates the ability of AA supplementation at physiologically achievable concentrations to reduce the normoxic overexpression of HIF-1α by approximately 50-60% in these cells. Protein expression was normalized to β-actin.

Using a metastatic human melanoma cell line (WM9), we compared the time and concentration dependent ability of AA or ascorbate-2-phosphate (A2P), an oxidation-resistant analog of AA, to decrease the level of HIF-1α protein (Fig. 8). At 15 minutes, HIF-1α protein is already

effectively reduced by approximately 50 % by the 50 μM concentration of AA and A2P. However, within 30 min, the amount of HIF-1α protein was decreased by >50 % by all concentration in both the AA and A2P-treated WM9 cells. The decrease was maintained during the 2 h time period. A2P treatment most effectively reduced HIF-1α protein levels below 15 % by 2 hours.

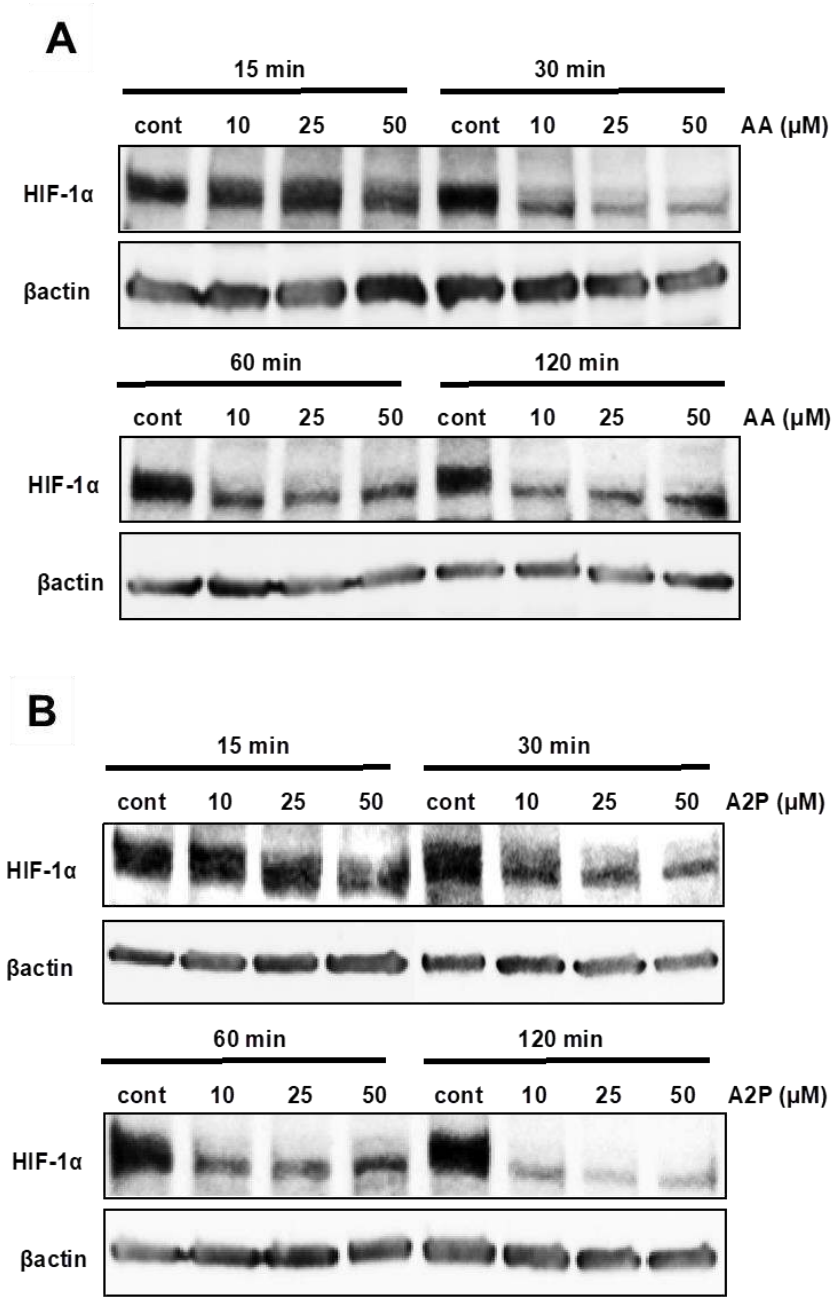

Figure 8. Effect of ascorbic acid and ascorbate 2-phosphate on HIF-1α stabilization in WM9 metastatic melanoma cells.

WM9 metastatic melanoma cells were treated with increasing concentrations (10, 25 or 50 μM) of ascorbic acid (AA), or the non-oxidizable analog ascorbate 2-phosphate (A2P) for 15-120 minutes under standard normoxic culture conditions. Western blot analysis of isolated nuclear fractions reveals that both A) AA and B) A2P cause nearly 50% reduction of normoxic stabilized HIF-1α in these cells as early as 30 min following treatment. Treatment with A2P provided nearly complete reduction in stabilized HIF-1α by 120 min. Protein expression was normalized to β-actin. All treatments were repeated a minimum of 2 additional times with similar results.

Ascorbic acid and A2P also inhibit hypoxia-mimetic-induced HIF-1α protein stabilization

It was important to determine whether AA and A2P could also decrease the much higher levels of HIF-1α found in hypoxic regions of tumors. We used $CoCl_2$ as a mimetic of hypoxia since this induced a minimum 15-20 fold increase in WM9 HIF-1α protein level (Fig. 9). Treatment of $CoCl_2$-treated cells with different concentrations of AA or A2P revealed that the latter compound was substantially more effective in reducing the amount of HIF-1α protein. Since 100 μM A2P eliminated essentially all of the HIF-1α protein, compared to 100 μM AA, which at this concentration had no effect on the protein, we titrated down the amount of A2P and found that 5 μM still reduced $CoCl_2$ induced HIF-1α by 90% (Fig. 9).

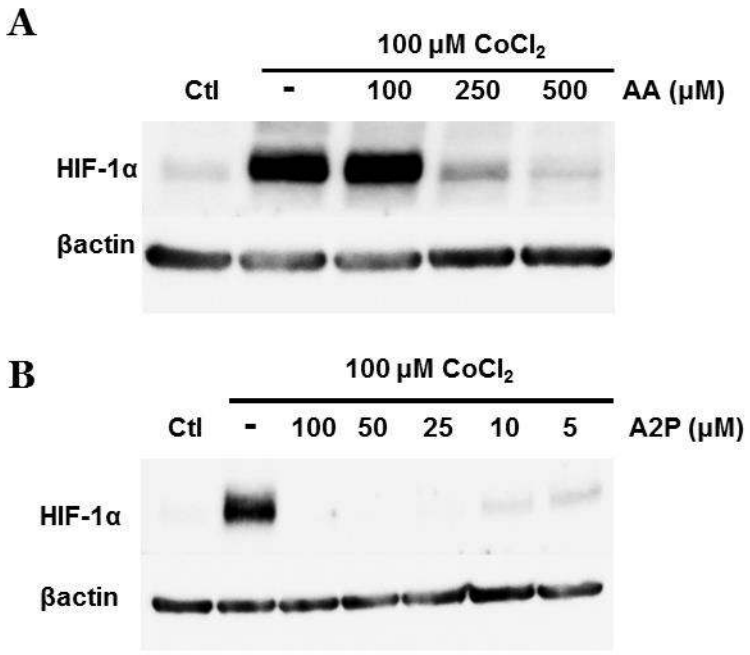

Figure 9. Effect of AA and A2P on cobalt chloride induced HIF-1α protein accumulation in metastatic melanoma.
WM9 metastatic melanoma cells were treated for 24 hours with the hypoxia mimetic cobalt chloride (100 μM) in the presence or absence of AA and A2P and nuclear extracts analyzed by western blot. A) The addition of AA (100-500 μM) reveals that 100μM AA is unable to reduce $CoCl_2$ induced HIF-1α accumulation. Higher concentrations of AA (250 and 500 μM) are necessary to reduce induced levels of HIF-1α. B) Cells treated with A2P (5.0-100 μM) show that A2P efficiently reduces $CoCl_2$ induced accumulation of HIF-1α at concentrations as low as 5μM. Protein expression was normalized to β-actin. All treatments were repeated a minimum of 2 additional times with similar results.

Ascorbic acid and A2P inhibit HIF-1α transcriptional activity in metastatic human melanoma

While high levels of HIF-1α imply induction of hypoxia-inducible genes, this needs to be verified by measuring HIF transcriptional activity. Therefore, we used a HIF-luciferase reporter plasmid transfected into WM9 metastatic human melanoma cells to examine the influence of AA and A2P on this activity. The hypoxia-mimetic $CoCl_2$ induced a time dependent increase in HIF activity and this was antagonized by both 100 μM AA and A2P, with the latter compound being

more effective (Fig. 10A). We also measured the concentration-dependent ability of AA and A2P to inhibit $CoCl_2$-induced HIF activity (Fig. 10 B, C, D). AA at 25 μM inhibited HIF activity by ~70 % with 50-250 μM causing a further decrease in activity (Fig. 10B). In contrast, 25 μM A2P eliminated >90 % of the HIF activity (Fig. 10C). Due to this potent effect, we treated the WM9 cells with 2.5-20 μM A2P. There is a significant decrease (>95 %) in HIF activity at a concentration of 10 μM A2P (Fig. 10D).

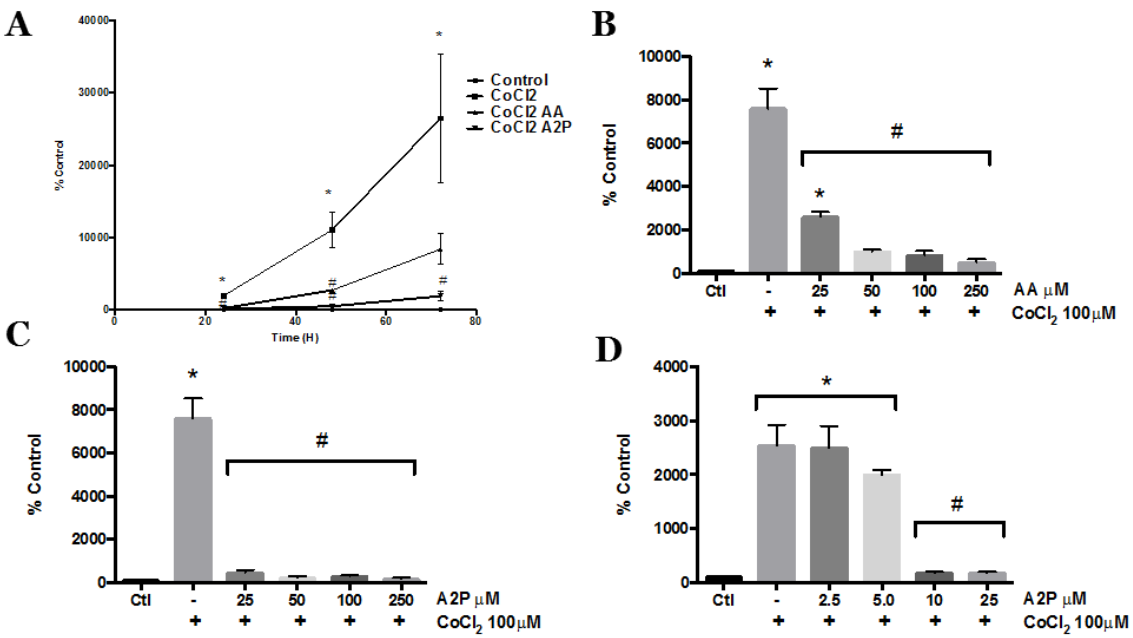

Figure 10. Effect of AA and A2P on HIF-1α transcriptional activity in metastatic melanoma.
WM9 metastatic melanoma cells were transiently transfected with an HIF-1 HRE-luciferase reporter vector. A) Transfected WM9 cells were treated with 100 μM $CoCl_2$ with or without AA (100 μM) or A2P (100 μM). Cells were collected and HIF-1 transcriptional activity was measured by luciferase assay at 24, 48, and 72 hours. Both AA and A2P significantly reduced HIF-1 transcriptional activity at 24 and 48 hours; at 72 hours, A2P significantly reduced $CoCl_2$ induced activity while AA began to show reduced efficacy by 72 hours. B) Dose dependent inhibition of $CoCl_2$ induced HIF-1 reporter activity using 25, 50, 100 and 250 μM AA. AA significantly reduced $CoCl_2$ induced HIF-1 activity at all concentrations, with 25 μM AA beginning to show reduced efficacy. C) Dose dependent inhibition of $CoCl_2$ induced HIF-1 reporter activity by 25, 50, 100 and 250 μM A2P. All concentrations of A2P significantly reduced $CoCl_2$ induced HIF-1 reporter gene activity. For this reason, lower doses of A2P were then tested. D) Low dose titration of A2P dependent inhibition of $CoCl_2$ induced HIF-1 reporter activity using 2.5, 5.0, 10 and 25 μM A2P. A2P demonstrated close to maximum inhibition of HIF-1 activity at concentrations as low as 10 μM, with 5 and 2.5 μM demonstrating little to no inhibition of activity. All HRE-luciferase activity was normalized to β-galactosidase activity. Data are represented as mean ± SEM of a minimum of n=3, analyzed by One-way ANOVA followed by Tukey's multiple comparisons test; * denotes significant difference from control, $p<0.0001$, # denotes significant difference from $CoCl_2$ treatment alone, $p<0.003$-0.0001.

PHD and AA/A2P inhibition of HIF-1α activity

AA serves as an essential co-factor for PHD enzyme activity that results in the targeted degradation of HIF-1α. Also, AA levels are frequently lower in human tumors than in the

surrounding uninvolved tissue (Kuiper *et al.*, 2014b). Therefore, we investigated whether AA and A2P decreased HIF-1α protein in WM9 metastatic melanoma cells through acting on PHD. First, we examined whether AA and A2P could counteract the effect of the PHD inhibitor ethyl 3,4-dihydroxybenzoate (EDHB). Fig. 11 illustrates the concentration dependent ability of EDHB to stimulate HIF reporter gene activity in WM9 cells. The maximum stimulation was achieved at 750 μM and this concentration was used in all further experiments. EDHB is thought to inhibit PDH activity through both binding to the AA site on the enzyme and by chelation of iron. Since AA has iron chelating activity, we tested the ability of AA to reverse the EDHB stimulation of HIF activity when added prior to EDHB treatment, or when the two compounds were simultaneously added to the cells. Cleary, AA is more effective in reversing EDHB stimulation of HIF activity (through inhibition of PHD activity) when pre-incubated with the cells (Fig. 11B, AA-EDHB) vs. adding it at the same time as EDHB (Fig. 11B, EDHB/AA).

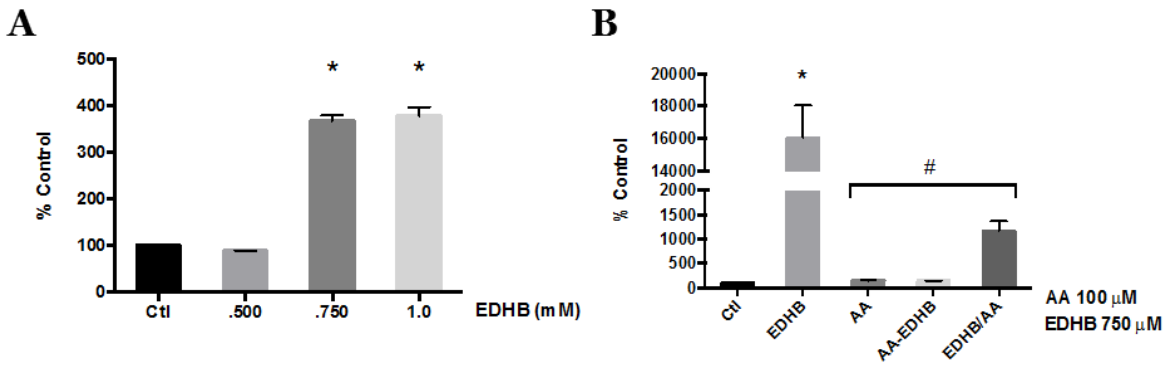

Figure 11. Effect of ascorbic acid on EDHB induced HIF-1α transcriptional activity in melanoma cells.
WM9 metastatic melanoma cells were transiently transfected with an HIF-1 HRE-luciferase reporter vector. A) Transfected cells were treated for 24 hours with 0.5, 0.75 and 1.0 mM EDHB. Induction of HIF-1 transcriptional activity was measured by luciferase assay. EDHB at 0.75 mM was found to be the lowest dose capable of generating near-maximal induction of HIF-1 transcriptional activity and was thus chosen for subsequent experiments. B) Cells were treated for 24 hours with 0.75 mM EDHB alone (EDHB), 100 µM AA alone (AA), pretreated with 100 µM AA for 4 hours prior to addition of EDHB (AA-EDHB), or treated with100 µM AA and EDHB concurrently (EDHB/AA). AA effectively reduces EDHB induced HIF-1 transcriptional activity; with AA pretreatment showing increased efficacy at inhibiting EDHB induced HIF-1 activity vs. concomitant treatment. All HRE-luciferase activity was normalized to β-galactosidase activity. Data are represented as mean ± SEM of n=3, analyzed by One-way ANOVA followed by Tukey's multiple comparisons test; * denotes significant difference from control, $p<0.0001$, # denotes significant difference from EDHB treatment alone, $p<0.0001$.

Next, we compared the concentration-dependent ability of A2P vs. AA to reverse the EDHB-stimulated HIF activity (Fig. 12). In contrast to the superior ability of A2P vs. AA to inhibit cobalt chloride induced HIF-1α levels (Fig. 10), the potency of A2P and AA to inhibit the EDHB-induction of HIF transcriptional activity was similar.

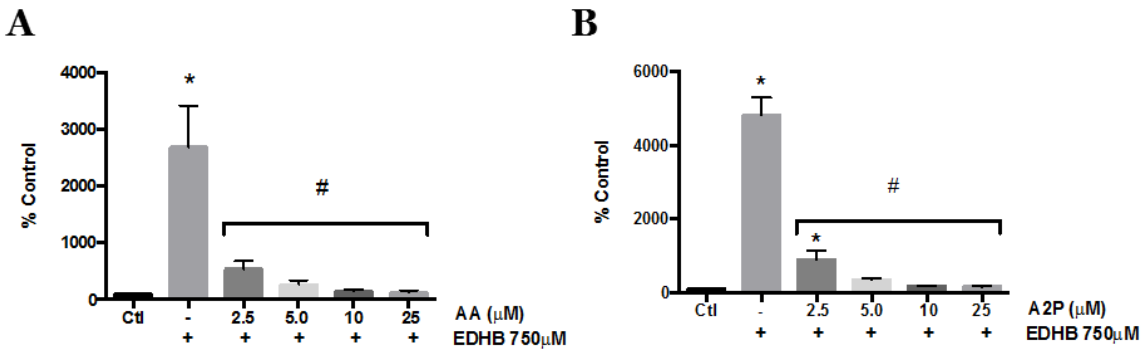

Figure 12. Effect of AA and A2P on EDHB induced HIF-1α transcriptional activity in melanoma cells.
WM9 metastatic melanoma cells were transiently transfected with an HIF-1 HRE-luciferase reporter vector. Transfected cells were treated for 24 hours with 750 μM EDHB in the presence of A) 2.5, 5.0, 10 or 25 μM AA or B) 2.5, 5.0, 10 or 25 μM A2P. HIF-1 transcriptional activity was measured by luciferase assay. Data are presented as the mean ± SEM of n=3, analyzed by One-way ANOVA followed by Tukey's multiple comparisons test; * denotes significant difference from control, $p<0.0001$, # denotes significant difference from EDHB treatment alone, $p<0.0001$.

To more directly determine whether PHD mediated the ability of AA to decrease HIF-1α protein levels, we first determined which PHD isoforms were expressed in the WM9 cells. Reverse transcription-PCR analysis shows that PHD2 had the highest RNA expression followed by PHD1 (Fig. 13B). mRNA expression of PHD3 was not detectable. We used PHD2 siRNA to knockdown the expression of PHD2. The amount of PHD2 protein was reduced by greater than 90 % in cells treated with the siPHD2 relative to cells treated with a control siRNA (Fig. 13A, D). Despite this dramatic decrease in PHD2 protein, AA treatment of the cells still markedly decreased HIF-1α protein levels. Note that the amount of HIF-1α is much higher in the siPHD2 vs. control siRNA treated cells likely due to the absence of PHD2 (Fig. 13A, C).

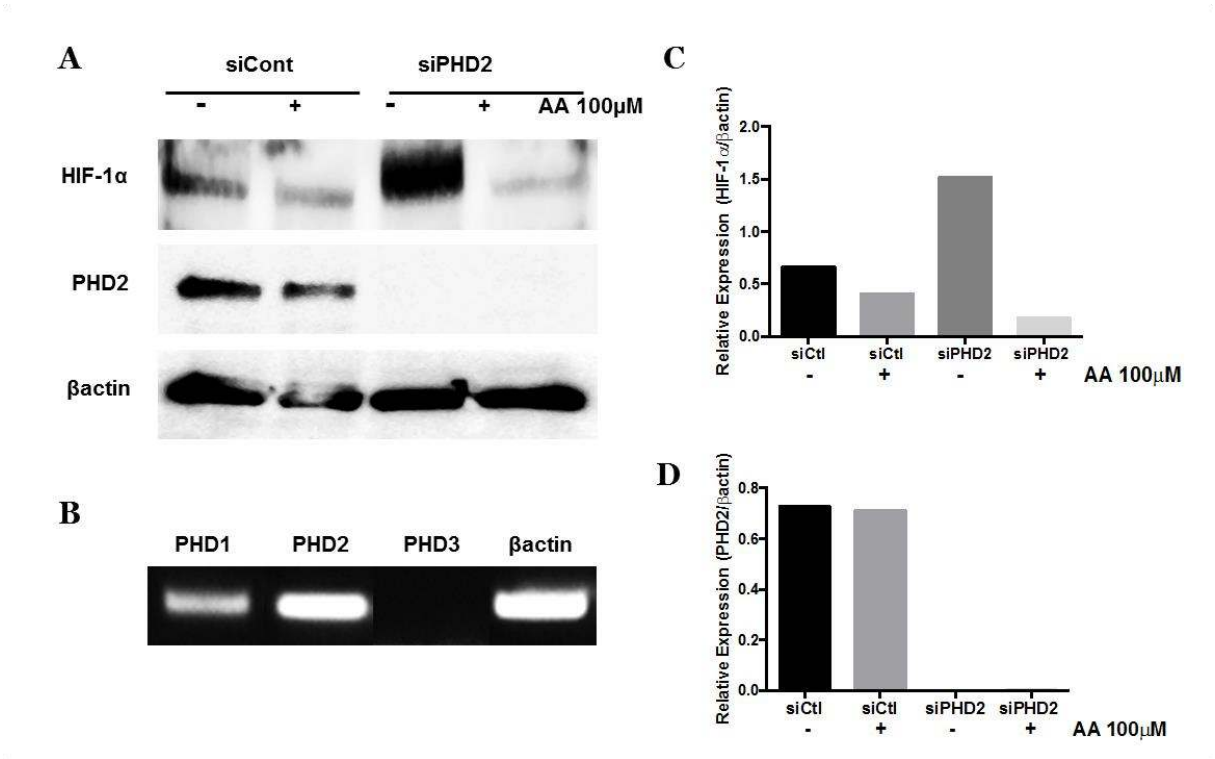

Figure 13. Effect of PHD2 knockdown on reduction of normoxic HIF-1α protein by AA in metastatic melanoma.
WM9 metastatic melanoma cells were transfected using non-targeting control siRNA or siGENOME SMARTpool siRNA against PHD2. A) siRNA transfected cells were treated for 24 hours with or without 100 μM AA under standard normoxic culture conditions. HIF-1α and PHD2 were analyzed by western blot, and normalized to β-actin. Knockdown of PHD2 caused an increase in stabilized HIF-1α protein; however, does not result in loss of effectiveness of AA to reduce accumulated HIF-1α. B) qPCR analysis of PHD1, 2, and 3 isoforms in untreated WM9 metastatic melanoma cells, normalized to β-actin expression. PHD2 appears is the prevalent isoform, however the presences of PHD1 may contribute to the retained activity of AA following PHD2 selective knockdown. C, D) Densitometry analysis of HIF-1α and PHD2 expression following PHD2 knockdown and AA treatment. siRNA experiments were repeated a minimum of 2 additional times with similar results.

A2P inhibits human metastatic melanoma cell invasion *in vitro*

Clinical samples of human melanoma express high levels of HIF-1α (Giatromanolaki *et al.*, 2003; Hanna *et al.*, 2013). Also, siRNA knockdown of HIF-1α decreases invasion of melanoma cell lines through Matrigel (Mills *et al.*, 2009). Therefore, we tested whether A2P would also decrease the invasion of WM9 human metastatic melanoma cells. Using the Matrigel

in vitro invasion assay (Fig. 14) we found that treatment of the WM9 cells with A2P decreased invasion by 50 %. In addition, supplementation with 225 µM A2P was able to decrease anchorage independent growth by approximately 20 % (Fig. 15).

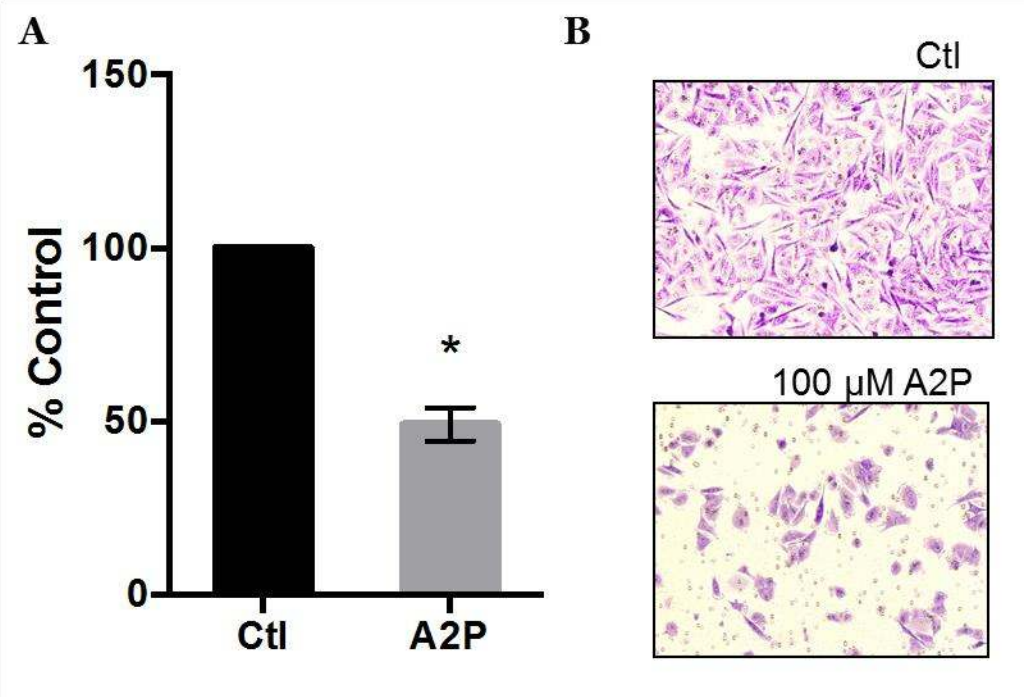

Figure 14. Effect of A2P treatment on invasive potential of metastatic melanoma cells. WM9 metastatic melanoma cells were maintained in 100 µM A2P for 5 days under standard normoxic culture conditions. Cells were seeded into Matrigel chambers and assayed for invasion after 24 hours. A) Matrigel invasion assay was completed as described in Methods and Materials. Cells grown in the presence of A2P demonstrated a 50% reduction in invasion. B) Representative photographs of Matrigel invasion chambers. Data are represented as mean ± SEM of n=3, analyzed by Student paired T-test; * denotes significant difference from control, $p<0.0087$.

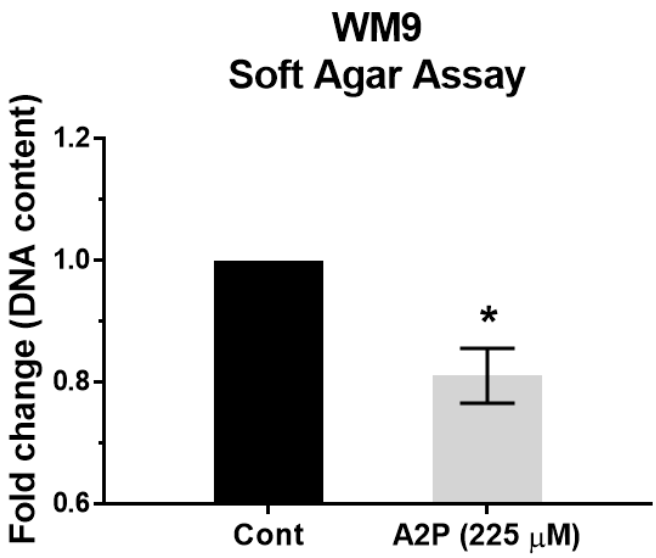

Figure 15. Anchorage independent growth of WM9 cells following A2P supplementation. Anchorage independent growth of WM9 cells treated with A2P was assessed using CytoSelect™ 96-Well Cell Transformation Assay (Soft Agar Colony Formation) (Cell Biolabs). Briefly, WM9 cells were pretreated with A2P (225 μM) for 4 days before being lifted with accutase and seeded into 96-well plates in agar according to manufacturer's protocol. Solidified agar was overlaid with 100 μL standard RPMI media containing 225 μM A2P and placed in humidified incubator (95 % air / 5 % CO_2) for a total of 9 days. On the 6th day media was exchanged with fresh media (225 μM). At time of collection, agar was solubilized and wells incubated with florescent DNA stain. Plate was read on a microplate reader Ex: 485/Em: 520 nm to determine total DNA content. Data was analyzed using Student's unpaired T test. Error bar represents ± SEM, n = 3. * denotes statistical significance from control, $p < 0.05$.

Discussion

Once melanoma progresses to the invasive and metastatic stage, it is very difficult to treat. Therefore, it is important to identify the molecular changes that contribute to the malignant progression of this disease. Hypoxia and acquisition of a vascular network together with reprograming of the cancer cell's metabolism have been noted as important events required for tumor progression (Kroemer and Pouyssegur, 2008; Semenza, 2002). Accumulation of HIF-1α and HIF-2α was measured via immunohistochemistry in 46 patient samples of nodular cutaneous malignant melanomas (Giatromanolaki *et al.*, 2003). Expression of HIF-1α and HIF-2α was

directly correlated with vascular endothelial growth factor accumulation (VEGF) and also associated with poor prognosis. A later study of 89 patients with primary cutaneous melanoma did not show a correlation between HIF-1α and overall survival or disease-free survival (Valencak *et al.*, 2009). However, the relative amount of HIF-1α and more importantly the activity of HIF as assessed by target gene expression in the samples was not assayed. HIF-1α was found under normoxic conditions in malignant melanoma cells, but not in normal human melanocytes. Further, the amount of HIF-1α was increased in cells from invasive and metastatic human melanomas relative to that found in cells from radial growth phase melanomas. Also, knockdown of HIF-1α in the metastatic cells led to marked decrease in anchorage-independent growth and the ability to invade through Matrigel (Mills *et al.*, 2009). Kuphal et al (Kuphal *et al.*, 2010) verified the constitutive expression of HIF-1α in malignant melanoma and their studies implicate ROS and the NF-κB pathways in contributing to this accumulation of HIF-1α.

Due to their role in tumor progression, HIF-1α and HIF are targets for the development of new small molecular inhibitors. However, most of the inhibitors to date work in an indirect fashion such as Bortezomib, a proteasome inhibitor and geldanamycin, a Hsp90 inhibitor (Hoelder, Clarke, and Workman, 2012). In fact, medicinal chemists have deemed that HIF is undrugable (Hoelder *et al.*, 2012).

AA (vitamin C) plays a direct role in regulating both the activity of PHD and thus the stability of HIF-1α and the activity of FIH, which inhibits the transcriptional activity of HIF. There are several reports that addition of AA to cancer cell lines decreases the amount of HIF-1α protein and also inhibits HIF activity (Knowles, Raval, Harris, and Ratcliffe, 2003; Kuiper, Dachs, Currie, and Vissers, 2014; Vissers, Gunningham, Morrison, Dachs, and Currie, 2007). Thus, AA might be useful as a direct inhibitor of the HIF pathway presumably through its action

on the family of Fe(II)-2-oxoglutarate-dependent oxygenases, of which PHD and FIH are members. We investigated this possibility in human malignant melanoma cells.

In agreement with the study of Knowles, et al (Knowles *et al.*, 2003) we found that AA decreased the amount of HIF-1α protein in malignant melanoma cells grown under either normoxic or hypoxic-mimetic ($CoCl_2$) conditions (Fig. 7, 8 and 9). Further, we showed that A2P, a less oxidizable analog of AA, was more potent than AA in reducing the amount of HIF-1α in the melanoma cells (Fig. 8 and 9). We could not find any other reports on the effect of A2P on HIF-1α levels, but several studies show that A2P inhibits tumor invasion (Liu, Nagao, Kageyama, and Miwa, 2000; Nagao, Nakayama, Etoh, Saiki, and Miwa, 2000), while it also inhibits melanogenesis in melanocytes (Kameyama *et al.*, 1996). A2P was also more potent than AA in reducing HIF reporter gene activity (Fig. 10). Also note that the ability of AA to inhibit HIF reporter gene activity was more potent than its ability to decrease HIF-1α protein levels (compare Fig. 10B with Fig. 9). This finding agrees with the report of Kuiper et al (Kuiper *et al.*, 2014) that AA preferentially suppresses the HIF-1 transcriptional response. The authors suggest that this preference is likely due to AA's ability to stabilize and reduce the iron atom in the PHD and FIH active sites, with FIH (asparagine hydroxylase) being more sensitive to fluctuations in intracellular ascorbate.

Next, we investigated the hypothesis that the effect of AA on decreasing HIF-1α in human melanoma cells was mediated through stimulation of PHD and or FIH activity. First, we used a pharmacological inhibitor of PHD and likely FIH, ethyl-3,4-dihydroxybenzoate (EDHB). This inhibitor decreases prolyl hydroxylase activity (Sasaki, Majamaa, and Uitto, 1987) through both competition for the AA binding site (Majamaa, Gunzler, Hanauske-Abel, Myllyla, and Kivirikko, 1986) and inducing an iron deficiency state in cells through a low affinity for ferric

iron (Wang, Buss, Chen, Ponka, and Pantopoulos, 2002). In our melanoma cells, EDBH at or above a concentration of 750 μM stimulated HIF reporter gene activity by 4-fold. Since AA pretreatment was more effective in blocking the EDHB stimulation of HIF-reporter gene activity than when EDHB and AA were added simultaneously to the cells, we suggest that there is competition for either the ferric iron or the AA binding site on the PHD/FIH enzymes (Fig. 11).

After defining the condition for maximum inhibition of PHD by EDBH as measured by HIF-reported gene activity, we then measured the concentration-dependent ability of pre-treatment with either AA or A2P to reverse the inhibition as determined by a decrease in HIF-reporter gene activity (Fig. 12). In contrast to the hypoxia mimetic $CoCl_2$ stimulation of HIF-reporter gene activity where A2P was more potent than AA in reversing this stimulation, AA and A2P were similar in their potency for reversing EDHB stimulation of HIF-reporter gene activity. Differential response may be due to off-target (non-PHD) effects of $CoCl_2$ causing greater stimulation of HIF-reporter gene activity relative to the activity induced by the PHD selective inhibitor, EDHB which may be more sensitive to AA supplementation.

Since chemical inhibitors can have off-target effects, we used siRNA to knock down the expression of the PHD2 isoform protein. An RT-PCR survey of the expression of PHD isozymes in the WM9 cells revealed that these cells express predominantly PHD2 and a small amount of PHD1, but we could not detect expression of PHD3. The PHD2 isozyme contributes the majority of the HIF-hydroxylase activity in cells with normal oxygen levels (Berra *et al.*, 2003) (Takeda *et al.*, 2008). Since PHD1 is localized exclusively in the nucleus (Metzen *et al.*, 2003), it should only be able to hydroxylate HIF-1α after it has been stabilized and transported into the nucleus. We were able to knockdown the expression of PHD2 in normoxic WM9 cells by greater than 90 %. As shown in Fig. 13, this knockdown resulted in a 2.3 fold higher amount of HIF-1α relative

to cells treated with the control siRNA. Despite the knockdown of PHD2 and the increase in the level of HIF-1α, the addition of 100 μM AA still decreased the amount of HIF-1α by nearly 90 %. There are at least two explanations for this unexpected result. One is that AA has additional modes of action, other than affecting prolyl hydroxylase, which result in a decrease in the HIF-1α protein. The other possibility is that in the absence of PHD2, the isozyme PHD1 can be stimulated by AA and result in the targeting of HIF-1α for degradation by the proteasome.

Regardless of the mechanism for the ability of AA and A2P to decrease HIF-1α levels and inhibit HIF transcriptional activity, the important question is whether blocking the HIF pathway decreases some of the malignant properties of the WM9 metastatic melanoma cells. We addressed this question by measuring the ability of WM9 cells to invade through Matrigel and form colonies in soft agar. A2P was able to inhibit invasion by 50 % (Fig. 14) and colony formation nearly 20 % (Fig. 15). A2P did not inhibit the proliferation of WM9 cells (data not shown). These findings, together with our previous work demonstrating that siRNA knockdown of HIF-1α also inhibits invasion through Matrigel (Mills *et al.*, 2009), suggests that AA affects the invasive ability of these metastatic cells through a decrease in HIF-1α/HIF activity.

Although our studies used established human melanoma cell lines, there are some *in vivo* studies that link AA levels to tumor aggressiveness. Low AA levels are associated with increased HIF-1α levels and HIF stimulated gene products in human endometrial tumors (Kuiper *et al.*, 2010). In contrast, increased tumor AA is associated with longer disease-free survival and decreased HIF-1α and HIF stimulated gene products in human colorectal tumors. Specifically in melanoma there is decreased plasma ascorbate levels in stage IV melanoma patients (Schleich *et al.*, 2013) while an epidemiological study found an association between dietary vitamin C (AA) and the risk of cutaneous melanoma in a Northern Italian population (Malavolti *et al.*, 2013). IL-

2 treatment of melanoma is unfortunately associated with severe toxicity and it causes a large decrease in circulating levels of AA. A clinical trial has been proposed to assess the use of intravenous AA as an adjuvant to IL-2 treatment of melanoma (Wagner *et al.*, 2014). Thus, AA has many potential roles and uses in human melanoma. The next step will be pre-clinical investigations of AA/A2P and HIF-1α/HIF activity in animal models that most closely recapitulate the initiation and progression of human melanoma.

Conclusion

Our studies suggest a positive role for ascorbic acid in regulating HIF-1α in melanoma. The addition of ascorbic acid can effectively reduce the amount of stabilized HIF-1α found under normoxic conditions in both vertical growth phase WM1366 and WM9 metastatic melanoma cells. The addition of ascorbic acid also significantly reduces the transcriptional activity of HIF-1α in WM9 metastatic melanoma cells, resulting in decreased invasive potential. Our data support the function of AA as a critical cofactor for PHD, restoring PHD function to reduce protein accumulation, and likely FIH activity resulting in significant reduction of HIF-1α transcriptional activity. However, there may also be non-PHD mediated mechanisms by which AA reduces the level of the HIF-1α protein. The overexpression of intra-tumor HIF-1α, as well as ascorbic acid deficiency has been noted not only in melanoma, but in other tumor types as well. Further studies to evaluate the causes of ascorbic acid deficiency and its role in the loss of HIF-1α regulation in malignancy are needed. The use of ascorbic acid as a non-toxic adjuvant therapy to aid in the inhibition of HIF-1α activity in order to reduce tumor progression and improve patient response to clinical therapy warrants further investigation.

CHAPTER 3
ASCORBIC ACID, BUT NOT DEHYDROASCORBIC ACID INCREASES INTRACELLULAR VITAMIN C CONTENT TO DECREASE HYPOXIA INDUCIBLE FACTOR -1 ALPHA ACTIVITY AND REDUCE MALIGNANT POTENTIAL IN HUMAN MELANOMA

The information in this chapter has been published in *Biomedicine & Pharmacotherapy, 86*, 502-513. doi: http://dx.doi.org/10.1016/j.biopha.2016.12.056

Fischer, and Miles. (2017a). Ascorbic acid, but not dehydroascorbic acid increases intracellular vitamin C content to decrease hypoxia inducible factor -1 alpha activity and reduce malignant potential in human melanoma. *Biomedicine & Pharmacotherapy, 86*, 502-513. doi:http://dx.doi.org/10.1016/j.biopha.2016.12.056

Adam P. Fischer
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: fischer35@marshall.edu

Sarah L. Miles Ph.D. *
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: kittlaus1@marshall.edu

*** Corresponding author**

Abstract

Background

Accumulation of hypoxia inducible factor-1 alpha (HIF-1α) in malignant tissue is known to contribute to oncogenic progression and is inversely associated with patient survival. Ascorbic acid (AA) depletion in malignant tissue may contribute to the aberrant normoxic activity of HIF-1α. While the restoration of physiological AA has been shown to attenuate aberrant HIF-1α function in malignant melanoma, the impact of dehydroascorbic acid (DHA; oxidized AA) has yet to be evaluated. We investigated and compared the ability of AA and DHA to decrease the malignant potential of human melanoma reducing the activity of HIF-1α and restoring intracellular vitamin C content.

Methods

HIF-1α protein accumulation was evaluated by western blot and transcriptional activity was evaluated by reporter gene assay using a HIF-1 HRE-luciferase plasmid. Protein expression and subcellular localization of vitamin C transporters were evaluated by western blot and confocal imaging. Intracellular vitamin C content following AA, ascorbate 2-phosphate (A2P), or DHA supplementation was determined using a vitamin C assay. Malignant potential was accessed using a 3D spheroid invasion assay. Data was analyzed by One or Two-way ANOVA with Tukey's multiple comparisons test as appropriate with $p < 0.05$ considered significant.

Results

Melanoma cells expressed sodium dependent vitamin C (SVCT2) and glucose (GLUT1) transporters necessary for the uptake of AA and DHA; however, advanced melanomas only responded favorably to AA, not DHA. Under physiological glucose (1 g/L) conditions, DHA supplementation resulted in reduced intracellular vitamin C content, contributing to elevated

HIF-1α activity and increased invasion when compared to AA/A2P supplemented cells. The ability of AA to regulate HIF-1α was dependent on SVCT2 function, and SVCT2 was not significantly inhibited at pH a representative of the tumor microenvironment.

Conclusions

The use of ascorbic acid as an adjuvant cancer therapy remains under investigated. While AA and A2P was capable of modulating aberrant HIF-1α protein accumulation/activity, DHA supplementation resulted in the eventual loss of vitamin C activity, contributing to its decreased ability to inhibit HIF-1α and invasion in advanced melanoma. Restoring AA dependent regulation of HIF-1α in malignant cells may prove beneficial in reducing chemotherapy resistance and improving treatment outcomes.

Background

The incidence of melanoma, a malignancy derived from pigment producing melanocytes, has continually risen over the past 30 years and accounts for 75 % of skin cancer deaths. In 2016 an estimated 76,000 new cases of skin melanoma will be diagnosed in the United States, contributing to over 10,000 fatalities by year's end (Siegel *et al.*, 2016). Most cutaneous melanomas can be readily cured by surgical excision (Bichakjian *et al.*, 2011; Tsao *et al.*, 2004), however once disseminated, metastatic melanoma is highly aggressive and difficult to treat. Five year survival of melanoma patients declines from 98 to 17 % following metastasis to a distant site (Siegel *et al.*, 2016). Even though clinical responses have improved through the use of immunotherapy, poor patient prognosis, attributed to chemotherapy resistance, highlights the need for alternative or adjuvant treatment options to improve survival in melanoma patients.

Melanoma oncogenesis is predominantly driven by the acquisition of BRAF mutations; 50 % of all melanomas contain either a V600E or V600D BRAF mutant (Ascierto *et al.*, 2012). This mutation results in the constitutive activation of signaling pathways leading to unchecked cell proliferation, invasion, and metastasis. Constitutive BRAF activation is also known to contribute to elevated gene expression of hypoxia inducible factor-1 alpha (HIF-1α) (Kumar *et al.*, 2007), the oxygen responsive subunit of the HIF-1 transcription factor. HIF-1α activity in malignant tissue contributes to increased expression of proteins that drive melanoma cell motility and invasion (Zbytek *et al.*, 2013). Elevated expression of HIF-1α protein is widespread in malignant tissue, including melanoma (Konstantina, Lazaris, Ioannidis, Liossi, and Aroni, 2011), and has been linked to poor patient outcomes in a variety of cancers (reviewed in (Semenza, 2010)). Ascorbic acid (AA; reduced vitamin C) is an essential cofactor for Fe II/2-oxoglutarate dioxygenase enzymes, including the prolyl hydroxylase (PHD1-3) and factor-inhibiting HIF

(FIH) hydroxylase enzymes that regulate HIF-1α protein stability and transcriptional activity respectively (Flashman *et al.*, 2010; Kuiper and Vissers, 2014c). Plasma AA concentration in healthy individuals is typically between 40 – 80 μM (Du *et al.*, 2012b). Interestingly, cancer patients, including those with melanoma (Schleich *et al.*, 2013), have been observed to have below normal levels of plasma AA (Fain *et al.*, 1998; Huijskens, Wodzig, Walczak, Germeraad, and Bos, 2016; Mayland, Bennett, and Allan, 2005; Mikirova, Casciari, Riordan, and Hunninghake, 2013). Likewise, tumor tissue has been found to contain decreased intracellular AA compared to paired non-transformed tissue from the same patient (Kuiper *et al.*, 2010). The degree of AA deficiency in malignant tissue also correlates with increased tissue accumulation of HIF-1α protein and with tumor stage (Kuiper *et al.*, 2014b; Kuiper *et al.*, 2010), suggesting that inadequate intracellular AA may contribute to the development or progression of a malignant phenotype.

The majority of current studies investigating AA as an anti-cancer therapy utilize I.V. administered mega or high dose (> 1 mM) AA supplementation to induce cytotoxic cell death; however, the potential benefit and use of AA supplementation at physiological concentrations to restore or support its cofactor functions in malignant cells has remained largely unexplored and uncharacterized. Recently, we reported that supplementation of human metastatic melanoma cells with physiological concentrations (10-100 μM) of ascorbic acid inhibits both normoxic and hypoxia-mimetic induced protein accumulation and transcriptional activity of HIF-1α, and reduces the malignant potential of these cells (Miles, Fischer, Joshi, and Niles, 2015; Mills *et al.*, 2009), emphasizing the functional importance of physiological levels of AA in malignant cells. *In vivo* studies using GULO -/- mice (a model of human AA dependency) inoculated with murine melanoma cells demonstrate that AA supplementation can decrease tumor accumulation

of HIF-1α (Campbell *et al.*, 2014) and decrease tumor volume (Campbell *et al.*, 2014; Cha *et al.*, 2011; Cha *et al.*, 2013). Furthermore, tumor ascorbate levels inversely correlated to the expression of HIF-1 target genes (Campbell *et al.*, 2014), providing further support for initiating the use of AA as an adjuvant cancer therapy.

Dietary vitamin C is comprised of both reduced AA and the fully oxidized form, dehydroascorbic acid (DHA). The ability of AA and DHA to provide equivalent intracellular vitamin C activity has been controversial for decades with conflicting reports on the ability of DHA intervention to prevent or treat scurvy in animal and human subjects (Frikke-Schmidt, Tveden-Nyborg, and Lykkesfeldt, 2016; Ogiri *et al.*, 2002; Otsuka, Kurata, and Arakawa, 1986; Todhunter, Mc, and Ehmke, 1950). Recently, McCarty (Mccarty, 2013) speculated that DHA would be a more effective cancer therapy than AA. The rationale for this idea was that advanced malignancies, particularly those with elevated HIF activity, often overexpress glucose transporters (GLUTs), a common observation in Warburg metabolism (Courtnay *et al.*, 2015). Interestingly, DHA uptake is facilitated by GLUTs, therefore elevated GLUT1 expression, which is a known HIF-1 target gene, would support increased DHA entry into malignant cells (Mccarty, 2013). DHA itself does not have any biological activity or act as an enzyme cofactor; however, once transported into the cell, it is reduced to AA in the cytosol, providing the functional form of vitamin C (Fig. 16) (Du *et al.*, 2012b). To our knowledge, the capacity of using DHA rather than AA as an effective clinical source for increasing intracellular AA levels has not been evaluated in malignant cells.

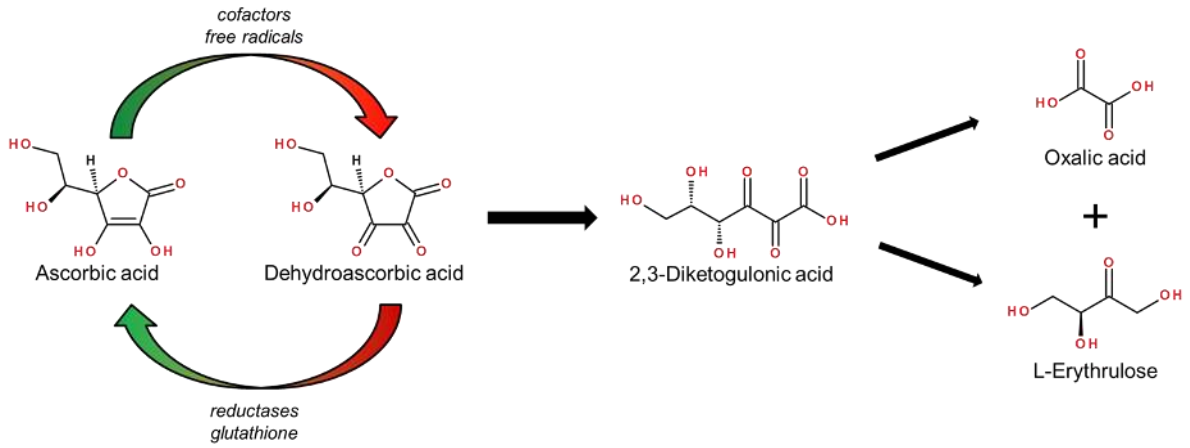

Figure 16. Major degradation products of ascorbic acid.
Ascorbic acid (AA) becomes fully oxidized to dehydroascorbic acid (DHA) following the donation of electrons to either enzyme cofactors or free radicals. Intracellular DHA can subsequently be reduced and recycled to AA by multiple DHA reductases or glutathione, facilitating comparatively higher levels of AA in tissues and plasma. Alternatively, impaired reductase or glutathione activity promotes the spontaneous and irreversible degradation of DHA to 2,3-diketogulonic acid (2,3-DKG) resulting in the loss of vitamin C. Cleavage between the second and third carbon atoms of 2,3-DKG causes the formation of L-erythrulose and oxalic acid, the latter being a primary metabolite of AA.

There are several physiological factors that may impair the delivery of adequate vitamin C to melanoma cells via AA or DHA supplementation. Some of these include the expression and subcellular localization of sodium dependent vitamin C transporters (SVCT1 and 2) and glucose transporters (GLUTs), which transport AA and DHA, respectively. DHA competes with glucose for entry into the cell through the GLUT transporters and is known to be unstable at physiological pH (Linster and Van Schaftingen, 2007). Therefore, the effect of glucose competition on DHA uptake poses a relevant concern for the use of DHA in the clinical setting as a means to promote intracellular vitamin C accumulation and warrants evaluation. The objective of this study was to compare the suitability of AA vs. DHA as a potential adjuvant cancer therapy. This objective was accomplished by examining the ability of AA and DHA to reduce the malignant potential of melanoma cells by increasing intracellular vitamin C content

and subsequently restoring regulation of aberrant normoxic HIF-1α protein accumulation and activity in human melanoma cells.

Materials and methods

Cell culture and reagents

WM3211, SbCl2, WM3248, WM1366, WM239A, and WM9 melanoma cell lines were a generous gift from Meenhard Herlyn's lab at the Wistar Institute (University of Pennsylvania). Human Epidermal Melanocytes, neonatal, lightly pigmented (HEMnLP) were purchased from Life Technologies. All cells were cultured in a humidified incubator with 5 % CO_2 / 95 % air at 37°C. SbCl2 cells were cultured in MCDB 153 media (Sigma) supplemented with 2 % fetal bovine serum (FBS), 5 µg/mL insulin (Sigma), 1.68 mM $CaCl_2$, and 1 % penicillin/streptomycin. WM3211 cells were cultured similarly except for 5 % FBS without $CaCl_2$. WM3248, WM1366, WM239A, and WM9 cells were cultured in standard RPMI 1640 media (Gibco; 1 g/L glucose as indicated in text) supplemented with 10 % FBS and 1 % penicillin/streptomycin. HEMnLP cells were cultured in Medium 254 (ThermoFisher) supplemented with Human Melanocyte Growth Supplement (HMGS; ThermoFisher) and 1 % gentamicin/amphotericin B. L-Ascorbic Acid (AA), Dehydroascorbic acid (DHA), L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A2P), Ethyl 3, 4-dihydroxbenozoate (EDHB), and Cobalt Chloride ($CoCl_2$) were purchased from Sigma. Glucose Transporter Inhibitor III; STF-31 was purchased from EMD Millipore.

Western blots

Membrane protein enriched fractions were prepared by trypsinizing and resuspending cells in a non-detergent buffer (250 mM sucrose, 10 mM Tris, 1 mM EDTA; pH 7.5) and passing through a 26G needle. The homogenate was centrifuged for 1 h at 16000 x g (4 degrees C). The resulting pellet was lysed (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.75 %

Trition X-100, 0.25 % SDS; pH 7.5) for 1 h on ice and centrifuged for 10 min at 16000 x g (4 degrees C) to collect the supernatant. Nuclear protein extracts were isolated using the NePER Nuclear and Cytoplasmic Extraction Kit (Pierce) following the manufacturers protocol. All buffers were supplemented with Complete Mini Protease Inhibitor Cocktail (Roche). Protein lysates were separated by SDS-PAGE on 4-20 % MP-TGX precast polyacrylamide gels (BioRad) and transferred to nitrocellulose using BioRad MINIProtean3 system. Membranes were immunoblotted with antibodies against SVCT2 (1:500; Santa Cruz), GLUT1 (1:5000; abcam), HIF-1α (1μL/mL; R&D Systems). Equal lane loading was accessed by visualization of total protein using MemCode™ Reversible Protein Stain Kit (Thermo Scientific) or probing for βactin (1:10000; 1.5 h at RT; Sigma). Immunoblots were visualized using an enhanced chemiluminescence detection kit (ECL Prime; GE Healthcare) and imaged on a PhotoDyne Imaging system (PhotoDyne Technologies). All western blot images are representative of three different experiments or biological repeats with similar results as indicated in figure legends.

Luciferase reporter assay

Reporter assays were conducted as previously described (Miles *et al.*, 2015). Briefly, 2.0 X 10^5 cells were seeded in 60 mm culture dishes 24 h prior to transfection. Cells were incubated overnight with transfection mixture containing 1.5 μg HIF-1 pTLLuc(5'-3': GTGACTACGTGCTGCCTAGGTGACTACGTGCTGCCTAGGTGACTACGT GCTGCCTAGGTGACTACGTGCTGCCTAG; Affymetrix, LR0128) and 0.1 μg pSV-β-galactosidase plasmids (Clontech) and eXtreme Gene 9 transfection reagent (Roche) following the manufacturers protocol in OptiMEM Reduced Serum media (ThermoFisher). Transfection media were replaced with standard RPMI the following day, and cells were treated as described. Luciferase activity was measured using the Luciferase Assay Kit (Promega) and normalized

against β-galactosidase activity that was measured using the β-galactosidase Assay Kit (Promega). Luciferase and β-gal were measured separately on a SpectraMax L and SpectraMax M2e plate reader (Molecular Devices) respectively.

Reverse transcription (RT) and PCR

Total RNA was isolated from WM9 cells using an RNeasy Mini Kit (Qiagen) following the manufacturers protocol. mRNA quality and quantity was assessed spectrophotometrically using a NanoDrop 200 UV/Vis spectrophotometer. cDNA was synthesized from total mRNA using the Advantage RT-for-PCR kit (Clonetech Laboratories) following the manufacturers protocol. PCR analysis of target sequences were generated using the Advantage cDNA kit (Clonetech Laboratories) with the following PCR primers; GLUT1: 5' - TCATCAACCGCAACGAGGAG – 3'(Forward), 5' – CAAAGATGGCCACGATGCTC – 3' (Reverse); BNIP3: 5' – TGGACGGAGTAGCTCCAAGA – 3' (Forward), 5' – TCATGACGCTCGTGTTCCTC – 3' (Reverse); βactin: 5' – GCTGCTCGTCGACAACGGCTC – 3' (Forward), 5' – CAAACATGATCTGGGTCATCTTCTC – 3' (Reverse). PCR condition: 95 °C 1 min; 25 cycles of 95 °C 30 sec, 57 °C 1 min, 72 °C 2 min; 72 °C 5 min. PCR products were separated on 1 % agarose gel containing GelRed™ nucleic acid stain (Biotium) and visualized by UV using a PhotoDyne Imaging system (PhotoDyne Technologies). cDNA and PCR products were generated using MJ Mini Personal Thermal Cycler (Biorad).

Confocal imaging

Melanoma cells were grown under standard conditions on chambered tissue culture slides (BD Falcon™). To visualize the plasma membrane, cells were incubated with wheat germ agglutinin (WGA) conjugated with Alexa Fluor® 594 (Invitrogen) for 10 min (2 µg/mL) before being fixed using 3.7 % formaldehyde in PBS (10 min). Cells were washed 3 X with PBS-T then

permeabilized using PBS-T containing 0.1 % Triton X-100 (5 min) and blocked with 5 % BSA for 1 h at RT. Fixed cells were incubated overnight at 4 degrees C with SVCT2 (1:50; Santa Cruz) or GLUT1 (1:250; Abcam) antibodies. Following PBS-T wash, cells were incubated with donkey anti-goat IgG-FITC (1:100; Santa Cruz) or donkey anti-rabbit IgG H&L (Alexa Fluor® 488) (1:500; Abcam) respectively for 2 h. Slides were mounted using VECTASHIELD Hard Set mounting medium (Vector Laboratories). Images were acquired at The Marshall University Molecular and Biological Imaging Center using a Leica SP5 TCSII (Leica Microsystems). A 63x glycerol lens (NA 1.3) was used to collect a series of images in z-axis with focus steps of 0.5 µm. Total thickness of z-series (approximation of cell mono-layer thickness) ranged from 2.5 – 4 µm. Z-series are shown here as single image maximum intensity projections. Image format is 2048 x 2048 pixels representing a square area of 156 µm x 156 µm (each pixel 76 nm x 76 nm). Emissions from Alexa Fluor® 594 (plasma membrane) were collected by photo-multiplier tube (PMT) through a slit passing light 575 – 650 nm with excitation from 561 nm solid state laser. Emissions from FITC and Alexa Fluor® (SVCT2 and GLUT1) were collected by PMT through a slit passing light 500 – 550 nm with excitation from 496 nm line of argon gas laser. Images were processed using ImageJ v 1.50g.

Intracellular vitamin C assay

Intracellular vitamin C (as AA and/or DHA) content was determined using a protocol adapted from Vislisel et al. (Vislisel, Schafer, and Buettner, 2007). In brief, cells were seeded similarly into 10 cm plates to ensure equivalent confluence at time of collection. At the time of vitamin C supplementation, cells were incubated in transport buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, pH 7.4) unless otherwise indicated in text, or in RPMI. After incubation, cells were trypsinized and cell pellets lysed on ice in methanol water

(60 MeOH:40 H_2O ratio) for 10 min before centrifugation at 12,000 x g for 10 min at 4 °C. Supernatant along with freshly prepared ascorbic acid standards (100 µL) were aliquoted into a 96-well plate with the addition of 100 µL 4-hydroxy-2,2,6,6-tetramethyl-piperidinooxy (TEMPOL; Acro Organics; 2.32 mM) and incubated for 10 min at RT. Immediately, 42 µL of o-phenylenediamine dihydrochloride (OPDA; Thermo Scientific; 5.5 mM) were added to samples and incubated 10 min at RT in the dark before endpoint fluorescence was read on a SpectraMax M2e plate reader (Molecular Devices) at Ex 345/Em 425 nM.

Tumor spheroid formation and 3D invasion assays

Multicellular tumor spheroid formation and 3D invasion assays were adapted from Vinci et al. (Vinci, Box, and Eccles, 2015). Briefly, WM9 cells were seeded as a monolayer and pretreated with or without A2P or DHA (100 µM) for four days. Thereafter, cells were detached and 1 X 10^4 cells were seeded into round bottom 96-well plates coated with agarose and incubated for 48 h under standard culture conditions to encourage spheroid formation. Spheroids were then suspended in basement membrane matrigel matrix (final conc. 3.5 mg/mL; BD Biosciences) and overlaid with RPMI (1 g/L glucose) with or without A2P or DHA (100 µM) for up to 96 h with images collected every 24 h using a Zeiss primovert microscope (Zeiss) to measure the diameter of the spheroid including the migrating cells. Cells and spheroids were cultured in RPMI media (1 g/L glucose) for the duration of the experiment (pre and post matrigel). Images were analyzed using ImageJ v 1.50g**.**

Statistical analysis

Data was analyzed using GraphPad Prism 6 software (version 6.0f; Graphpad Software, Inc.). Statistical significance was determined by One-way or Two-way ANOVA followed by Tukey multiple comparison tests as appropriate. Three individual trials were performed for each

experiment and data represented as mean ± SEM. $p < 0.05$ was considered statistically significant. Specific p values are indicated in figure legends.

Results

A2P and AA inhibit hypoxia-mimetic induced HIF-1α protein stability and transcriptional activity more effectively than DHA in human melanoma cells

In our initial experiment, we wanted to compare the ability of ascorbate 2-phosphate (A2P) vs. DHA to inhibit the hypoxia-mimetic ethyl 3, 4-dihydroxbenozoate (EDHB) induced stability of HIF-1α protein in WM9 and WM239A metastatic melanoma cells following a 24 h treatment. EDHB is a specific inhibitor of PHDs, binding the ascorbate and 2-oxoglutarate sites of the enzyme (Majamaa *et al.*, 1986). AA is known to have a short half-life in culture media due to rapid oxidation (Frikke-Schmidt and Lykkesfeldt, 2010; Michels and Frei, 2013); therefore A2P, which is an oxidation resistant analog of AA, was utilized as our source for vitamin C in longer time point studies. Similar to our previous report (Miles *et al.*, 2015), we demonstrated that all concentrations of A2P (100-500 μM) cause nearly complete abolishment of EDHB induced HIF-1α protein expression at 24 h, while only by the highest concentration of DHA (500 μM) was capable of reducing EDHB induced HIF-1α (Fig. 17).

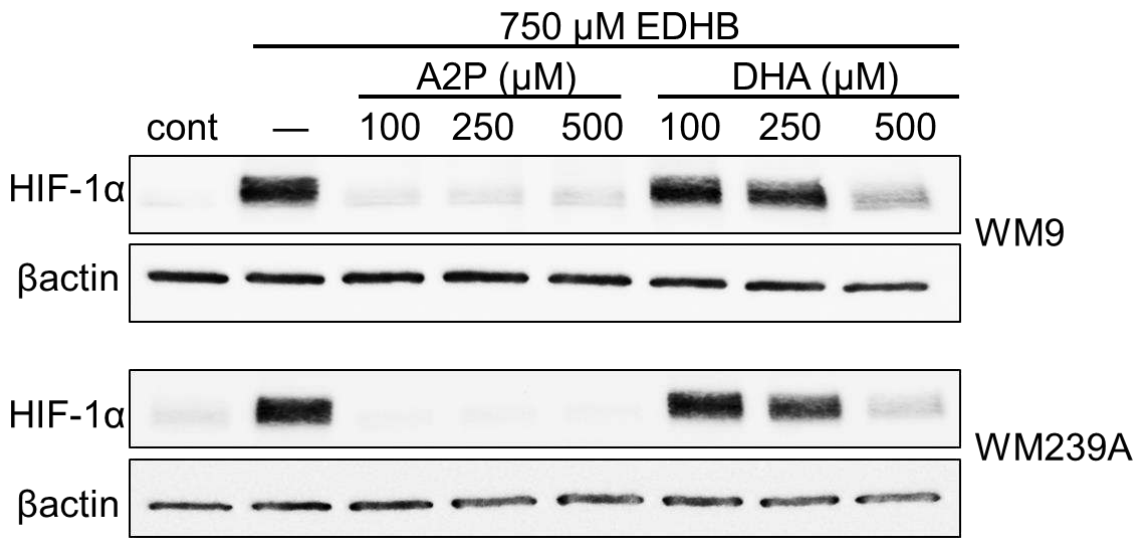

Figure 17. Comparison of A2P and DHA to reduce EDHB induced HIF-1α protein accumulation in metastatic melanoma.
WM9 and WM239A metastatic melanoma cells were incubated in RPMI (1 g/L glucose) containing EDHB with or without increasing concentrations (100 – 500 μM) of A2P or DHA as indicated for 24 h. Our data demonstrates A2P supplementation is more effective than DHA in decreasing HIF-1α stability and accumulation. Blots are representative of three experiments with similar results.

Since AA also functions as a cofactor for FIH to regulate the transcriptional activity of HIF-1α, we wanted to compare the ability of various vitamin C compounds to decrease the transcriptional activity of HIF-1α in melanoma cells. Therefore, we transiently transfected normal human melanocytes (HEMnLP) and a panel of five established human melanoma cell lines with a HIF-1 HRE-luciferase reporter plasmid prior to being stimulated with the hypoxia mimetic cobalt chloride ($CoCl_2$) with or without 100 μM A2P, AA, or DHA for 24 h. As expected, HIF-1α activity was not inducible in HEMnLP cells (Fig, 18A). Interestingly, all compounds demonstrated a similar ability to decrease transcriptional activity in WM3211 and SbCl2 cells (Fig. 18B-C), which demonstrated only modest induction of HIF-1α activity. Conversely, DHA was unable to decrease HIF-1α activity in WM3248, WM1366, and WM9

cells while AA, in particular A2P, significantly suppressed $CoCl_2$ induced HIF-1α transcriptional activity (Fig. 18D-F).

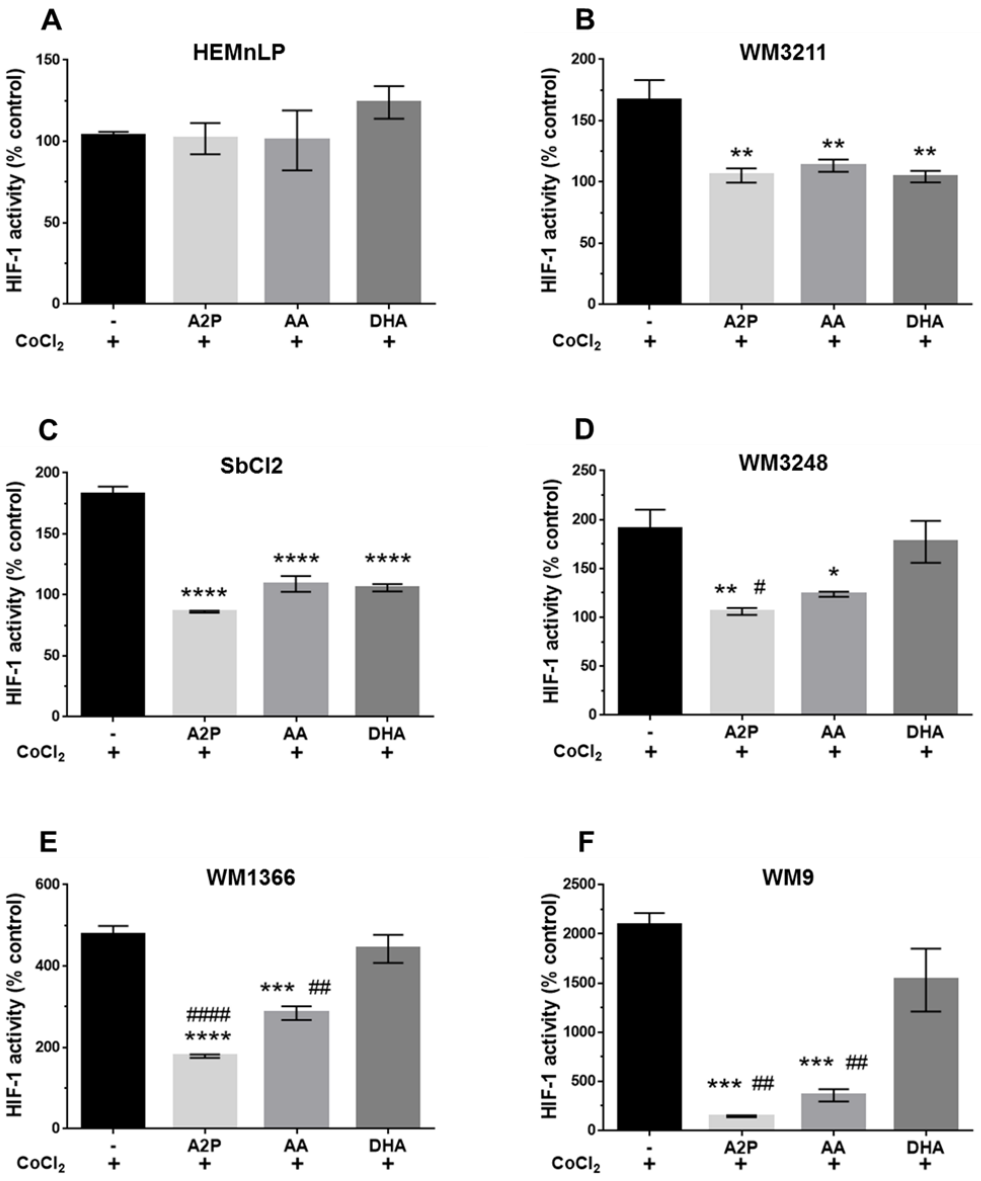

Figure 18. Survey of HIF-1α transcriptional activity following supplementation of vitamin C compounds in melanoma cells.

Normal melanocytes (HEMnLP) and a panel of melanoma cells were transiently transfected with an HIF-1 HRE-luciferase reporter vector. Following overnight transfection, cells were treated with the hypoxia-mimetic $CoCl_2$ (100 µM) with or without 100 µM A2P, AA, or DHA for 24 h. Our results demonstrate A) HEMnLP cells do not have inducible HIF-1 activity, while B) WM3211 and C) $SbCl_2$ cells are responsive to all vitamin C compounds. D) WM3248, E) WM1366, and F) WM9 cells were all responsive to A2P and AA, but not DHA. Data was analyzed using One-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. * denotes statistical significance from $CoCl_2$ induced cells $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. # denotes statistical significance from DHA treated cells $p < 0.05$; ## $p < 0.01$; #### $p < 0.0001$. Data was normalized as percent of control (control = 100 %; not shown).

Consistent with the about results (Fig. 18F), we also found that incubation of WM9 cells with 100 µM A2P for 24 h was more effective than DHA in inhibiting HIF-1α induced mRNA expression of GLUT1 and BNIP3, classic HIF-1 target genes (Fig. 19A-C).

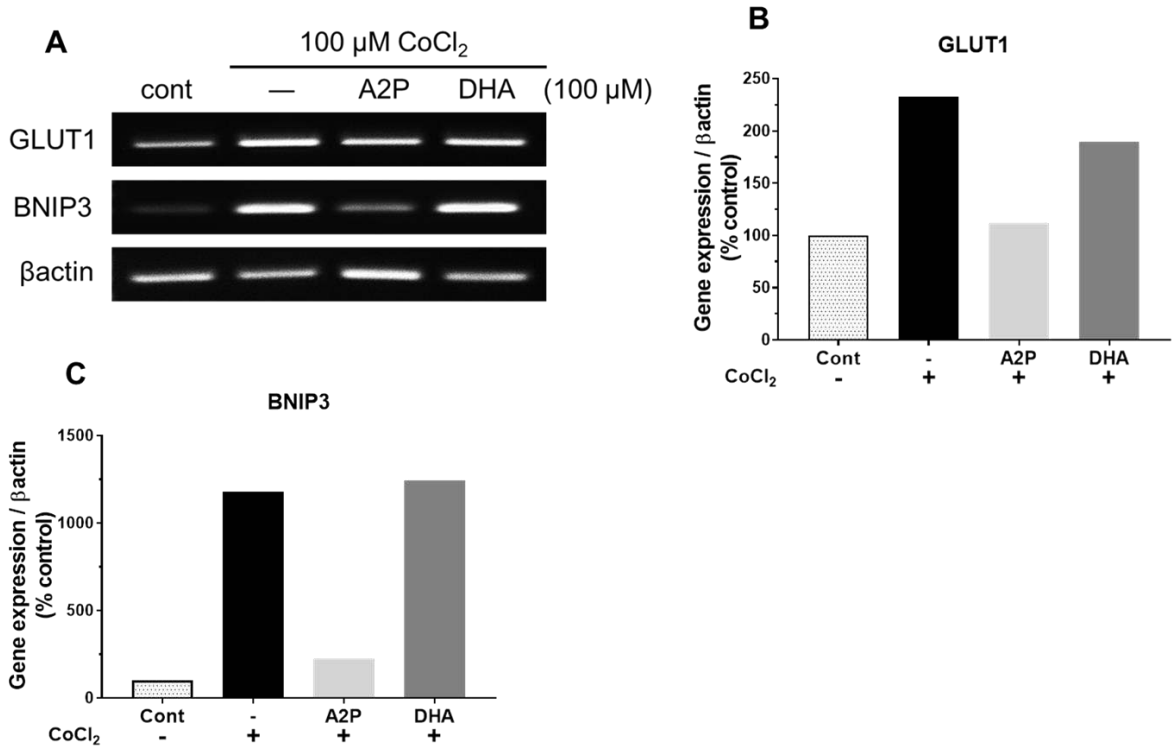

Figure 19. Regulation of HIF-1α target gene expression by A2P or DHA.
To evaluate the impact of vitamin C compounds on HIF-1 target gene mRNA expression, HIF-1 transcriptional activity was stimulated in WM9 cells using $CoCl_2$ (100 µM) and co-treated with 100 µM A2P or DHA for 24 h before cDNA was generated from isolated total RNA. A) Traditional PCR using primers for classic HIF-1 target genes (GLUT1 and BNIP3) reveals A2P supplementation more effectively decreases HIF-1 target gene mRNA expression compared to DHA. B, C) Densitometry of GLUT1 and BNIP3 mRNA expression. Data is representative of multiple individual experiments with similar results.

Human melanoma cells express SVCT2 and GLUT1 transporters necessary for AA and DHA transport

The inability of DHA supplementation to reduce HIF-1α transcriptional activity among the melanoma cell lines may be due to differences in the expression and subcellular localization

of vitamin C transporters in the different cell lines. Sodium dependent vitamin C transporters (SVCT1 and 2) facilitate cellular transport of reduced vitamin C (ascorbic acid; AA). The primary function of SVCT1 is dietary absorption and reabsorption, therefore expression of this isoform is typically confined to intestinal enterocytes and renal tubule cells, as well as liver and lung tissue, providing adequate circulating AA in the plasma (Corti *et al.*, 2010; Lindblad *et al.*, 2013; May, 2011). SVCT2 expression is wide spread throughout most tissues and organ systems, facilitating uptake and intracellular accumulation of AA from the plasma (Corti *et al.*, 2010; Lindblad *et al.*, 2013). The oxidized form of AA (DHA) is transported by glucose transporters (GLUTs), primarily GLUT1 and 3 (Du *et al.*, 2012b; Liang *et al.*, 2001; Lindblad *et al.*, 2013). Following uptake, DHA can be reduced back to AA enzymatically by DHA reductases or, to a lesser extent, non-enzymatically by glutathione (Fig. 16) (Linster and Van Schaftingen, 2007). To evaluate the potential contribution of differential transporter expression and localization on aberrant HIF-1α activity and subsequent ability of AA and DHA to modulate HIF-1α stability and activity, we first evaluated the protein expression of SVCT2 and GLUT1 transporters in HEMnLP and melanoma cells by western blot. All cell lines were found to express both SVCT2 and GLUT1 proteins (Fig. 20A). Subsequent PCR analyses confirmed that SVCT2 and GLUT1 are the primary isoforms expressed in these cell lines (data not shown).

To effectively transport extracellular AA or DHA into the cell, SVCT2 and GLUT1 transporters must be localized to the plasma membrane. Therefore, we examined the subcellular localization of SVCT2 and GLUT1 in WM3211 and WM9 melanoma cells. These cells were chosen for this experiment because they possess similar protein expression of GLUT1 (Fig. 20A), yet were either responsive (WM3211) or non-responsive (WM9) to DHA. Our results demonstrate that the majority of SVCT2 protein appears to be internalized in both cell lines (Fig.

20B) while GLUT1 appears to be localized primarily to the plasma membrane (Fig. 20C). Thus, the ability of melanoma to utilize DHA as a source of AA to reduce HIF-1α activity does not appear to be a consequence of altered or differential GLUT1 protein expression or localization.

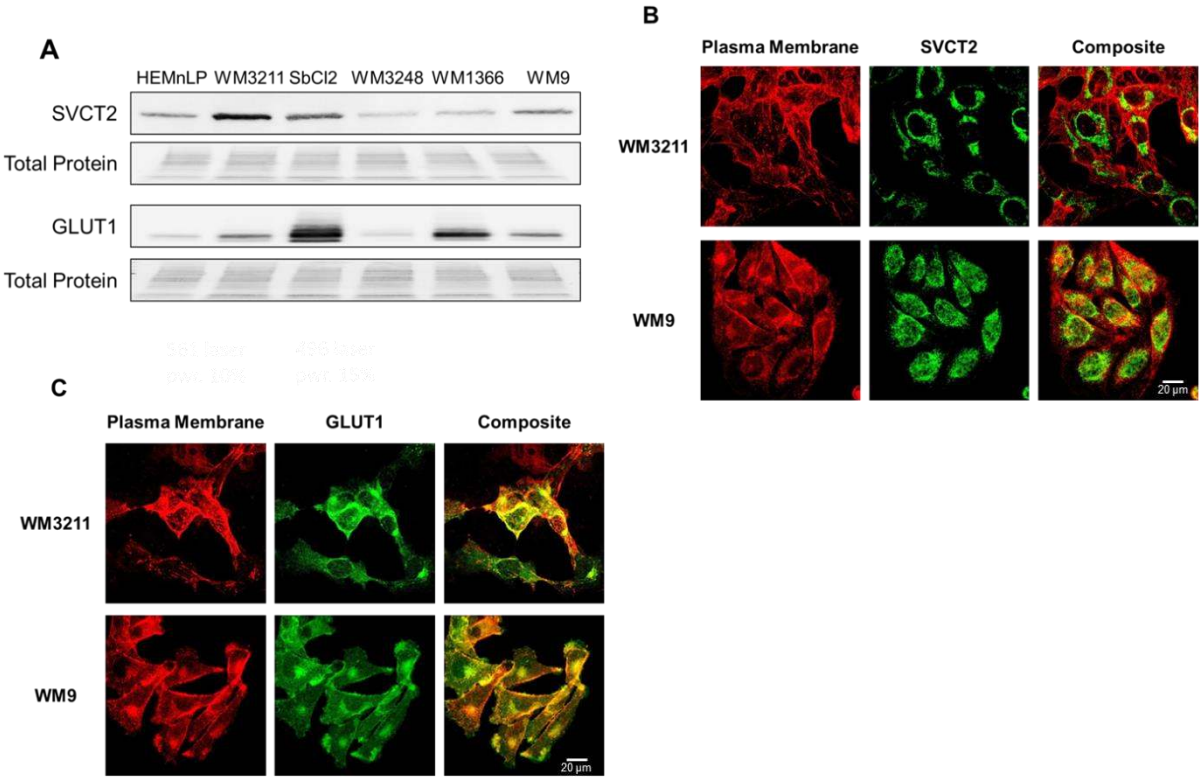

Figure 20. Protein expression and subcellular localization of SVCT2 and GLUT1 in human melanoma.
A) Western blot analysis of cell membrane enriched protein extracts reveal varying expression of SVCT2 and GLUT1 isolated from melanocytes (HEMnLP) and a variety of melanoma cell lines. B) Confocal analysis of WM3211 and WM9 cells depicts the majority of SVCT2 protein is internalized and not present within the plasma membrane. Conversely, C) the expression of GLUT1 is primarily limited to the plasma membrane. Confocal images are representative of multiple experiments.

AA and DHA supplementation leads to the accumulation of vitamin C in WM9 metastatic melanoma cells

To further elucidate why DHA appeared to be ineffective at enhancing the intracellular cofactor function of AA, WM9 cells were used as the study model in the remainder of this

investigation because they represent an advanced metastatic phenotype, providing a more clinically relevant model for evaluating the use of AA or DHA as an adjuvant therapy in melanoma. To evaluate intracellular vitamin C accumulation, we compared the ability of AA and DHA to increase intracellular vitamin C content in WM9 metastatic melanoma cells. Cells were incubated for 30 min with AA or DHA (100 μM). Cells were treated with AA rather than A2P because A2P must first be dephosphorylated by cell surface phosphatases or esterases before it can be transported by SVCTs (Chepda, Cadau, Girin, Frey, and Chamson, 2001; Fujiwara *et al.*, 1997). This dependence on phosphatases is known to delay the initial transport of AA (Vislisel *et al.*, 2007) an observation our laboratory also confirmed (data not shown). Mechanistically, the vitamin C assay operates by oxidizing the AA present in the sample to DHA before the addition of o-phenylenediamine (OPDA). DHA and OPDA then react to form a fluorescent condensation product (Vislisel *et al.*, 2007), thus this assay quantifies total intracellular vitamin C (AA and DHA) content and does not distinguish between AA and DHA. At short time points, intracellular vitamin C content was significantly higher following DHA incubation compared to AA (Fig. 21A), supporting previous findings (Spielholz, Golde, Houghton, Nualart, and Vera, 1997). However, the effect of glucose competition on DHA transport was not accounted for under these transport conditions. Therefore, studies were repeated in the presence of physiological glucose (1 g/L; 5.5 mM) and demonstrated that glucose significantly attenuated the accumulation of intracellular vitamin C in the presence of DHA while having no impact on the ability of AA to raise intracellular vitamin C levels. The addition of glucose to the incubation buffer resulted in similar initial accumulation of intracellular vitamin C using either the SVCT2/AA or GLUT1/DHA transport system (Fig. 21A). To validate that vitamin C accumulation following DHA incubation was due to DHA uptake by GLUT1 transport, vitamin C accumulation studies

were also done in the presence of the highly selective GLUT 1 inhibitor STF-31 (10 μM). Inhibition of GLUT1 significantly reduced DHA uptake and confirmed that DHA transport was mediated by GLUT 1 (Fig. 21A).

To confirm that AA or DHA supplementation results in functionally equivalent intracellular AA, the protein accumulation of HIF-1α in WM9 cells was evaluated by western blot. During a short incubation (30 min), all concentrations (10-50 μm) of AA nearly eliminated hypoxia-mimetic (EDHB) induced HIF-1α accumulation, while only the highest concentration of DHA (50 μM) was able to reduce HIF-1α protein levels (Fig. 21B).

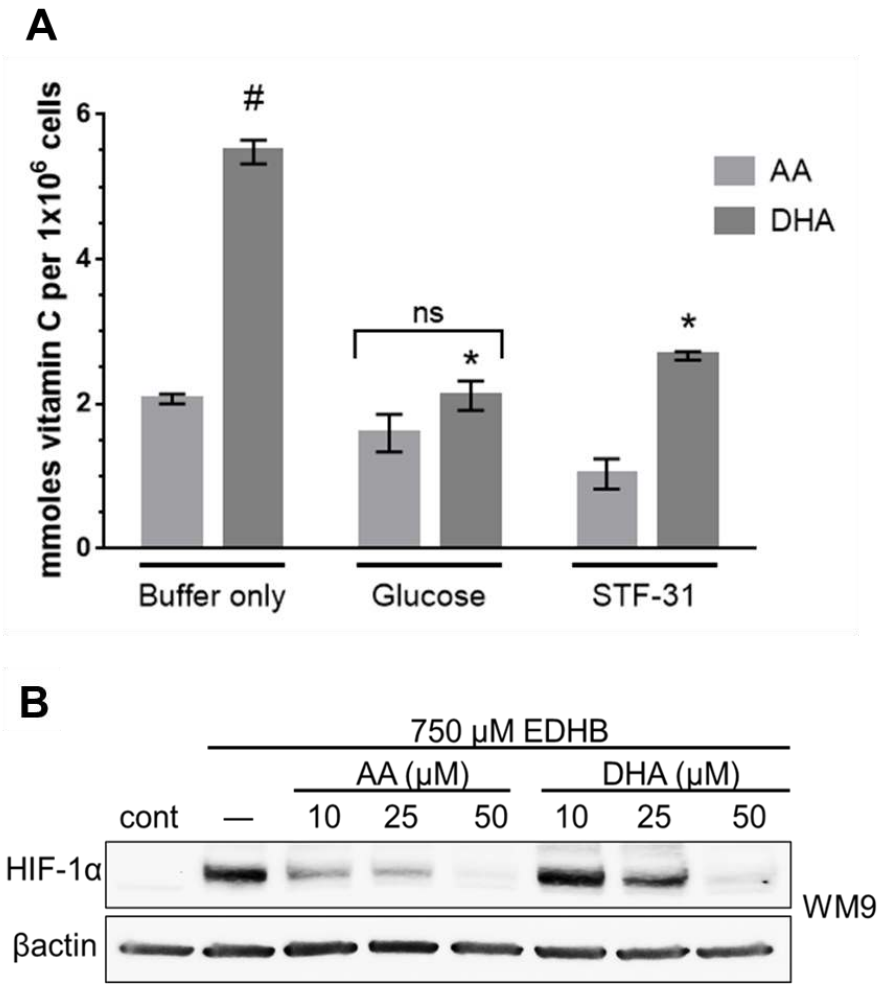

Figure 21. Initial transport and activity of AA or DHA in WM9 melanoma cells.
To evaluate the contribution of GLUT1 to vitamin C accumulation in metastatic melanoma, A) WM9 cells were incubated with AA or DHA (100 μM) for 30 min. in a standard transport buffer with or without glucose (1 g/L) or the GLUT1 inhibitor STF-31 (10 μM), before being collected and intracellular vitamin C measured. B) To compare the initial ability of AA or DHA to decrease HIF-1α protein stability and accumulation WM9 cells were incubated with EDHB (750 μM) for 2 h before the media was exchanged with fresh RPMI (1 g/L glucose) containing low concentrations (10 – 50 μM) of AA or DHA for 30 min before nuclear protein extracts were analyzed by western blot. Our results demonstrate that in the absence of glucose DHA appears to be preferentially transported across the plasma membrane over AA, resulting in a higher initial intracellular vitamin C content. However, the addition of glucose significantly attenuates DHA transport as seen by loss of intracellular accumulation of vitamin C. The use of STF-31 similarly inhibits DHA transport confirming GLUT1 is the primary mechanism for DHA uptake. Western blot analysis indicates that at all concentrations AA was more effective at regulating HIF-1α protein stability than DHA. Data was analyzed using Two-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. # denotes statistical difference from buffer only AA, $p < 0.0001$. * denotes statistical difference from buffer only DHA, $p < 0.0001$. ns = not significant. AA content was not detectable in control cells so significance is not indicated.

Prolonged incubation with DHA results in the loss of intracellular vitamin C

Our results demonstrated that the presence of glucose attenuated the initial DHA transport into cells; therefore, we wanted to determine if this would impact the ability of cells to accumulate and maintain intracellular vitamin C over time, thus representing a more physiologically and clinically relevant scenario for assessing the therapeutic potential of AA and DHA. To evaluate the accumulation of vitamin C at longer time points, WM9 cells were supplemented with 100 µM A2P, AA, or DHA for 24, 48, and 72 h under standard culture conditions (Table 2). To maintain the health of the cells during the extended time point incubations, transport buffer was substituted with standard RPMI media containing (1 g/L) glucose. Following incubation, cells were collected and the total vitamin C content measured using the vitamin C assay. Our results show supplementation with A2P resulted in significantly higher intracellular vitamin C compared to either AA or DHA at both 24 and 48 h. Intriguingly, in contrast to shorter time points, incubation with DHA at all longer time points (24 – 72 h) resulted in minimal vitamin C content in our cells (Table 2), suggesting a limited therapeutic capacity for DHA compared to AA and A2P.

	Vitamin C (100 µM)	mmoles per 10^6 cells
24 h	**A2P**	4.700 ± 0.333
	AA	2.907 ± 0.184 **
	DHA	0.163 ± 0.029 *** #
48 h	**A2P**	2.500 ± 0.411
	AA	0.847 ± 0.428 *
	DHA	0.020 ± 0.020 **
72 h	**A2P**	0.390 ± 0.189
	AA	ND
	DHA	0.033 ± 0.015

Table 2. Intracellular accumulation of vitamin C following incubation with various vitamin C compounds.
WM9 cells were incubated with 100 µM A2P, AA, or DHA for the indicated time period in RPMI media containing 1 g/L glucose. Incubation with A2P resulted in the greatest vitamin C content compared to AA and DHA. Interestingly, minimal vitamin C accumulation resulted from DHA incubation. Data was analyzed using One-way ANOVA with a Tukey multiple comparison test within each time period. Data shown as ± SEM, n = 3. * denotes significance from A2P at the indicated time period, $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. # denotes significance from AA at the indicated time period, $p < 0.001$. ND = not detectable.

A2P reduces HIF-1α activity and invasive potential more effectively than DHA

Since our data demonstrated that incubation with A2P results in significantly higher intracellular accumulation of vitamin C compared to DHA over an extended time period, we wanted to evaluate the possible therapeutic potential of A2P and DHA by assessing any changes in malignant potential during that time. Examination of the ability of 100 µM A2P, AA, or DHA to inhibit HIF-1α transcriptional activity at 48 and 72 h in WM9 cells indeed demonstrated persistent inhibition of HIF activity following A2P treatment, while DHA treatment proved to be ineffective (Fig. 22A-B).

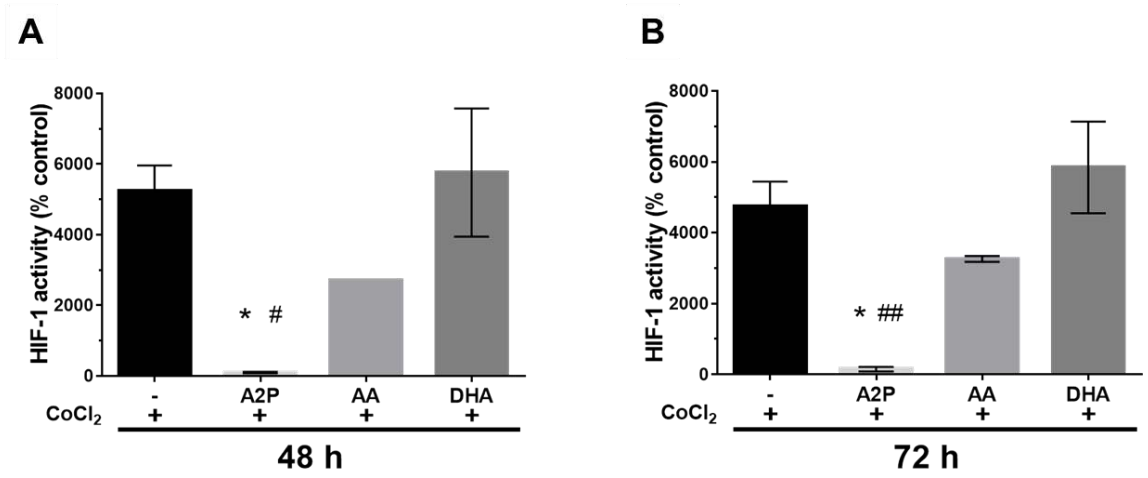

Figure 22. Comparison of vitamin C compounds to decrease HIF-1α transcriptional activity over time in WM9 melanoma cells.
Similar to Fig. 3F, WM9 were transiently transfected with an HIF-1 HRE-luciferase reporter vector. Following overnight transfection, cells were treated with the hypoxia-mimetic $CoCl_2$ (100 µM) with or without 100 µM A2P, AA, or DHA for A) 48 h and B) 72 h. Our findings show A2P supplementation significantly attenuates HIF-1 activity up to 72 h. Data was analyzed using One-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. * denotes statistical significance from $CoCl_2$ induced cells $p < 0.05$. # denotes statistical significance from DHA treated cells $p < 0.05$; ## $p < 0.01$. Data was normalized as percent of control (control = 100 %; not shown).

In order to assess and compare the ability of A2P vs. DHA to decrease the malignant potential of metastatic WM9 cells, we conducted 3D tumor spheroid invasion assays. Cells were incubated in the presence or absence of A2P or DHA (100 µM) for four days prior to the generation of spheroids and throughout spheroid formation. Following seeding and formation of tumor spheroids, spheroids were directly imbedded in matrigel matrix (with the addition of 100 µM of the appropriate vitamin C) and the migration of invasive cells into the matrix was monitored over the next 96 h. Our findings show that A2P effectively prevented the migration and invasion of cells, limiting spheroid progression to approximately 3.0 %, while control and DHA supplemented cells demonstrated a 20 % and 23 % increase in spheroid diameter respectively (Fig. 23A-B). These results further demonstrate the reduced efficacy of using DHA as a potential

therapeutic agent to re-establish intracellular AA levels. Cell counts prior to spheroid formation following the four day pretreatment of cells confirmed vitamin C treatment did not result in altered cell proliferation (data not shown).

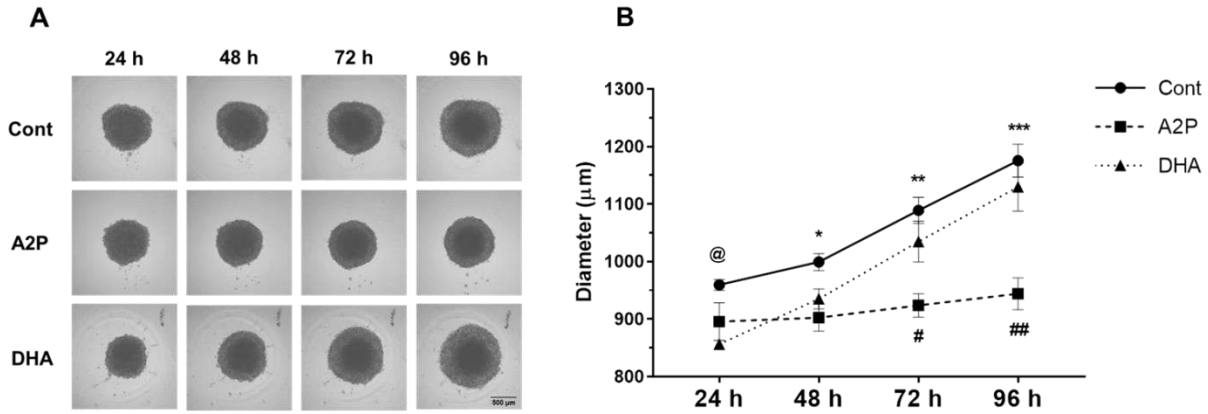

Figure 23. A2P, but not DHA, inhibits the invasive potential of human melanoma. WM9 cells were pretreated with or without A2P or DHA (100 μM) for four days prior to the generation of tumor spheroids. Following their suspension in matrigel, A) images of the tumor spheroids were collected up to 96 h to visualize cell migration/invasion. B) The diameter of the expanding tumor spheroids, including the migrating cells, was measured to access the impact of A2P or DHA on cancer cell migration/invasion. Our results demonstrate supplementation with A2P is capable of limiting the invasive potential of WM9 metastatic melanoma cells. Not surprising, DHA was largely ineffective at inhibiting cell migration/invasion. Images are representative of multiple experiments. Data was analyzed using Two-way ANOVA with a Tukey multiple comparison test. Error bars represent SEM, n = 3. @ denotes statistical significance between control and DHA treated cells $p < 0.05$. * denotes statistical significance between control and A2P treated cells $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. # denotes statistical significance between A2P and DHA treated cells $p < 0.05$; ## $p < 0.0001$.

AA mediated regulation of HIF-1α is dependent on SVCT2 activity

After confirming that the non-responsiveness of WM9 cells to DHA is not due to the absence of GLUT1 expression or function, we wanted to confirm that modulation of HIF activity by AA is dependent on SVCT2 activity, resulting in increased intracellular accumulation of vitamin C (as AA) rather than unrelated extracellular stimuli triggered by AA supplementation. To demonstrate the role of SVCT2 in AA mediated HIF-1α regulation, the activity of SVCT2, a

sodium dependent transporter, was functionally inhibited by the replacement of sodium chloride (NaCl) with choline chloride (ChCl) in the transport buffer. Since SVCTs are the only transporters capable of transporting AA (May, 2011), the impact of sodium deprivation from the media would effectively prevent any extracellular transport of reduced vitamin C. To measure the impact of sodium removal on AA accumulation, WM9 cells were incubated for 30 min in either NaCl or ChCl containing transport buffer with 100 μM AA before intracellular vitamin C content was determined. Substitution of Na in the transport buffer resulted in complete inhibition of vitamin C accumulation in the cells (Fig. 24A). To demonstrate the functional impact of SVCT transporter inhibition on AA mediated HIF-1α regulation, cells were pretreated with EDHB (750 μM) for 2 h in standard RPMI media to induce HIF-1α before the media was exchanged with NaCl or ChCl transport buffer with or without AA (100 μM) for 30 min. As determined by western blot, inhibition of SVCT2 function also prevented the AA mediated decrease in HIF-1α protein stability (Fig. 24B), demonstrating that the functionality of SVCT2 is directly associated with AA accumulation and anticancer activity within these cells.

The tumor microenvironment of aggressive malignancies is frequently found to be slightly acidic (pH 6.5 – 6.9) (Estrella *et al.*, 2013). Because SVCT2 function can be diminished by decreasing pH (Liang *et al.*, 2001), potentially contributing to reduced tumor AA levels, as well as presenting a challenge for the implementation of AA therapy, we wanted to examine the impact of pH on the ability of AA vs. DHA to be transported across the plasma membrane. To do this WM9 cells were pretreated for 30 min with a concentration of AA or DHA (100 μM; empirically determined from Fig. 21B) known to inhibit HIF-1α protein stability in a standard transport buffer (1 g/L glucose) adjusted to pH 6.5, 7.0, or 7.4. Following pretreatment, buffer was exchanged with fresh RPMI containing EDHB (750 μM) for 2 h before cell collection.

Using HIF-1α protein degradation as an indicator for transporter function, western blot analysis shows that reduced pH, representative of the tumor microenvironment, did not alter the activity of SVCT2 or GLUT1 (Fig. 24C), suggesting that the reduced pH of the microenvironment would likely have a negligible impact on the uptake of therapeutic AA or DHA.

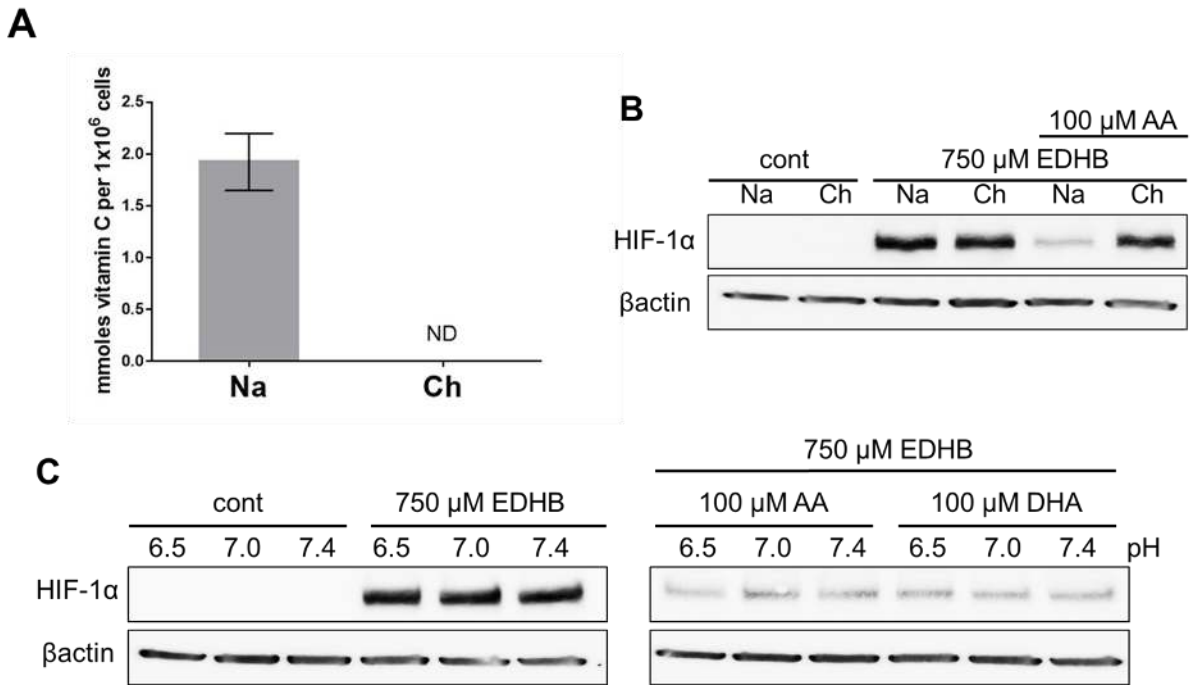

Figure 24. SVCT2 function mediates the regulation of HIF-1α by ascorbic acid.
A) WM9 cells were incubated with AA (100 μM) for 30 min. in a transport buffer containing NaCl (Na) or choline chloride (Ch) before the cells were collected and intracellular AA accumulation was measured. Error bars represent SEM, n = 3. ND = not detectable. B) WM9 cells were incubated with AA for 30 min. in transport buffer containing either NaCl (Na) or choline chloride (Ch). Following incubation, buffer was exchanged for fresh RPMI containing EDHB (750 μM) for 2 h. Western blot analysis demonstrates that SVCT2 activity is required to mediate AA regulation of HIF-1α stability. C) WM9 cells were incubated with AA or DHA for 30 min. in standard transport buffer containing 1 g/L glucose and adjusted to pH 6.5, 7.0, or 7.4 to examine the impact of pH on transporter function. After AA or DHA pretreatment, buffer was exchanged with RPMI containing EDHB for 2 h as indicated. Using the status of HIF-1α protein as a measurement of sufficient vitamin C transporter activity, western blots indicate lower pH does not impair the ability of SVCT2 or GLUT1 to increase functional intracellular vitamin C content in our cells. Blots are representative of three different experiments each with similar results.

Inhibition of HIF-1α depends on cofactor function and not antioxidant properties

Since intracellular AA can function as an enzyme cofactor or as an antioxidant (free radical scavenger) we wanted to confirm that the AA associated inhibition of HIF-1α protein stability is a result of its HIF hydroxylase (PHD1-3, FIH) cofactor function rather than an indirect function of its antioxidant properties. Therefore we examined the protein stability of EDHB-induced HIF-1α following short (30 min) or long term (24 h) incubation with increasing concentrations (10-500 μM) of N-acetyl cysteine (NAC), another common antioxidant. Supplementation with NAC failed to reduce HIF-1α protein accumulation at all concentrations and time points (Fig. 25A-B) confirming AA mediated decrease of HIF-1α is due to its function as a PHD/FIH cofactor and is not the result of its antioxidant properties.

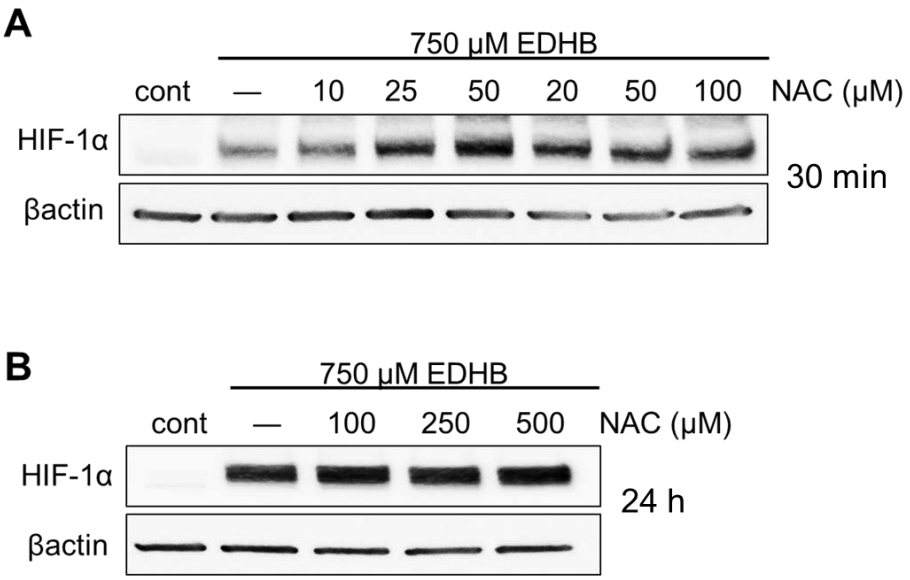

Figure 25. Ability of NAC to decrease EDHB induced HIF-1α protein stability.
A) WM9 cells were incubated with EDHB (750 μM) for 2 h before the media was exchanged with fresh RPMI containing low concentrations (10 – 100 μM) of NAC for 30 min. before nuclear protein extracts were analyzed by western blot. B) WM9 cells were incubated in RPMI containing EDHB with or without increasing concentrations (100 – 500 μM) of N-acetyl cysteine (NAC) for 24 h. Western blot analysis for both experiments indicates NAC is unable to regulate the protein stability of HIF-1α. Blots are representative of biological repeats with similar results.

Discussion

The colonization of malignant melanoma to distant tissues coincides with a dramatic decrease in patient survivability (Siegel *et al.*, 2016). One of the defining characteristics of metastatic melanoma is aberrant increased expression and activity of the HIF-1 transcription factor (Konstantina *et al.*, 2011; Kuphal *et al.*, 2010). Widespread evidence suggests elevated HIF activity promotes the acquisition of several cancer hallmarks including, but not limited to, rapid tumor invasion, induction of angiogenesis, resistance to cell death, and the procurement of classic Warburg metabolism (Courtnay *et al.*, 2015; Greijer and Van Der Wall, 2004; Hanahan and Weinberg, 2011; Lu and Kang, 2010; Zimna and Kurpisz, 2015). Several factors may contribute to aberrant HIF activity in melanoma including inadequate intratumoral AA, which has previously been associated with tumor accumulation of HIF-1α in a variety of different malignancies (Kuiper *et al.*, 2014b; Kuiper *et al.*, 2010). Our previous findings demonstrating that supplementing metastatic melanoma cells with physiological concentrations of AA decreases their malignant potential, suggests that AA deficiency plays a biologically relevant role in oncogenesis, and highlights the need to evaluate its efficacy and use in the clinical setting to improve patient response and survival. The goal of this study was to evaluate and compare the efficacy of AA and DHA supplementation (≤ 0.5 mM) as therapeutic means to promote increased intracellular AA; promote AA mediated regulation of aberrant HIF-α protein stabilization and transcriptional activity and reduce the malignant potential of human melanoma cells.

Circulating plasma and tissue vitamin C is maintained by the dietary ingestion and intestinal absorption of AA and/or DHA. Following absorption, enterocytes reduce DHA to AA and the pool of intracellular AA is released into the plasma by diffusion or hypothesized volume-

sensitive anion channels (Lindblad *et al.*, 2013; Wilson, 2002). The rapid reduction of DHA in the enterocytes, as well as its relative instability at physiological pH (Fig. 16), results in very low levels of plasma DHA (2-5 μM) (Liang *et al.*, 2001), while under the same conditions, AA exists as an ascorbate monoanion (Du *et al.*, 2012b) (commonly referred to as AA; 60-80 μM). The ability of oral DHA supplementation to treat or prevent scurvy has been vigorously contested for decades with some reports showing complete reversal of scurvy symptoms yet others demonstrate low vitamin C activity following oral DHA administration (Frikke-Schmidt *et al.*, 2016; Ogiri *et al.*, 2002; Otsuka *et al.*, 1986; Todhunter *et al.*, 1950). Regardless of the contradictory reports, it is likely that any increased vitamin C activity after DHA ingestion is derived from elevated plasma AA levels stemming from the reduction of DHA and subsequent secretion of AA from the intestinal lumen. This implies that oral DHA supplementation would be unlikely to result in elevated plasma DHA levels, thus limiting the use of DHA as a therapeutic agent to intravenous administration. Intravenous administration immediately demonstrates a disadvantage for any oncology patient prescribed DHA treatment because they will require additional medical assistance. Conversely, oral vitamin C supplementation in the form of AA would likely be sufficient to restore physiological AA in cancer patients. Therefore, intravenous injection of DHA would only be preferred if it provided a significant therapeutic advantage over AA.

To evaluate and compare the efficacy and functional impact of AA vs. DHA supplementation as a potential therapeutic option in melanoma, we compared the ability of A2P and DHA to decrease the protein stability and accumulation of HIF-1α in WM9 and WM239A metastatic melanoma cells. After discovering that DHA was less effective in decreasing HIF-1α protein than A2P (Fig. 17), we wanted to evaluate the impact of the various vitamin C

compounds on the overall transcriptional activity of HIF-1α in a collection of human melanoma cell lines representing various stages of melanoma progression and HIF-1α expression/stability (Fig. 18). While AA acts as a cofactor for several enzymes, its impact on PHD and FIH activity is particularly important because of its regulation of HIF-1α activity, which is known to contribute to oncogenic progression not only in melanoma but other malignancies as well. Our data demonstrated all melanoma cells were sensitive to AA supplementation, particularly via A2P, while only cell lines with low HIF-1α induction (WM3211 and SbCl2; Fig. 18B-C) were significantly responsive to DHA. Alternatively, WM1366 and WM9 cells, representative of advanced stage melanoma, were non-responsive to DHA (Fig. 18E-F).

Other studies in breast cancer, demonstrate a correlation between SVCT2 expression and sensitivity to AA treatment (Hong *et al.*, 2013). To elucidate factors that may contribute to the differential response of melanoma to AA and DHA, we investigated the prevalence and subcellular localization of SVCT2 and GLUT1 protein in our melanoma cell lines since SVCT2 specifically transports reduced vitamin C (AA), and GLUT1 transports the oxidized form of vitamin C (DHA). SVCT2 appeared to be primarily internalized, with limited localization to the plasma membrane, which would suggest a limited capacity for AA uptake (Fig. 20B). This finding was somewhat unexpected given our previous and current findings following treatment of these cells with AA (Miles *et al.*, 2015), yet this is not entirely unreasonable. Previous research has found SVCT1 and 2 can localize to various organelle membranes within the cell (Munoz-Montesino *et al.*, 2014; Reidling, Subramanian, Dahhan, Sadat, and Said, 2008). The exact purpose of intracellular localization is not fully understood, but the possibility of extracellular AA promoting the insertion of SVCTs into the plasma membrane of malignant cells is yet to be evaluated. Surprisingly, we observed both WM3211 and WM9 cells have similar

expression and localization of GLUT1 (Fig. 20A, C), suggesting that other factors govern the potential anti-cancer activity of DHA.

It has been suggested that overexpression of GLUT transporters would facilitate increased uptake of both glucose and DHA by malignant tissue, however the influence of glucose competition on DHA transport was not fully considered (Mccarty, 2013). Recent reports show the impact of glucose on DHA uptake varies greatly between cell types (Corti *et al.*, 2010). Therefore, it was important to evaluate the impact of physiological glucose (1 g/L) on DHA transport to determine if it would limit the ability of melanoma cells to utilize DHA to reestablish functional intracellular AA. Our data clearly demonstrates physiological glucose concentrations drastically decreased the initial uptake of DHA resulting in a nearly 60 % decrease in intracellular vitamin C, yielding results comparable to incubation with AA (Fig. 21A). However, when we examined the ability of AA and DHA to decrease HIF-1α protein accumulation under the same conditions we found AA was more effective (Fig. 21B). Equally significant is the absence of intracellular vitamin C following DHA incubation for 24 – 72 h (Table 2). This finding suggests that although WM9 cells are capable of initially transporting equimolar concentrations of AA and DHA across the plasma membrane (Fig. 21A), they are unable to effectively recycle intracellular DHA to AA, resulting in decreased activity likely through the irreversible degradation of DHA and loss of AA necessary for downstream functions over an extended period of time (Fig. 16). The inability of DHA to be recycled is likely attributed, at least in part, to deficiencies in DHA reductase activity or glutathione. The observation that DHA is still transported into the cell after treatment with a GLUT1 inhibitor, STF-31, is likely due to the presence of GLUT3 protein, which is also known to transport DHA (Rumsey *et al.*, 1997). GLUT3 protein expression and function was not extensively examined in this study because of

the minimal expression of GLUT3 mRNA compared to GLUT1 in our cell lines (data not shown).

The inability of WM9 cells to accumulate and maintain vitamin C levels following incubation with DHA raises considerable concerns as to whether clinical DHA supplementation could increase intracellular AA content sufficiently to promote its cofactor function and anticancer activity. To compare the biological significance of DHA vs. A2P supplementation we used 3D tumor spheroid invasion assays to evaluate the ability of DHA and A2P to decrease invasive potential of WM9 cells. 3D invasion assays were chosen because they more accurately model physiological tumor conditions *in vitro*. Supporting our previous findings in 2D invasion chambers (Miles *et al.*, 2015), these assays demonstrate that incubation of WM9 cells with A2P significantly inhibits cell migration/invasion; however, DHA supplementation failed to impede cell migration/invasion, resulting in spheroid formations nearly identical to non-treated control cells (Fig. 23A-B). While our WM9 tumor spheroids do not display single cell projections typically associated with an invasive phenotype in 3D culture, the morphogenesis of our spheroids are reminiscent of collective cancer cell migration/invasion (Friedl, Locker, Sahai, and Segall, 2012). We were able to exclude changes in cell proliferation as the cause of increased or decreased spheroid diameter by conducting cell counts following A2P or DHA incubation prior to the formation of spheroids (data not shown).

While the response to DHA is not solely dependent on the expression and function of GLUT1, we sought to investigate the influence of SVCT2 activity on the modulation of HIF-1α following AA intervention. In an earlier report, Hong *et al*. observed SVCT2 expression was an indicator of cytotoxicity for high dose (> 1 mM) AA treatment in breast cancer (Hong *et al.*, 2013). By evaluating the impact of inhibiting the function of SVCT2 on AA mediated HIF-1α

regulation in WM9 cells, we found the ability of AA to regulate HIF-1α was entirely dependent on SVCT2 transporter activity (Fig. 24A-B). Also, our data demonstrated that incubation with AA under acidic conditions representative of the tumor microenvironment (pH 6.5) did not impede the ability of AA to decrease HIF-1α protein stability, indicating any inhibition of SVCT2 function by a pH change did not result in a biologically significant decrease in the ability of melanoma cells to accumulate and use AA (Fig. 24C). Lastly, Fig. 25 demonstrated incubation with the common antioxidant NAC is unable to decrease the protein stability of HIF-1α, providing evidence that regulation of HIF-1 by AA was a result of its function as a PHD/FIH cofactor and not a general antioxidant property, supporting the dependence of enzyme function on the presence of AA.

Previously we have shown that melanoma progression is associated with increasing normoxic (aberrant) expression of HIF-1α (Mills *et al.*, 2009) and that supplementing cells with physiological concentrations of AA not only decreased HIF activity but also reduced malignant properties of metastatic cells (Miles *et al.*, 2015). These studies demonstrate that AA may be a beneficial adjuvant therapy for melanoma patients, as well as patients with other types of malignancies, particularly those demonstrating elevated HIF activity. Previous studies have determined that individuals with plasma AA levels < 28 μM had a 62 % increased likelihood of dying from cancer compared to those with AA levels ≥ 74 μM, and that mortality risk decreased dose-dependently with increasing plasma AA (Goyal *et al.*, 2013; Loria *et al.*, 2000). While the evidence for using AA as a cancer therapy continues to mount, it is speculative as to whether the use of DHA, either intravenous or dietary, would provide a therapeutic benefit to cancer patients. Most of the dispute surrounding the efficacy of DHA as a therapeutic source for AA involves its ability to alleviate scurvy, not cancer. One of the only studies examining the anti-cancer effects

of high-dose DHA found that DHA (IC_{50} = 12.7 – 30 mM) was much less effective than high-dose AA ($IC_{50} \approx 4 – 8$ mM) in decreasing the viability of several different malignant cell lines (Fromberg *et al.*, 2011). However, the cytotoxic mechanism of high dose AA (> 1 mM) is dependent on the generation of H_2O_2 to induce cell death, rather than restoration of its cofactor function resulting in the down regulation of HIF activity, making the significance of this comparison difficult to interpret. In our present study we clearly demonstrated the improved efficacy of low dose AA vs. DHA ($\leq$ 0.5 mM) to decrease invasive potential in metastatic melanoma conceivably by increasing intracellular vitamin C content and decreasing HIF activity through optimization of PHD/FIH function. The inability of DHA intervention to regulate HIF activity, and therefore invasion, is likely attributed to a combination of several factors, 1) the insufficient uptake of DHA in the presence of physiological glucose, 2) the failure of metastatic melanoma cells to reduce intracellular DHA to AA, possibly due to inefficient DHA reductase activity or a high endogenous oxidative burden depleting glutathione, and 3) the rapid and spontaneous irreversible degradation of non-recycled DHA within the cell.

Conclusion

These studies provide evidence to suggest that AA supplementation would in fact be more effective than DHA as an adjuvant therapy in the treatment of advanced cancer, particularly in malignancies such as melanoma, which demonstrate aberrant regulation of HIF-1α. Adequate delivery of intracellular AA is crucial for restoring or augmenting its cofactor function for critical enzymes such as the HIF hydroxylases, which regulate the protein stability and accumulation, as well as the transcriptional activity of the HIF-1α/HIF-1 transcription factor. Human melanoma is collectively sensitive to AA therapy; however in our studies, only early stage non-aggressive melanoma is significantly responsive to DHA intervention, while providing

little impact in metastatic melanoma which typically requires chemotherapeutic intervention. Melanoma cells express the vitamin C transporters necessary for the uptake of both AA and DHA; however, in metastatic cells the fact that the initial uptake/transport of DHA across the plasma membrane is impeded by the presence of physiological glucose potentially impacts its clinical efficacy. This observation, accompanied by the probable degradation of unrecycled DHA, likely contributes to the inability of DHA supplementation to increase intracellular vitamin C content this leads to ineffective regulation of HIF-1α, ultimately resulting in failure to attenuate melanoma cell invasion, in comparison to AA. We also determined that the ability of AA to regulate HIF-1α is dependent on SVCT2 activity; however the ability of SVCT2 to modify HIF-1α is unaffected by decreasing pH. These findings are clinically relevant when considering interventions to restore physiological AA levels in patients diagnosed with advanced cancer and highlight the need for further investigation, particularly for clinical trials examining the benefit of restoring physiological AA not only in melanoma, but other cancer types as well.

CHAPTER 4
SILENCING HIF-1α INDUCES TET2 EXPRESSION AND AUGMENTS ASCORBIC ACID INDUCED 5-HYDROXYMETHYLATION OF DNA IN HUMAN METASTATIC MELANOMA CELLS.

The information in this chapter has been published in *Biochemical and Biophysical Research Communications*, *490*(2), 176-181. doi: https://doi.org/10.1016/j.bbrc.2017.06.017

Fischer, and Miles. (2017b). Silencing HIF-1α induces TET2 expression and augments ascorbic acid induced 5-hydroxymethylation of DNA in human metastatic melanoma cells. *Biochem Biophys Res Commun, 490*(2), 176-181. doi:https://doi.org/10.1016/j.bbrc.2017.06.017

Adam P. Fischer
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: fischer35@marshall.edu

Sarah L. Miles Ph.D. *
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University,
One John Marshall Drive
Huntington, WV 25755
Email: kittlaus1@marshall.edu

*** Corresponding author**

Abstract

Expression and function of Ten-eleven translocation (TET) enzymes, which initiate DNA demethylation by catalyzing the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) on methylated DNA, are frequently lost in malignant tissue. Inactivity of TETs ultimately results in lost expression of methylated tumor suppressor genes. Many malignancies, including melanoma, also aberrantly overexpress the oncogenic hypoxia inducible factor-1α (HIF-1α) transcription factor; however the association between HIF-1α and TET enzyme expression is largely uninvestigated. Interestingly, ascorbic acid, a critical cofactor for optimal TET enzyme function and normoxic regulation of HIF-1α protein stability, is frequently depleted in malignant tissue, and may further contribute to the malignant phenotype. In our studies, we found supplementation of WM9 human metastatic melanoma cells with ascorbic acid significantly increased 5hmC content, which was abrogated by TET2 knockdown. Moreover, knockdown of HIF-1α increased TET2 gene and protein expression, and further augmented ascorbic acid-induced TET2 dependent 5-hydroxymethylation in both WM9 and T98G glioblastoma cells. Our data provides novel evidence that HIF-1α is involved in regulating TET expression and 5hmC status of malignant cells. Furthermore, therapeutic intervention to inhibit HIF-1α in conjunction with adjuvant ascorbic acid may promote DNA demethylation and reexpression of critical tumor suppressor genes in malignant cells and warrants further investigation.

Background

DNA methylation is essential for the regulation of cellular differentiation and development. It is now understood that modifications to the epigenome, including aberrant DNA methylation, are common in cancer. For years DNA methylation was thought to be an irreversible process, managed only by DNA methyltransferase (DNMT) inhibition and DNA dilution following replication (Bochtler, Kolano, and Xu, 2017). However, the recently identified Ten-eleven translocation (TET1-3) enzymes are now recognized to contribute to demethylation by oxidizing 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), promoting active demethylation and the subsequent excision and replacement with unmodified cytosine in the DNA (Huang and Rao, 2014; Rasmussen and Helin, 2016).

TET enzyme dysfunction was first associated with cancer following the discovery of TET1 and numerous TET2 mutations in hematological malignancies. Accumulating evidence suggests aberrant TET function may also contribute to several solid malignancies as well. Loss of function of multiple TET isoforms by mutation/deletion, reduced protein expression, or enzyme inhibition have been identified in clear-cell renal cell carcinoma, colorectal, gastric, prostate, liver, lung, and breast cancers, in addition to glioblastoma and melanoma, with the latter six malignancies exhibiting a corresponding loss of 5hmC (Huang and Rao, 2014; Rasmussen and Helin, 2016). While current evidence suggests decreased TET function and/or 5hmC are associated with increased tumorigenesis and metastasis in multiple malignancies (Chen *et al.*, 2017; Hsu *et al.*, 2012; Song *et al.*, 2013), the consequences of 5hmC loss may be most exemplified in melanoma. Primary and metastatic melanomas demonstrate decreased expression of TET enzymes, particularly TET2, when compared to non-neoplastic nevi (Lian *et*

al., 2012). Strikingly, the subsequent loss of 5hmC correlates with increasing malignancy and poor clinical outcomes, whereas melanomas retaining 5hmC are more typical of lower tumor stage (≤ 1) and increased survivability (Gambichler, Sand, and Skrygan, 2013; Lee *et al.*, 2014; Lian *et al.*, 2012; Uchiyama *et al.*, 2014). The loss of 5hmC is so significant it has been declared an epigenetic hallmark of melanoma (Lian *et al.*, 2012).

Like all other enzymes in the Fe II/2-oxoglutarate dioxygenase family, TET enzymes require a common set of co-substrates and cofactors, including enzyme-bound non-heme ferrous iron, 2-oxoglutarate, ascorbic acid, and oxygen (O_2) to maintain optimal activity. The dependence of TET function on oxygen availability has been suggested to contribute to 5hmC loss within dense hypoxic tumors; however, other environments with equivalent low oxygenation, such as those surrounding embryonic tissues and the bone marrow, maintain high TET activity (Ito *et al.*, 2010; Tahiliani *et al.*, 2009), suggesting other factors related to pathological hypoxia may contribute to the regulation of TET function within malignant cells. While the impact of hypoxia on TET function has been previously described (Thienpont *et al.*, 2016), the influence of stabilized Hypoxia Inducible Factor-1α (HIF-1α) transcription factor remains largely under investigated. In malignant tissue, including melanoma (Konstantina *et al.*, 2011), HIF-1α, the subunit that confers HIF-1 transcription factor activity, is often aberrantly overexpressed (Mills *et al.*, 2009), contributing to the expression of genes that facilitate an aggressive malignant phenotype similar to melanomas presenting with low 5hmC content. The notable occurrence of HIF-1α overexpression and loss of 5hmC in high grade melanomas may suggest a relationship between both pathways that contributes to the progression of melanoma. Interestingly, both pathways are regulated by Fe II/2-oxoglutarate dioxygenase enzymes, which require ascorbic acid as a cofactor. Accordingly, ascorbic acid supplementation has been shown

to inhibit HIF-1α accumulation and stimulate increased 5hmC content (Blaschke *et al.*, 2013; Miles *et al.*, 2015), suggesting a possible dual benefit for ascorbic acid therapy in patients with advanced melanoma. The objective of this study was to investigate the influence of HIF-1α on TET enzyme expression and function and its subsequent impact on the capacity of ascorbic acid to modulate the 5hmC content through enhanced TET activity in metastatic melanoma. To examine the effects of HIF-1α on TET, independently of hypoxia, we conducted *in vitro* studies using WM9 human metastatic melanoma cells which have been demonstrated to aberrantly stabilize and accumulate elevated levels of HIF-1α protein under normoxic conditions (Mills *et al.*, 2009). Here we report for the first time, evidence that HIF-1α negatively regulates TET2 gene and protein expression and may contribute to the oncogenic epigenetic landscape in malignant tissue.

Materials and methods

Cell culture and reagents

WM9 human metastatic melanoma and T98G human glioblastoma cells were incubated in a humidified incubator at 5 % CO_2 / 95 % air at 37 °C while cultured in standard RPMI 1640 media (Lonza) supplemented with 10 % fetal bovine serum (FBS) and 0.5 % penicillin/streptomycin. All cell treatments with ascorbic acid were done using 100 μM ascorbate 2-phosphate (A2P) for the times indicated. A2P is an oxidation resistant analog of ascorbic acid with increased stability in cell culture (Fischer and Miles, 2017). WM9 cells were a generous gift from Dr. Meenhard Herlyn at the Wistar Institute (University of Pennsylvania). L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A2P) and cobalt chloride ($CoCl_2$) were purchased from Sigma.

Reverse transcription and qPCR

Total RNA was isolated from cells using an All prep DNA/RNA kit (Qiagen) following the manufacturers protocol. mRNA quality and quantity was assessed spectrophotometrically using a NanoDrop 200 UV/Vis spectrophotometer. cDNA was synthesized from total mRNA using the Advantage RT-for-PCR kit (Clonetech Laboratories) following the manufacturers protocol. Following the generation of standard curves, Taqman Gene expression assays were conducted using primer/probe sets for TET1, 2, and 3 (hs00286756_m1; hs00325999_m1; hs00379125_m1; Applied Biosystems) on a QuantStudio® 3 Real-Time PCR system (Applied Biosystems). Experiments were run in triplicate and data normalized to 18S mRNA expression ($Ct_{target} - Ct_{18S}$).

Western blots

Nuclear protein extracts were prepared using a NePER Nuclear and Cytoplasmic Extraction Kit (Pierce) following the manufacturers protocol. Protein lysates were separated by SDS-PAGE on 4-20 % MP-TGX precast polyacrylamide gels (BioRad) and transferred to nitrocellulose using BioRad MINIProtean3 system. Membranes were immunoblotted with antibodies against TET1 (1:250; Santa Cruz), TET2 (1:5000; Novus), TET3 (1:1000; EMD Millipore), and HIF-1α (1 μg/mL; R&D Systems) overnight at 4°C. Equal lane loading was accessed by probing for Lamin B1 (1:1000; CST) or total protein using a MemCode™ Reversible Protein Stain Kit (Thermo Scientific). Following secondary incubation, blots were visualized using an enhanced chemiluminescence detection kit (ECL Prime; GE Healthcare) and imaged on a Fotodyne imaging system (Fotodyne Technologies). Densitometry analysis was done using ImageJ v 1.50. All blots are representative of three individual experiments.

DNA dot blot

Following cell treatment, DNA dot blots were completed using a 96-well Dot Blot apparatus (Biorad). Briefly, total DNA was isolated from collected cells using an All prep DNA/RNA kit (Qiagen) according to manufacturer's protocol and assessed spectrophotometrically using a NanoDrop 200 UV/Vis spectrophotometer. DNA was denatured by heating to 99 °C for 10 min and diluted to desired concentrations in 2 X SSC buffer (see below) immediately preceding experiments. Nitrocellulose membranes were presoaked in 20 X SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) prior to apparatus assembly and sample loading. After drying at 80 °C for 2 h, membranes were blocked in 5 % milk for 1 h at RT and incubated overnight in an anti-5hmC antibody (1:1000; Zymo Research). Following secondary incubation, blots were visualized using an enhanced chemiluminescence detection kit (ECL Prime; GE Healthcare) and imaged on a Fotodyne imaging system (Fotodyne Technologies). Total DNA loading was determined by methylene blue staining and used to normalize dot blots for densitometry analysis (ImageJ v 1.50g). All blots are representative of three individual experiments.

Small interfering RNA (siRNA) transfection

Cells were transfected as previously described (Miles *et al.*, 2015). Briefly, cells were transfected at time of seeding with 10 nM non-targeting control siRNA, HIF-1α siGENOME SMARTpool siRNA, or 20 nM TET2 siGENOME SMARTpool siRNA (GE Dharmacon) using Lipofectamine RNAimax in standard RPMI media following manufactures protocol. $1.5X10^5$ cells were seeded into 35 mm plates with a fresh media change after attachment. Cells were collected for analysis 48 h after transfection.

Statistical analysis

Data was analyzed using GraphPad Prism 7 software (version 7.03; GraphPad Software Inc.). Statistical significance was determined by One-way or Two-way ANOVA followed by Tukey's multiple comparison tests as appropriate. Three individual trials were performed for each experiment and data represented as ± SEM. $p < 0.05$ was considered statistically significant. Specific p values are indicated in figure legends.

Results

Ascorbic acid augments TET activity and increases 5hmC in melanoma

Published reports have indicated that decreased expression and function of TET enzymes inhibits 5hmC levels in malignant cells (Huang and Rao, 2014; Rasmussen and Helin, 2016). Our initial experiments surveyed the expression of TET isoforms in WM9 metastatic melanoma cells by qPCR and western blot. All three TET isoforms appear to be equally expressed at the gene level (Fig. 26A). Furthermore, all three isoforms also appear to be expressed at the protein level (Fig. 26B) in these cells. To confirm that the TET proteins expressed in these cells have functional activity, we examined the ability of ascorbic acid, a known critical TET enzyme cofactor, to promote the formation of 5hmC over time (24 – 72 h). In agreement with previous findings (Gustafson *et al.*, 2015), we found that supplementation of cells with 100 μm A2P, which represents physiological levels of ascorbate (Du, Cullen, and Buettner, 2012a; Fischer and Miles, 2017a; Miles *et al.*, 2015), significantly increased global 5hmC levels in our cells (Fig. 26C-D). We also observed that maintaining cells continuously in A2P for three weeks resulted in even greater 5hmC content, showing 5-fold higher 5hmC levels than non-treated controls and 2

fold higher than shorter time point (24-72hr) treatments of standard cultured cells with A2P (Fig. 26C-D).

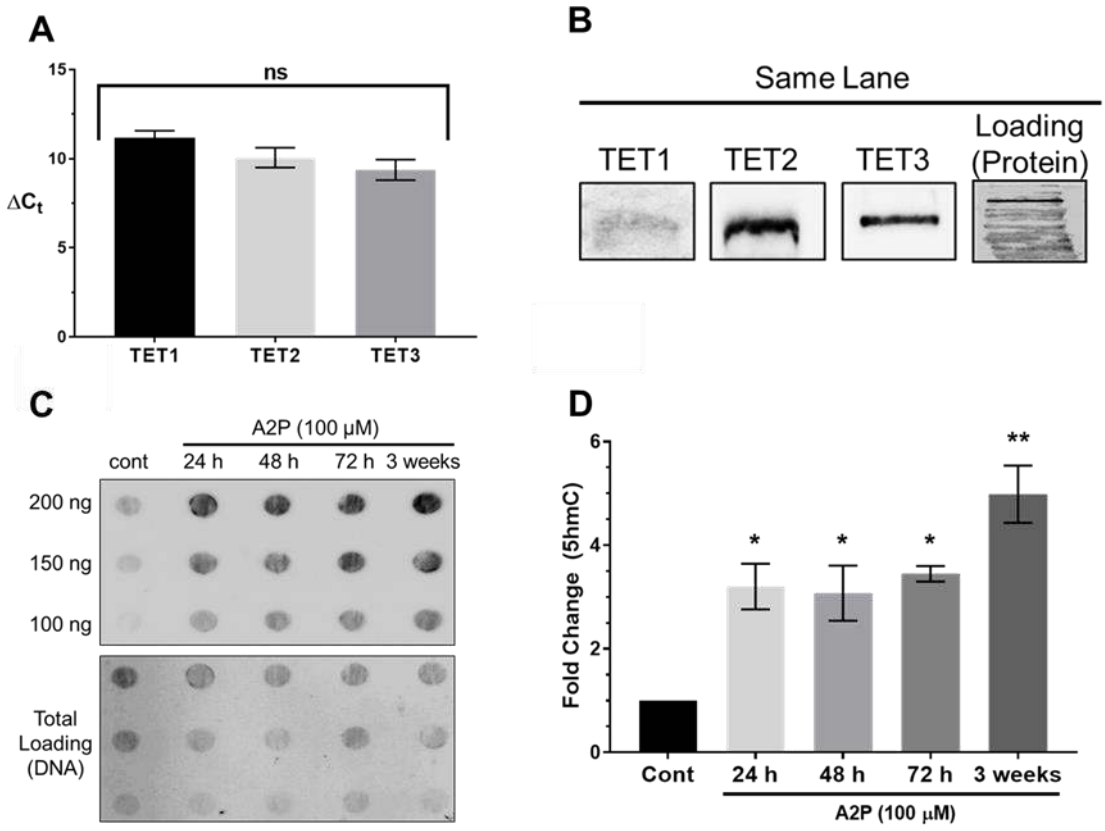

Figure 26. Ascorbic acid increases global 5hmC content in WM9 metastatic melanoma cells. A) mRNA expression of TET1-3 using qualitative real-time PCR (qPCR) and B) protein expression by western blot. Protein expression was examined within the same lane. Total protein loading for lane visualized using protein stain. C) WM9 cells were incubated with A2P (100 μM) for 24 – 72 h before global 5hmC content analyzed by DNA dot blot. 5hmC content was also accessed in cells maintained in A2P (100 μM; changed every 48 h) for three weeks. D) Densitometry of DNA dot blots. Data was analyzed by One-way ANOVA with a Tukey's multiple comparisons test. Error bars represent SEM, n = 3. * denotes statistical significances from control, $p < 0.05$; ** $p < 0.001$. ns = not significant.

Silencing of HIF-1α increases 5hmC by promoting increased TET2 expression

Extensive literature evidence suggests 5hmC content in melanoma is primarily regulated by TET2 (Gambichler *et al.*, 2013; Lee *et al.*, 2014; Lian *et al.*, 2012). To validate the role of TET2 as a principal contributor to the generation of 5hmC in WM9 metastatic melanoma cells,

and to determine its suitability as a target to evaluate the potential regulation of TET enzymes by HIF-1α, we first examined global 5hmC content by DNA dot blot analysis following siRNA knockdown of TET2. Silencing of TET2 alone resulted in a marked decrease in 5hmC levels (Fig. 27A), demonstrating that the TET2 isoform notably contributes to the epigenetic landscape of DNA in these cells. Since aberrantly elevated expression of HIF-1α and reduced 5hmC levels appear concurrently in multiple types of malignancies, including melanoma, we wanted to evaluate whether HIF-1α contributes to the "hallmark" 5hmC profile seen in melanoma via regulation of TET expression and/or activity. To do this we examined mRNA and protein expression of TET2 following siRNA knockdown of HIF-1α. We found that silencing of HIF-1α significantly increased both TET2 gene and protein expression (Fig. 27B-D). Additionally, treatment with 100 μM A2P did not appear to alter the gene or protein level of TET2 (Fig. 27B-D) suggesting that the modulation of 5hmC by ascorbic acid is due solely to its function as an enzymatic cofactor of TET2.

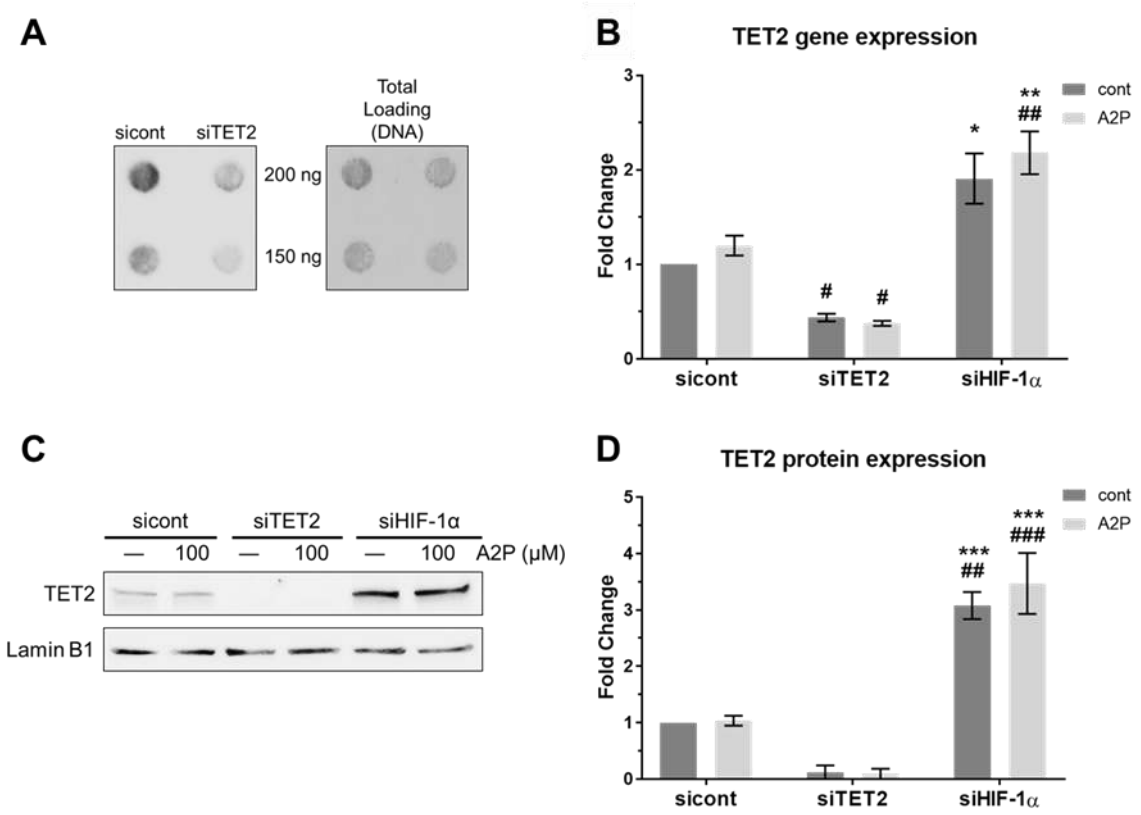

Figure 27. Silencing of HIF-1α increases TET2 mRNA and protein expression in WM9 cells. A) Global 5hmC levels were evaluated by DNA dot blot following siRNA knockdown of TET2. B) TET2 mRNA expression was evaluated by qPCR and C) protein expression by western blot following siRNA knockdown of HIF-1α and incubation with or without A2P (100 μM). D) Densitometry data for western blot. Data was analyzed by Two-way ANOVA with a Tukey's multiple comparisons test. Error bars represent SEM, n = 3. * denotes statistical significance from non-treated sicont, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. # denotes statistical significance from A2P treated sicont, $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$.

To further assess the roles of ascorbic acid and HIF-1α on TET function we examined 5hmC content of WM9 cells following siRNA knockdown of TET2 or HIF-1α in the presence or absence of A2P (100 μM). To validate that the increase in 5hmC following A2P supplementation (Fig. 26C-D) is in fact due to its function as a TET2 cofactor, TET2 siRNA knockdown cells were treated with or without 100 μM A2P, and global 5hmC was measured by DNA dot blot. Knockdown of TET2 not only eliminated basal 5hmC, but also completely abrogated the response to A2P, showing no induction of 5hmC levels by A2P (Fig. 28A-B). Remarkably, while

silencing of HIF-1α significantly increased TET2 protein, it did not significantly increase 5hmC levels (Fig. 28A-B). However, knockdown of HIF-1α in addition to A2P supplementation resulted in global 5hmC content approximately 3.3 times higher than A2P treatment alone (Fig. 28A-B). This data suggests that while TET2 expression may be regulated in part by HIF-1α, global 5hmC content is dependent on TET2 enzyme activity, which is significantly augmented by the presence of ascorbic acid. To determine if these findings were specific to melanoma, we repeated this experiment using T98G glioblastoma cell line (also known to accumulate normoxic HIF-1α protein (Jensen, Ragel, Whang, and Gillespie, 2006)) with comparable results (Fig. 28C-D), further supporting a role for HIF-1α in modulating the epigenetic landscape by regulation of TET2.

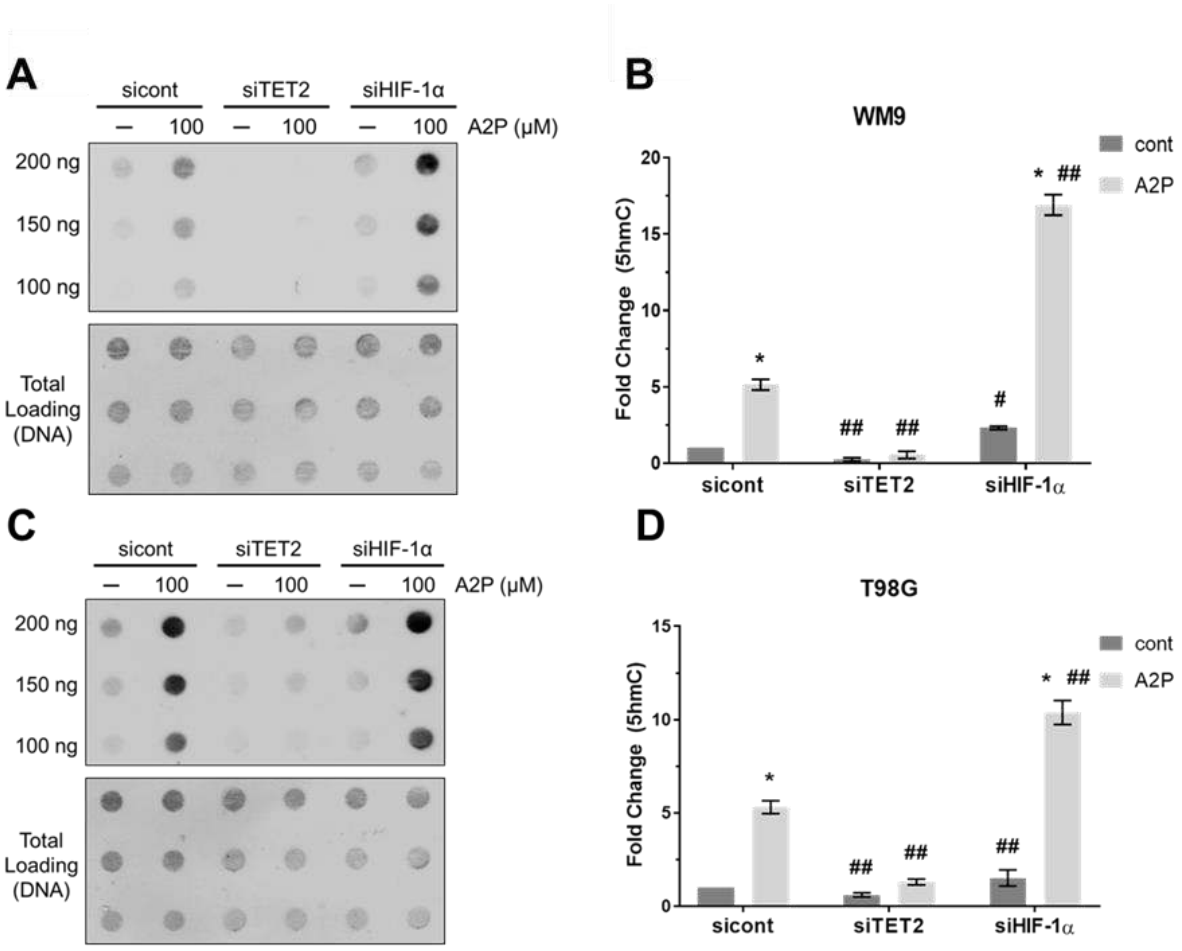

Figure 28. Silencing HIF-1α significantly increases ascorbic acid induced 5hmC content in melanoma and glioblastoma cells.
A) DNA dot blots of 5hmC content were used to evaluate the effects of silencing TET2 and HIF-1α gene expression on A2P supplemented WM9 cells. B) Densitometry data for DNA dot blot. C and D) Experiment was repeated using T98G glioblastoma cells with comparable results. Data was analyzed by Two-way ANOVA with a Tukey's multiple comparisons test. Error bars represent SEM, n = 3. * denotes statistical significance from non-treated sicont, $p < 0.0001$. # denotes statistical significance from A2P treated sicont, $p < 0.001$; ## $p < 0.0001$.

Discussion

The discovery of TET enzymes less than 10 years ago has led to a richer understanding of cellular DNA methylation control and the importance of epigenetic regulation in various disease states including cancer. Aberrant hypermethylation is commonly observed within CpG islands of tumor suppressor promoters, allowing silencing of these genes, and supporting malignant

transformation and progression. TET enzymes promote DNA demethylation by inducing the formation of 5hmC from 5mC, which is then further oxidized by TET enzymes to 5-formylcytosine and 5-carboxylcytosine before being excised and replaced with unmodified cytosine by DNA repair enzymes (Huang and Rao, 2014; Rasmussen and Helin, 2016). Currently, TET1-3 are the only enzymes known to induce hydroxylation of 5mC thus (Ficz and Gribben, 2014), low levels of 5hmC correspond with impaired TET expression or function. Loss of 5hmC correlates with advanced cancer and poor survival in numerous malignancies including melanoma (Chen *et al.*, 2017; Uchiyama *et al.*, 2014), suggesting treatments to improve TET activity and increase 5hmC content may be a valuable treatment strategy.

As the significance of 5hmC in both normal and malignant tissue becomes more apparent, the need to understand its regulation, particularly through the activity and regulation of TET enzymes, is paramount. While evidence implicates oxygen availability as a major regulator of TET activity, kinetic studies have revealed TET1 and 2 have a Km value for O_2 of 0.31 and 0.53 % respectively (Thienpont *et al.*, 2016), which is significantly lower than the reported 1.4 % median oxygen content typical of solid malignancies (Mckeown, 2014). These studies suggest complete inhibition of TET function may not actually occur during pathological hypoxia (0.3 – 4.2 % O_2 depending on tumor type) (Mckeown, 2014). However, prolyl hydroxylase 2 (PHD2), the oxygen-sensing enzyme that primarily regulates the stability and accumulation of HIF-1α, has a Km for O_2 that is about eight times higher than TET1 and 2 (Laukka *et al.*, 2016) suggesting intratumoral hypoxia would not be limiting for TET activity, but would be inhibitory to PHD function and promote the accumulation of HIF-1α.

The possible influence of elevated or aberrantly stabilized HIF-1α on TET function has remained largely uninvestigated, although a recent report has found evidence that TET1 may be

necessary to elicit a full hypoxic response in neuroblastoma cells by demethylating the promoters of hypoxic response genes (Mariani *et al.*, 2014), warranting further examination of the interactions between HIF-1 and TET enzymes in malignant cells. In the present study for the first time, we provide evidence for a novel interaction between HIF-1α and TET2 expression in human melanoma and glioblastoma. Remarkably, we found that silencing gene expression of HIF-1α in WM9 metastatic melanoma cells, which are known to aberrantly stabilize and accumulate high levels of HIF-1α protein (Mills *et al.*, 2009) even under normoxic conditions, significantly increased both the mRNA and protein expression of TET2 (Fig. 27B-D). Elevated TET2 expression via HIF-1α inhibition, in conjunction with A2P supplementation (which augments the activity of both TET and PHD enzymes), significantly increased global 5hmC content in metastatic melanoma as well as glioblastoma cells (Fig. 28). Given the frequency of malignant tissues which express both elevated HIF-1α expression and diminished 5hmC levels, our data suggests that regulation of TET2 expression by HIF-1α may be prevalent across multiple cancer types and impact the malignant potential of those tissues.

Widespread evidence indicates accumulation and transcriptional activation of HIF-1 drive the progression of malignant melanoma (Zbytek *et al.*, 2013). Concurrently, advanced melanomas also present with low levels of 5hmC (Gambichler *et al.*, 2013; Lee *et al.*, 2014; Lian *et al.*, 2012; Uchiyama *et al.*, 2014). In the current study, we demonstrated that silencing of HIF-1α in metastatic melanoma cells with aberrant normoxic HIF-1α activity not only increased the expression of TET2 mRNA and protein, but also significantly increased global 5hmC content when combined with A2P compared to cells treated with A2P alone. While further investigation is required, our data provides novel evidence that high aberrant HIF-1α activity may play a role in diminishing 5hmC content, independent of oxygen tension, through inhibition of TET2

expression. Loss of TET2 may be caused by the expression of a yet unidentified HIF-1α-dependent TET2 gene repressor or possibly the direct binding of the HIF-1 transcription factor may prevent TET2 transcription. In our studies, silencing of HIF-1α had no effect on protein expression of TET1 or TET3 (data not shown). Intriguingly, a specific decrease in TET2 expression within advanced melanomas has been observed (Gambichler *et al.*, 2013; Lian *et al.*, 2012). While studies have also shown advanced melanomas frequently accumulate HIF-1α, studies have not investigated a possible association between elevated HIF-1α expression and low TET2 expression. Published studies have however demonstrated that overexpression of TET2 resulted in increased 5hmC levels, decreased melanoma cell invasion, and reduced tumor size in murine models compared to a mutant TET2 vector (Lian *et al.*, 2012), highlighting the significance of restoring TET2 expression/activity in malignant cells. These studies suggest a combination of HIF-1α inhibition in conjunction with adjuvant ascorbic acid therapy could prove beneficial in melanoma patients by promoting DNA demethylation of tumor suppressor genes.

CHAPTER 5
CONCLUSION

Dating back to the early 1950's, oncologists began to speculate the existence of a relationship between AA status in humans and malignant disease when a study found that plasma AA levels in patients with uterine, gastrointestinal and breast cancer were significantly lower than plasma levels in healthy individuals (Bodansky *et al.*, 1952). In 1959, Dr. W.J. McCormick postulated that the deterioration of collagen surrounding carcinomas, which would likely contribute to malignant invasion and metastasis, may be caused by a nutrient deficiency and suggested vitamin C as the primary candidate (McCormick, 1959). Strikingly, besides knowing AA is needed to prevent scurvy, only minimal advancements in understanding the role of AA in human physiology had been made by the end of the twentieth century. In fact, SVCT1 and 2, the only transporters known to carry AA across the cell membrane, were not identified and cloned until 1999 in rats (May, 2011; Tsukaguchi *et al.*, 1999), underscoring the slow progression of AA research.

Today, much more is known about the metabolism and the importance of AA for overall wellbeing. Possibly the greatest advancement in vitamin C research is the understanding that AA is an essential cofactor for the Fe II/2-oxoglutarate dioxygenase superfamily. Aberrant function of many enzymes within the superfamily, including the HIF hydroxylases and TET enzymes, are thought to contribute to malignant development and progression (Lian *et al.*, 2012; Zhong *et al.*, 1999). Unfortunately, inadequate intracellular AA, which is not uncommon for those diagnosed with cancer (Huijskens *et al.*, 2016; Mikirova *et al.*, 2013; Schleich *et al.*, 2013), can impair the activity of these enzymes and potentially propagate aggressive malignancies.

Melanoma is the deadliest form of skin cancer and is attributed to 75 % of skin cancer deaths (Siegel *et al.*, 2016). While primary melanomas can be easily treated, there are few

treatment options for those with metastatic disease. Therapeutic treatment of metastatic melanoma frequently results in acquired resistance, thus the discovery of new and adjuvant therapies is necessary to improve disease prognosis. Melanoma is also one of many cancer types that has been observed to be associated with an AA depletion in patients (Schleich *et al.*, 2013). Interestingly, advanced melanomas frequently exhibit aberrant overactivation of HIF-1 and the loss of 5hmC (Konstantina *et al.*, 2011; Lian *et al.*, 2012), both of which can be explained by diminished activity of HIF hydroxylases and TET enzymes due to inadequate AA. In these studies, we sought to determine if supplementation of human melanoma, particularly metastatic melanoma, with physiological levels of AA could decrease the malignant potential of cells by inhibiting the aberrant accumulation/activation of HIF-1α and increasing 5hmC content under normoxic conditions by augmenting the function of HIF hydroxylases and TET enzymes respectively.

Throughout chapters 2 and 3 we examine the ability of different forms of vitamin C (AA, A2P, DHA) to inhibit hypoxia mimetic induced and normoxic HIF-1α protein accumulation and transcriptional activity using multiple melanoma cell lines. In our findings, supplementation of cells with both AA and A2P was able to consistently decrease HIF-1α accumulation and activity across multiple cell lines, although A2P was more effective in most cells (Fig. 7-12, 18, 22). The ability of AA to reduce HIF-1α protein accumulation and activity is most likely due to its function as a cofactor and not an anti-oxidant, since treatment with NAC was unable to inhibit HIF-1α protein accumulation (Fig. 25). Incubation of WM9 cells with 100 μM AA or A2P for 24 – 72 h results in significantly higher total intracellular vitamin C content when cells were supplemented with A2P (Table 2). Differences in vitamin C content is likely due to the increased stability of A2P over AA. Under cell culture conditions, AA is rapidly auto-oxidized, likely due

to high oxygen content and components of culture media such as free unbound iron. Therefore, A2P is frequently used for *in vitro* experiments because its stability in a cell culture system more accurately represents AA in physiological conditions. This rationale provides a sensible explanation for the improved performance of A2P over AA in cell culture conditions.

In a recent hypothesis, it has been speculated that treatment of cells with DHA may be a better therapeutic option than AA for advanced cancers (Mccarty, 2013). In these cancers, alterations to energy metabolism, frequently caused by overactive oncogenes, can increase the expression of GLUT1, one of the transporters responsible for DHA uptake (Courtnay *et al.*, 2015; Mccarty, 2013). Overexpression of GLUT1 may promote increased uptake of DHA by cancer cells, resulting in a higher intracellular AA content to be used either as an antioxidant or enzyme cofactor following intracellular reduction of DHA to AA in the cytoplasm. Differences in intracellular AA accumulation are possible because AA and DHA enter the cell via two different transport systems (SVCTs or GLUTs) (Lindblad *et al.*, 2013). Therefore, we also investigated the ability of DHA to decrease HIF-1α accumulation and activity compared AA or A2P. Our studies found incubation of WM9 cells with DHA along with physiological glucose concentrations (1 g/L) resulted in the rapid depletion of intracellular vitamin C in as little as 24 h (Table 1), even though vitamin C content was similar after a 30 min incubation of AA or DHA (Fig. 21). Rapid loss of intracellular vitamin C following DHA incubation may be caused by the inability of WM9 cells to reduce and recycle DHA to AA and may be caused by a high endogenous oxidative burden or deficiencies of DHA reductases. Regardless, given that DHA is relatively unstable at a physiological pH (Linster and Van Schaftingen, 2007), inhibition of DHA recycling would likely result in the degradation of DHA into metabolites that are excreted from the cell (Fig. 16). Not surprisingly, HIF-1α activity of most melanoma cell lines was not

significantly decreased following supplementation with DHA (Fig. 17, 18, 22). This data suggests elevated GLUT1 expression in malignant melanoma cannot significantly increase DHA uptake to an extent to overcome competition with glucose uptake and inefficient DHA recycling, limiting DHA's usefulness as an adjuvant therapy.

The ability of physiological AA to decrease HIF-1α activity is significant because HIF-1 is known to contribute to the acquisition of multiple cancer hallmarks (Courtnay *et al.*, 2015; Hanahan and Weinberg, 2011; Lu and Kang, 2010; Zimna and Kurpisz, 2015). Because of our success using AA to restore activity of the HIF hydroxylases, we wanted to assess its ability to augment the function of other 2-OG dioxygenases that are relevant to cancer. Hence in chapter 4, we choose to investigate TET enzyme activity following supplementation with A2P. TET enzymes are able to facilitate demethylation of gene promoters by oxidizing 5mC to 5hmC, commencing a series of reactions ending with cytosine demethylation (Huang and Rao, 2014; Ito *et al.*, 2011). Reduced 5hmC content has been linked to an increased malignant phenotype and poor patient prognosis in several cancers (Chen *et al.*, 2017); however, it is most prominent in melanoma where low 5hmC has been described as an epigenetic hallmark (Lian *et al.*, 2012). In our studies, supplementation of WM9 cells with 100 μM A2P dramatically increased global 5hmC content as evident by DNA dot blots (Fig. 26). Knockdown of TET2 using siRNA prevented A2P-dependent generation of 5hmC, confirming TET2 in our cells was the primary enzyme regulating 5hmC status (Fig. 27). Surprisingly, we found that silencing HIF-1α using siRNA increased TET2 gene and protein expression (Fig. 27). While HIF-1α knockdown alone was unable to significantly increase 5hmC content in cells via elevated TET2, the addition of A2P (100μM) drastically and significantly increased global 5hmC content in WM9 and T98G cells (Fig. 28). Increased 5hmC content is undoubtedly due to the augmented activity of elevated

TET2 protein. This finding was novel; therefore, a mechanism explaining how the absence of HIF-1α was able to increase TET2 expression is unknown at this time. However, I can speculate that the HIF-1 transcription factor may either enhance transcription of a TET2 gene repressor or direct binding of HIF-1 to the genome may inhibit TET2 transcription. Regardless of the mechanism, these findings provide exciting evidence that HIF-1 may modify DNA methylation in malignant cells, particularly in melanoma. This evidence may suggest elevated HIF-1 activity and loss of 5hmC, eventually contributing to hypermethylation and silencing of tumor suppressors, may not be independent, but instead sequential events that are initiated by HIF-1.

Finally and most significantly, we have demonstrated that supplementation with A2P was capable of significantly decreasing the invasive potential of WM9 cells as determined by transwell matrigel invasion assays (Fig. 14). Likewise, A2P also inhibited anchorage independent growth of WM9 cells (Fig. 15). Our investigation into which form of vitamin C (A2P or DHA) would be more effective in preventing invasion of WM9 tumor spheroids found once again A2P decreased invasion while DHA had little to no effect (Fig. 23).

The canonical loss of 2-OG dioxygenase activity, such as HIF hydroxylases, occurs due to the lack of O_2. Here, we choose to investigate changes in the hydroxylation of 2-OG dioxygenase substrates under aberrant normoxic conditions as to better isolate and understand the contribution of AA on dioxygenase function. Previous studies examining hypoxic cell cultures observed little to no increase in PHD hydroxylase activity follow AA supplementation, likely due to insufficient oxygenation to allow enzyme function, although this is not the case for all cells types (Kuiper *et al.*, 2014). Our investigation of WM9 and WM239A cells under hypoxic conditions (1.5 % O_2) found that 100 µM A2P was unable to decrease protein accumulation of HIF-1α; however total transcriptional activity, mediated by FIH, was decreased

by approximately 20 % in both cells lines. The biological relevance of this finding was not assessed (unpublished observation) but warrants future investigation. While physiological AA therapy may not inhibit canonical accumulation of HIF-1α within hypoxic tumor tissue, it may still be effective in preventing metastasis by inhibiting HIF activity of invasive cells that enter the circulation, and are under normoxic conditions. Metastasis prevention may be possible because aberrant activation of HIF-1 influences gene expression that promotes systemic dissemination, extravasation, and colonization (Lu and Kang, 2010). Additionally, not all 2-OG dioxygenases are as sensitive to hypoxia as the HIF hydroxylases. In fact, the TET enzymes have an approximately eight times higher affinity for O_2 than PHD2, suggesting TET activity or other 2-OG dioxygenases may be augmented by AA supplementation in both hypoxic and non-hypoxic tissues, possibly providing continual anti-cancer activity in all tissues.

In our studies, we focused on the ability of AA to act as a cofactor for HIF hydroxylase and TET enzymes. However, it must be recognized that AA is a cofactor for all enzymes within the 2-OG dioxygenase superfamily. There are several other members within the superfamily whose dysfunction can contribute to malignant transformation and progression which may be alleviated by restoring physiological levels of AA (Kuiper and Vissers, 2014c). Therefore, decreased malignant potential of our cells may not exclusively be a result of promoting proper HIF hydroxylase and TET enzyme activity, but may be caused from enhancing activity of multiple 2-OG dioxygenases. To evaluate the role of additional cancer related 2-OG dioxygenases, such as histone demethylases and carnitine biosynthesis enzymes, similar experiments, as described in this work, could be conducted by isolating the prospective pathways and examining the hydroxylation status of affiliated substrates in response to AA. The most interesting finding in our study is the novel interaction between HIF-1α and TET2 gene and

protein expression. Since there is no previous literature documenting a connection between HIF-1α and TET2, extensive *in vitro* investigations are needed to further elucidate the extent and impact of this relationship. These investigations would include establishing the mechanism through which silencing HIF-1α increases TET2 expression and determine whether overexpression of HIF-1α could facilitate a reciprocal response. While a global increase in DNA 5hmC content should ensure DNA demethylation, methylation specific PCR including promoter sequencing and gene specific reverse transcription-PCR or RNA sequencing should be evaluated to confirm gene demethylation and reexpression in response to AA.

The studies presented herein strongly support the ability of physiological concentrations of AA to reduce malignant properties, particularly in melanoma, is based in part on its ability to support HIF hydroxylase and TET enzyme activity. This work justifies the need for future investigations to evaluate the possible anti-cancer effects of AA through augmenting the function of other 2-OG dioxygenases. The conceivable simplicity of using AA as an adjuvant anti-cancer therapy cannot be overstated. Currently, most researchers and clinicians conducting vitamin C cancer research are investigating the cytotoxic effects of mega dose (> 1 mM) intravenous administration of AA as a cancer therapy. Here we have provided evidence that restoring healthy physiological levels of AA to patients could in-fact decrease the malignancy of their disease. Physiological levels could easily be achieved by oral supplementation or dietary changes with careful monitoring by a patient's primary care physician. These changes may not only be physiologically advantageous, but also encourage emotional and mental health as well. Frequently, oncology patients suffer from harmful feelings of helplessness and defeat (Van Laarhoven *et al.*, 2011). Prescribing specific dietary changes and supplements can allow patients to take back a certain degree of control in their lives and promote emotional well-being. AA

supplementation may also be valuable as a preventive cancer treatment to inhibit transformation events. Shockingly, measurement of plasma AA is not a part of routine laboratory screenings for oncology or primary care patients, meaning most individuals and their physicians do not have a known measurement of their plasma AA levels. This lack of screening underscores the wide-ranging underappreciation for the importance of AA in the maintenance of normal cellular function and homeostasis.

As more information related to the cofactor function of AA is discovered, the next logical step is to investigate the importance of AA cofactor functions *in vivo* and in human trials. However, vitamin C research has potential limitations and pitfalls that if not recognized by investigators could present challenges not only in designing and conducting experiments but also in interpreting their results. To date, a vast amount of cancer research has been done using cell lines (primary or transformed) derived from a wide range of cancers. It is important to note that most commercially available cell culture media and FBS commonly used for these *in vitro* studies are devoid of AA (Michels and Frei, 2013). The lack of AA may be particularly worrisome for established cell lines that have likely been cultured without AA since their initial isolation. Cell lines can survive without AA because it is not essential for growth and division, and thus cells have conceivably adapted to life without AA. While AA is necessary for optimal 2-OG dioxygenase function, it is not needed to directly facilitate hydroxylation of substrates, but instead to reduce enzyme associated Fe^{3+} to Fe^{2+} when electron transfer becomes uncoupled during the hydroxylation reaction (Kuiper and Vissers, 2014c). It is speculated that cell lines have developed increased protein turnover of 2-OG dioxygenases to ensure enzyme activity without AA (Michels and Frei, 2013). The most common animal models used for *in vivo* cancer research are mice and rats. Unfortunately, because mice and rats are capable of synthesizing AA,

it may make it difficult to extrapolate information that can be applied to human physiology. More recently, investigators conducting *in vivo* vitamin C research have used GULO -/- knockout mice. Lacking the enzyme L-gulonolactone oxidase, these mice are similarly dependent on vitamin C consumption for survival. However, even in these models, intestinal transport (uptake) of AA and DHA may still be differently regulated presumably because mice are not normally dependent on intestinal absorption of vitamin C via SVCT1 and GLUTs as in humans (Michels and Frei, 2013). Currently, the degree by which gut absorption of AA differs between mice and humans is unknown. Therefore, study models that lack endogenous AA synthesis, such as guinea pigs, should be preferred particularly for studies involving oral administration of vitamin C. In clinical research trials, there is not a standard procedure for the collection and determination of AA content in plasma and tissues. Different vacutainers and anticoagulants have been shown to affect the stability of AA in various samples (Lykkesfeldt, 2012). In addition, exposure to heat and light accelerates AA oxidation to DHA as well as DHA degradation (Michels and Frei, 2013), making it difficult to accurately quantitate AA levels in plasma or extracted from tissue samples.

Although there are limitations to vitamin C research, that does not mean it is not valuable. However, it is imperative that investigators are aware of these challenges when designing studies and more importantly when analyzing results. As previously stated, investigations into the usefulness of AA as a cancer therapy have been mired in controversy for decades. Yet, the conflicting results of past studies may simply be caused by misinterpretation of data, stemming from the lack of vitamin C knowledge and poor or variable study designs, suggesting reevaluation of past studies may be warranted and could possibly shed light on a consensus that has previously eluded investigators. Here in this work we have shown that

vitamin C in the form of AA (administered as A2P), not DHA, preferentially increases intracellular vitamin C content; we have also demonstrated that supplementation with physiological concentrations of AA, that are achievable via oral supplementation, can decrease the malignant properties of vitamin C depleted human metastatic melanoma cells (Fig. 29).

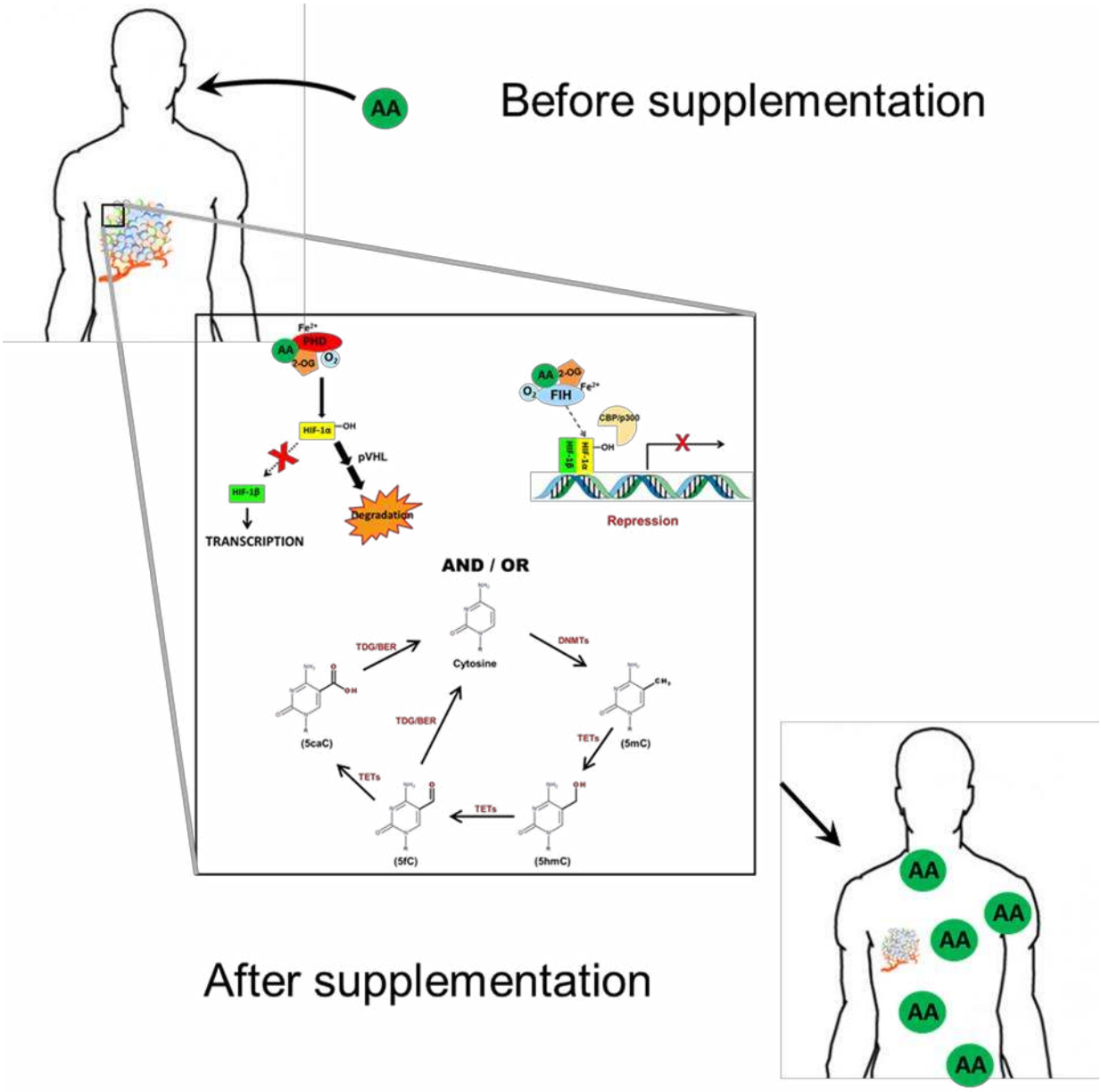

Figure 29. Possible before and after effects of AA supplementation in clinical patients. Individuals with cancer, including melanoma, are commonly found to have below normal levels of plasma AA. Our results in melanoma cells have demonstrated that physiological concentrations of AA decrease the protein accumulation and activity of HIF-1α and increase global DNA 5hmC content, likely resulting in demethylation of tumor suppressor gene promoters. Together, these factors decrease the malignant properties of metastatic melanoma cells, supporting the use of AA as a potential adjuvant anti-cancer therapy.

Our evidence suggests reduction of malignant properties in these cells is likely attributed to the reestablishment of optimal activity of several cancer associated 2-OG dioxygenases, such

as the HIF hydroxylases and TET enzymes. We have also provided novel evidence that the HIF-1α transcription factor can modulate the methylation of the genome by influencing the expression of TET2. It is our hope that these studies will inspire a closer look at past clinical AA investigations and further stimulate and support future inquiries into the clinical use of AA as an adjuvant anti-cancer therapy.

REFERENCES

Aik, Chowdhury, Clifton, Hopkinson, Leissing, Mcdonough, Nowak, Schofield, and Walport. (2015). Chapter 2 introduction to structural studies on 2-oxoglutarate-dependent oxygenases and related enzymes *2-oxoglutarate-dependent oxygenases* (pp. 59-94): The Royal Society of Chemistry.

Aik, Mcdonough, Thalhammer, Chowdhury, and Schofield. (2012). Role of the jelly-roll fold in substrate binding by 2-oxoglutarate oxygenases. *Curr Opin Struct Biol, 22*(6), 691-700. doi:10.1016/j.sbi.2012.10.001

Ascierto, Kirkwood, Grob, Simeone, Grimaldi, Maio, Palmieri, Testori, Marincola, and Mozzillo. (2012). The role of BRAF V600 mutation in melanoma. *J Transl Med, 10*, 85-85. doi:10.1186/1479-5876-10-85

Atkins, Gollob, Sosman, Mcdermott, Tutin, Sorokin, Parker, and Mier. (2002). A phase II pilot trial of concurrent biochemotherapy with cisplatin, vinblastine, temozolomide, interleukin 2, and IFN-alpha 2b in patients with metastatic melanoma. *Clin Cancer Res, 8*(10), 3075-3081.

Bedogni, and Powell. (2009). Hypoxia, melanocytes and melanoma - survival and tumor development in the permissive microenvironment of the skin. *Pigment Cell Melanoma Res, 22*(2), 166-174. doi:10.1111/j.1755-148X.2009.00553.x

Bennett. (2008). How to make a melanoma: What do we know of the primary clonal events? *Pigment Cell Melanoma Res, 21*(1), 27-38. doi:10.1111/j.1755-148X.2007.00433.x

Berra, Benizri, Ginouves, Volmat, Roux, and Pouyssegur. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of hif-1alpha in normoxia. *Embo j, 22*(16), 4082-4090. doi:10.1093/emboj/cdg392

Berrocal, Cabanas, Espinosa, Fernandez-De-Misa, Martin-Algarra, Martinez-Cedres, Rios-Buceta, and Rodriguez-Peralto. (2014). Melanoma: Diagnosis, staging, and treatment. Consensus group recommendations. *Adv Ther*. doi:10.1007/s12325-014-0148-2

Bhagat, Ghone, Suryakar, and Hundekar. (2011). Lipid peroxidation and antioxidant vitamin status in colorectal cancer patients. *Indian J Physiol Pharmacol, 55*(1), 72-76.

Bichakjian, Halpern, Johnson, Foote Hood, Grichnik, Swetter, Tsao, Barbosa, Chuang, Duvic, Ho, Sober, Beutner, Bhushan, and Smith Begolka. (2011). Guidelines of care for the management of primary cutaneous melanoma. American academy of dermatology. *J Am Acad Dermatol, 65*(5), 1032-1047. doi:10.1016/j.jaad.2011.04.031

Blaschke, Ebata, Karimi, Zepeda-Martinez, Goyal, Mahapatra, Tam, Laird, Hirst, Rao, Lorincz, and Ramalho-Santos. (2013). Vitamin C induces TET-dependent DNA demethylation and a blastocyst-like state in es cells. *Nature, 500*(7461), 222-226. doi:10.1038/nature12362

Bochtler, Kolano, and Xu. (2017). DNA demethylation pathways: Additional players and regulators. *Bioessays, 39*(1), 1-13. doi:10.1002/bies.201600178

Bodansky, Wroblewski, and Markardt. (1952). Concentrations of ascorbic acid in plasma and white blood cells of patients with cancer and noncancerous chronic disease. *Cancer, 5*(4), 678-684.

Bowman. (2012). Ascorbic acid, cognitive function, and alzheimer's disease: A current review and future direction. *Biofactors, 38*(2), 114-122. doi:10.1002/biof.1002

Cameron, and Campbell. (1974). The orthomolecular treatment of cancer. II. Clinical trial of high-dose ascorbic acid supplements in advanced human cancer. *Chem Biol Interact, 9*(4), 285-315.

Cameron, and Pauling. (1976). Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. *Proc Natl Acad Sci U S A, 73*(10), 3685-3689.

Campbell, Vissers, Bozonet, Dyer, Robinson, and Dachs. (2014). Restoring physiological levels of ascorbate slows tumor growth and moderates hif-1 pathway activity in GULO−/− mice. *Cancer Medicine*, n/a-n/a. doi:10.1002/cam4.349

Carpenter. (1986). *The history of scurvy and vitamin C*. Cambridge; New York: Cambridge University Press.

Cha, Roomi, Ivanov, Kalinovsky, Niedzwiecki, and Rath. (2011). Ascorbate depletion increases growth and metastasis of melanoma cells in vitamin C deficient mice. *Exp Oncol, 33*(4), 226-230.

Cha, Roomi, Ivanov, Kalinovsky, Niedzwiecki, and Rath. (2013). Ascorbate supplementation inhibits growth and metastasis of B16FO melanoma and 4T1 breast cancer cells in vitamin c-deficient mice. *Int J Oncol, 42*(1), 55-64. doi:10.3892/ijo.2012.1712

Chen, Espey, Krishna, Mitchell, Corpe, Buettner, Shacter, and Levine. (2005). Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc Natl Acad Sci U S A, 102*(38), 13604-13609. doi:10.1073/pnas.0506390102

Chen, Espey, Sun, Lee, Krishna, Shacter, Choyke, Pooput, Kirk, Buettner, and Levine. (2007). Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci U S A, 104*(21), 8749-8754. doi:10.1073/pnas.0702854104

Chen, Shi, Guo, Li, Luo, and He. (2017). Decreased 5-hydroxymethylcytosine levels correlate with cancer progression and poor survival: A systematic review and meta-analysis. *Oncotarget, 8*(1), 1944-1952. doi:10.18632/oncotarget.13719

Chepda, Cadau, Girin, Frey, and Chamson. (2001). Monitoring of ascorbate at a constant rate in cell culture: Effect on cell growth. *In Vitro Cell Dev Biol Anim, 37*(1), 26-30. doi:10.1290/1071-2690(2001)037<0026:moaaac>2.0.co;2

Clifton, Mcdonough, Ehrismann, Kershaw, Granatino, and Schofield. (2006). Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta-helix fold proteins. *J Inorg Biochem, 100*(4), 644-669. doi:10.1016/j.jinorgbio.2006.01.024

Corti, Casini, and Pompella. (2010). Cellular pathways for transport and efflux of ascorbate and dehydroascorbate. *Arch Biochem Biophys, 500*(2), 107-115. doi:10.1016/j.abb.2010.05.014

Courtnay, Ngo, Malik, Ververis, Tortorella, and Karagiannis. (2015). Cancer metabolism and the warburg effect: The role of HIF-1 and PI3K. *Mol Biol Rep, 42*(4), 841-851. doi:10.1007/s11033-015-3858-x

Creagan, Moertel, O'fallon, Schutt, O'connell, Rubin, and Frytak. (1979). Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial. *N Engl J Med, 301*(13), 687-690. doi:10.1056/nejm197909273011303

Culos, and Cuellar. (2013). Novel targets in the treatment of advanced melanoma: New first-line treatment options. *Ann Pharmacother, 47*(4), 519-526. doi:10.1345/aph.1R614

Dickson, Gustafson, Young, Zuchner, and Wang. (2013). Ascorbate-induced generation of 5-hydroxymethylcytosine is unaffected by varying levels of iron and 2-oxoglutarate. *Biochem Biophys Res Commun, 439*(4), 522-527. doi:10.1016/j.bbrc.2013.09.010

Drouin, Godin, and Pagé. (2011). The genetics of vitamin C loss in vertebrates. *Current Genomics, 12*(5), 371-378. doi:10.2174/138920211796429736

Du, Cullen, and Buettner. (2012a). Ascorbic acid: Chemistry, biology and the treatment of cancer. *Biochim Biophys Acta, 1826*(2), 443-457. doi:10.1016/j.bbcan.2012.06.003

Du, Cullen, and Buettner. (2012b). Ascorbic acid: Chemistry, biology and the treatment of cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1826*(2), 443-457. doi:http://dx.doi.org/10.1016/j.bbcan.2012.06.003

Esme, Cemek, Sezer, Saglam, Demir, Melek, and Unlu. (2008). High levels of oxidative stress in patients with advanced lung cancer. *Respirology, 13*(1), 112-116. doi:10.1111/j.1440-1843.2007.01212.x

Estrella, Chen, Lloyd, Wojtkowiak, Cornnell, Ibrahim-Hashim, Bailey, Balagurunathan, Rothberg, Sloane, Johnson, Gatenby, and Gillies. (2013). Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res, 73*(5), 1524-1535. doi:10.1158/0008-5472.can-12-2796

Fain, Mathieu, and Thomas. (1998). Scurvy in patients with cancer. *BMJ, 316*(7145), 1661-1662.

Farrow, and Facchini. (2014). Functional diversity of 2-oxoglutarate/Fe(II)-dependent dioxygenases in plant metabolism. *Frontiers in Plant Science, 5*, 524. doi:10.3389/fpls.2014.00524

Ficz, and Gribben. (2014). Loss of 5-hydroxymethylcytosine in cancer: Cause or consequence? *Genomics, 104*(5), 352-357. doi:http://dx.doi.org/10.1016/j.ygeno.2014.08.017

Fischer, and Miles. (2017a). Ascorbic acid, but not dehydroascorbic acid increases intracellular vitamin C content to decrease hypoxia inducible factor -1 alpha activity and reduce malignant potential in human melanoma. *Biomedicine & Pharmacotherapy, 86*, 502-513. doi:http://dx.doi.org/10.1016/j.biopha.2016.12.056

Fischer, and Miles. (2017b). Silencing HIF-1α induces TET2 expression and augments ascorbic acid induced 5-hydroxymethylation of DNA in human metastatic melanoma cells. *Biochem Biophys Res Commun, 490*(2), 176-181. doi:https://doi.org/10.1016/j.bbrc.2017.06.017

Flashman, Davies, Yeoh, and Schofield. (2010). Investigating the dependence of the hypoxia-inducible factor hydroxylases (factor inhibiting hif and prolyl hydroxylase domain 2) on ascorbate and other reducing agents. *Biochem J, 427*(1), 135-142. doi:10.1042/bj20091609

Flashman, and Schofield. (2007). The most versatile of all reactive intermediates? *Nat Chem Biol, 3*(2), 86-87. doi:10.1038/nchembio0207-86

Fraenkel-Conrat, Stoy, and Tsai. (1967). Investigation of ascorbic acid levels in blood of cancer patients compared to normal subjects. *Mo Med, 64*(12), 1001-1002.

Friedl, Locker, Sahai, and Segall. (2012). Classifying collective cancer cell invasion. *Nat Cell Biol, 14*(8), 777-783. doi:10.1038/ncb2548

Frikke-Schmidt, and Lykkesfeldt. (2010). Keeping the intracellular vitamin C at a physiologically relevant level in endothelial cell culture. *Anal Biochem, 397*(1), 135-137. doi:10.1016/j.ab.2009.09.040

Frikke-Schmidt, Tveden-Nyborg, and Lykkesfeldt. (2016). L-dehydroascorbic acid can substitute L-ascorbic acid as dietary vitamin C source in guinea pigs. *Redox Biol, 7*, 8-13. doi:10.1016/j.redox.2015.11.003

Fromberg, Gutsch, Schulze, Vollbracht, Weiss, Czubayko, and Aigner. (2011). Ascorbate exerts anti-proliferative effects through cell cycle inhibition and sensitizes tumor cells towards cytostatic drugs. *Cancer Chemother Pharmacol, 67*(5), 1157-1166. doi:10.1007/s00280-010-1418-6

Fujiwara, Nagao, Monden, Misumi, Kageyama, Yamamoto, and Miwa. (1997). Enhanced protection against peroxidation-induced mortality of aortic endothelial cells by ascorbic acid-2-o-phosphate abundantly accumulated in the cell as the dephosphorylated form. *Free Radic Res, 27*(1), 97-104.

Gambichler, Sand, and Skrygan. (2013). Loss of 5-hydroxymethylcytosine and ten-eleven translocation 2 protein expression in malignant melanoma. *Melanoma Res, 23*(3), 218-220. doi:10.1097/CMR.0b013e32835f9bd4

Giatromanolaki, Sivridis, Kouskoukis, Gatter, Harris, and Koukourakis. (2003). Hypoxia-inducible factors 1alpha and 2alpha are related to vascular endothelial growth factor expression and a poorer prognosis in nodular malignant melanomas of the skin. *Melanoma Res, 13*(5), 493-501. doi:10.1097/01.cmr.0000056268.56735.4c

Goda, Dozier, and Johnson. (2003). HIF-1 in cell cycle regulation, apoptosis, and tumor progression. *Antioxid Redox Signal, 5*(4), 467-473. doi:10.1089/152308603768295212

Gokce, Keaney, Frei, Holbrook, Olesiak, Zachariah, Leeuwenburgh, Heinecke, and Vita. (1999). Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation, 99*(25), 3234-3240. doi:10.1161/01.cir.99.25.3234

Goyal, Terry, and Siegel. (2013). Serum antioxidant nutrients, vitamin A, and mortality in U.S. Adults. *Cancer Epidemiology Biomarkers & Prevention, 22*(12), 2202-2211. doi:10.1158/1055-9965.epi-13-0381

Greijer, and Van Der Wall. (2004). The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol, 57*(10), 1009-1014. doi:10.1136/jcp.2003.015032

Gustafson, Yang, Dickson, Shao, Van Booven, Harbour, Liu, and Wang. (2015). Epigenetic reprogramming of melanoma cells by vitamin C treatment. *Clin Epigenetics, 7*, 51. doi:10.1186/s13148-015-0087-z

Gyorki, Spillane, Speakman, Shackleton, and Henderson. (2014). Current management of advanced melanoma: A transformed landscape. *ANZ J Surg, 84*(9), 612-617. doi:10.1111/ans.12673

Hanahan, and Weinberg. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674.

Hanna, Krishnan, Bailey, Moschos, Kuan, Shimamura, Osborne, Siegel, Duncan, O'brien, Superfine, Miller, Simon, Wong, and Kim. (2013). HIF1alpha and HIF2alpha independently activate SRC to promote melanoma metastases. *J Clin Invest, 123*(5), 2078-2093. doi:10.1172/jci66715

Harding, Wareham, Bingham, Khaw, Luben, Welch, and Forouhi. (2008). Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: The european prospective investigation of cancer--norfolk prospective study. *Arch Intern Med, 168*(14), 1493-1499. doi:10.1001/archinte.168.14.1493

Hausinger. (2004). FeII/alpha-ketoglutarate-dependent hydroxylases and related enzymes. *Crit Rev Biochem Mol Biol, 39*(1), 21-68. doi:10.1080/10409230490440541

He, Li, Li, Liu, Wang, Tang, Ding, Jia, Chen, Li, Sun, Li, Dai, Song, Zhang, He, and Xu. (2011). TET-mediated formation of 5-carboxylcytosine and its excision by tdg in mammalian DNA. *Science, 333*(6047), 1303-1307. doi:10.1126/science.1210944

Hegg, and Que. (1997). The 2-his-1-carboxylate facial triad--an emerging structural motif in mononuclear non-heme iron(II) enzymes. *Eur J Biochem, 250*(3), 625-629.

Hemilä. (2006). *Do vitamins C and E affect respiratory infections?* (Dissertation), University of Helsinki, Finland.

Hewitson, Granatino, Welford, Mcdonough, and Schofield. (2005). Oxidation by 2-oxoglutarate oxygenases: Non-haem iron systems in catalysis and signalling. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 363*(1829), 807.

Hirsila, Koivunen, Gunzler, Kivirikko, and Myllyharju. (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J Biol Chem, 278*(33), 30772-30780. doi:10.1074/jbc.M304982200

Hoelder, Clarke, and Workman. (2012). Discovery of small molecule cancer drugs: Successes, challenges and opportunities. *Mol Oncol, 6*(2), 155-176. doi:10.1016/j.molonc.2012.02.004

Hong, Lee, Moon, Hwang, Kim, Ko, Kim, Cho, Kang, Kim, Kim, Shin, Jung, Jeong, Cho, Kim, Lee, Kang, Hwang, Noh, Jin, and Lee. (2013). SVCT-2 in breast cancer acts as an indicator for l-ascorbate treatment. *Oncogene, 32*(12), 1508-1517. doi:10.1038/onc.2012.176

Hsu, Peng, Kang, Chen, Yang, Tsai, Chu, Jeng, Chen, Lin, Huang, Lu, Teng, Lin, Lin, Tang, Lee, Hsu, Yu, Hsiao, and Juan. (2012). TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases. *Cell Rep, 2*(3), 568-579. doi:10.1016/j.celrep.2012.08.030

Huang, and Rao. (2014). Connections between TET proteins and aberrant DNA modification in cancer. *Trends Genet, 30*(10), 464-474. doi:10.1016/j.tig.2014.07.005

Huijskens, Wodzig, Walczak, Germeraad, and Bos. (2016). Ascorbic acid serum levels are reduced in patients with hematological malignancies. *Results Immunol, 6*, 8-10. doi:10.1016/j.rinim.2016.01.001

Inamdar, Madhunapantula, and Robertson. (2010). Targeting the mapk pathway in melanoma: Why some approaches succeed and other fail. *Biochemical pharmacology, 80*(5), 624-637. doi:10.1016/j.bcp.2010.04.029

Institute of Medicine. (2000). *Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids*. Washington, DC: The National Academies Press.

Ito, D'alessio, Taranova, Hong, Sowers, and Zhang. (2010). Role of tet proteins in 5mc to 5hmc conversion, es-cell self-renewal and inner cell mass specification. *Nature, 466*(7310), 1129-1133. doi:10.1038/nature09303

Ito, Shen, Dai, Wu, Collins, Swenberg, He, and Zhang. (2011). TET proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science, 333*(6047), 1300-1303. doi:10.1126/science.1210597

Jensen, Ragel, Whang, and Gillespie. (2006). Inhibition of hypoxia inducible factor-1α (HIF-1α) decreases vascular endothelial growth factor (VEGF) secretion and tumor growth in malignant gliomas. *Journal of Neuro-Oncology, 78*(3), 233-247. doi:10.1007/s11060-005-9103-z

Jiang, and Feng. (2006). Hypoxia-inducible factor 1alpha (HIF-1alpha) correlated with tumor growth and apoptosis in ovarian cancer. *Int J Gynecol Cancer, 16 Suppl 1*, 405-412. doi:10.1111/j.1525-1438.2006.00310.x

Jokilehto, and Jaakkola. (2010). The role of HIF prolyl hydroxylases in tumour growth. *Journal of Cellular and Molecular Medicine, 14*(4), 758-770. doi:10.1111/j.1582-4934.2010.01030.x

Kallio, Pongratz, Gradin, Mcguire, and Poellinger. (1997). Activation of hypoxia-inducible factor 1alpha: Posttranscriptional regulation and conformational change by recruitment of the arnt transcription factor. *Proc Natl Acad Sci U S A, 94*(11), 5667-5672.

Kameyama, Sakai, Kondoh, Yonemoto, Nishiyama, Tagawa, Murata, Ohnuma, Quigley, Dorsky, Bucks, and Blanock. (1996). Inhibitory effect of magnesium L-ascorbyl-2-phosphate (vc-pmg) on melanogenesis in vitro and in vivo. *J Am Acad Dermatol, 34*(1), 29-33.

Kimbro, and Simons. (2006). Hypoxia-inducible factor-1 in human breast and prostate cancer. *Endocr Relat Cancer, 13*(3), 739-749. doi:10.1677/erc.1.00728

Knowles, Raval, Harris, and Ratcliffe. (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Res, 63*(8), 1764-1768.

Koh, Yabuuchi, Rao, Huang, Cunniff, Nardone, Laiho, Tahiliani, Sommer, Mostoslavsky, Lahesmaa, Orkin, Rodig, Daley, and Rao. (2011). TET1 and TET2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell, 8*(2), 200-213. doi:10.1016/j.stem.2011.01.008

Koivunen, Hirsila, Remes, Hassinen, Kivirikko, and Myllyharju. (2007). Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: Possible links between cell metabolism and stabilization of hif. *J Biol Chem, 282*(7), 4524-4532. doi:10.1074/jbc.M610415200

Konstantina, Lazaris, Ioannidis, Liossi, and Aroni. (2011). Immunohistochemical expression of VEGF, HIF1-a, and PIGF in malignant melanomas and dysplastic nevi. *Melanoma Res, 21*(5), 389-394. doi:10.1097/CMR.0b013e328347ee33

Krieg, Haas, Brauch, Acker, Flamme, and Plate. (2000). Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von hippel-lindau tumor suppressor gene loss of function. *Oncogene, 19*(48), 5435-5443. doi:10.1038/sj.onc.1203938

Kroemer, and Pouyssegur. (2008). Tumor cell metabolism: Cancer's achilles' heel. *Cancer Cell, 13*(6), 472-482. doi:10.1016/j.ccr.2008.05.005

Kuiper, Dachs, Currie, and Vissers. (2014). Intracellular ascorbate enhances hypoxia-inducible factor (hif)-hydroxylase activity and preferentially suppresses the hif-1 transcriptional response. *Free Radical Biology and Medicine, 69*, 308-317. doi:http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.033

Kuiper, Dachs, Munn, Currie, Robinson, Pearson, and Vissers. (2014b). Increased tumor ascorbate is associated with extended disease-free survival and decreased hypoxia-inducible factor-1 activation in human colorectal cancer. *Front Oncol, 4*, 10. doi:10.3389/fonc.2014.00010

Kuiper, Molenaar, Dachs, Currie, Sykes, and Vissers. (2010). Low ascorbate levels are associated with increased hypoxia-inducible factor-1 activity and an aggressive tumor phenotype in endometrial cancer. *Cancer Res, 70*(14), 5749-5758. doi:10.1158/0008-5472.can-10-0263

Kuiper, and Vissers. (2014c). Ascorbate as a co-factor for Fe- and 2-oxoglutarate dependent dioxygenases: Physiological activity in tumor growth and progression. *Front Oncol, 4*, 359. doi:10.3389/fonc.2014.00359

Kumar, Yu, Edwards, Chen, Kazianis, Brafford, Acs, Herlyn, and Xu. (2007). Mutant V600E BRAF increases hypoxia inducible factor-1alpha expression in melanoma. *Cancer Res, 67*(7), 3177-3184. doi:10.1158/0008-5472.can-06-3312

Kuphal, Winklmeier, Warnecke, and Bosserhoff. (2010). Constitutive HIF-1 activity in malignant melanoma. *Eur J Cancer, 46*(6), 1159-1169. doi:10.1016/j.ejca.2010.01.031

Laukka, Mariani, Ihantola, Cao, Hokkanen, Kaelin, Godley, and Koivunen. (2016). Fumarate and succinate regulate expression of hypoxia-inducible genes via TET enzymes. *J Biol Chem, 291*(8), 4256-4265. doi:10.1074/jbc.M115.688762

Lee, Murphy, and Lian. (2014). Melanoma epigenetics: Novel mechanisms, markers, and medicines. *Lab Invest, 94*(8), 822-838. doi:10.1038/labinvest.2014.87

Levine, Conry-Cantilena, Wang, Welch, Washko, Dhariwal, Park, Lazarev, Graumlich, King, and Cantilena. (1996). Vitamin C pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. *Proc Natl Acad Sci U S A, 93*(8), 3704-3709.

Levine, Padayatty, and Espey. (2011). Vitamin c: A concentration-function approach yields pharmacology and therapeutic discoveries. *Adv Nutr, 2*(2), 78-88. doi:10.3945/an.110.000109

Lian, Xu, Ceol, Wu, Larson, Dresser, Xu, Tan, Hu, Zhan, Lee, Hu, Lian, Kleffel, Yang, Neiswender, Khorasani, Fang, Lezcano, Duncan, Scolyer, Thompson, Kakavand, Houvras, Zon, Mihm, Kaiser, Schatton, Woda, Murphy, and Shi. (2012). Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell, 150*(6), 1135-1146. doi:10.1016/j.cell.2012.07.033

Liang, Johnson, and Jarvis. (2001). Vitamin C transport systems of mammalian cells. *Mol Membr Biol, 18*(1), 87-95.

Lindblad, Tveden-Nyborg, and Lykkesfeldt. (2013). Regulation of vitamin C homeostasis during deficiency. *Nutrients, 5*(8), 2860-2879. doi:10.3390/nu5082860

Linster, and Van Schaftingen. (2007). Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J, 274*(1), 1-22. doi:10.1111/j.1742-4658.2006.05607.x

Liu, Nagao, Kageyama, and Miwa. (2000). Anti-metastatic effect of an autooxidation-resistant and lipophilic ascorbic acid derivative through inhibition of tumor invasion. *Anticancer Res, 20*(1a), 113-118.

Loria, Klag, Caulfield, and Whelton. (2000). Vitamin C status and mortality in U.S. adults. *Am J Clin Nutr, 72*(1), 139-145.

Lu, and Kang. (2010). Hypoxia and hypoxia-inducible factors: Master regulators of metastasis. *Clin Cancer Res, 16*(24), 5928-5935. doi:10.1158/1078-0432.ccr-10-1360

Lykkesfeldt. (2012). Ascorbate and dehydroascorbic acid as biomarkers of oxidative stress: Validity of clinical data depends on vacutainer system used. *Nutr Res, 32*(1), 66-69. doi:10.1016/j.nutres.2011.11.005

Majamaa, Gunzler, Hanauske-Abel, Myllyla, and Kivirikko. (1986). Partial identity of the 2-oxoglutarate and ascorbate binding sites of prolyl 4-hydroxylase. *J Biol Chem, 261*(17), 7819-7823.

Malavolti, Malagoli, Fiorentini, Longo, Farnetani, Ricci, Albertini, Lanzoni, Reggiani, Virgili, Pagliarello, Santini, Fanti, Dika, Sieri, Krogh, Pellacani, and Vinceti. (2013). Association between dietary vitamin C and risk of cutaneous melanoma in a population of northern Italy. *Int J Vitam Nutr Res, 83*(5), 291-298. doi:10.1024/0300-9831/a000171

Malo, and Wilson. (2000). Glucose modulates vitamin c transport in adult human small intestinal brush border membrane vesicles. *J Nutr, 130*(1), 63-69.

Mariani, Vasanthakumar, Madzo, Yesilkanal, Bhagat, Yu, Bhattacharyya, Wenger, Cohn, Nanduri, Verma, Prabhakar, and Godley. (2014). TET1-mediated hydroxymethylation facilitates hypoxic gene induction in neuroblastoma. *Cell Rep, 7*(5), 1343-1352. doi:http://dx.doi.org/10.1016/j.celrep.2014.04.040

Masson, and Ratcliffe. (2014). Hypoxia signaling pathways in cancer metabolism: The importance of co-selecting interconnected physiological pathways. *Cancer & Metabolism, 2*(1), 3. doi:10.1186/2049-3002-2-3

May. (2011). The SLC23 family of ascorbate transporters: Ensuring that you get and keep your daily dose of vitamin c. *Br J Pharmacol, 164*(7), 1793-1801. doi:10.1111/j.1476-5381.2011.01350.x

Mayland, Bennett, and Allan. (2005). Vitamin C deficiency in cancer patients. *Palliat Med, 19*(1), 17-20.

Mccarty. (2013). Expression and/or activity of the SVCT2 ascorbate transporter may be decreased in many aggressive cancers, suggesting potential utility for sodium bicarbonate and dehydroascorbic acid in cancer therapy. *Med Hypotheses, 81*(4), 664-670. doi:10.1016/j.mehy.2013.07.023

McCormick. (1959). Cancer: A collagen disease, secondary to a nutritional deficiency. *Arch Pediatr, 76*(4), 166-171.

McDonough, Loenarz, Chowdhury, Clifton, and Schofield. (2010). Structural studies on human 2-oxoglutarate dependent oxygenases. *Curr Opin Struct Biol, 20*(6), 659-672. doi:10.1016/j.sbi.2010.08.006

McKeown. (2014). Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *Br J Radiol, 87*(1035), 20130676. doi:10.1259/bjr.20130676

Mendiratta, Qu, and May. (1998). Erythrocyte ascorbate recycling: Antioxidant effects in blood. *Free Radical Biology and Medicine, 24*(5), 789-797. doi:https://doi.org/10.1016/S0891-5849(97)00351-1

Metzen, Berchner-Pfannschmidt, Stengel, Marxsen, Stolze, Klinger, Huang, Wotzlaw, Hellwig-Burgel, Jelkmann, Acker, and Fandrey. (2003). Intracellular localisation of human HIF-1 alpha hydroxylases: Implications for oxygen sensing. *J Cell Sci, 116*(Pt 7), 1319-1326.

Michels, and Frei. (2013). Myths, artifacts, and fatal flaws: Identifying limitations and opportunities in vitamin C research. *Nutrients, 5*(12), 5161-5192. doi:10.3390/nu5125161

Mikirova, Casciari, Riordan, and Hunninghake. (2013). Clinical experience with intravenous administration of ascorbic acid: Achievable levels in blood for different states of inflammation and disease in cancer patients. *J Transl Med, 11*, 191. doi:10.1186/1479-5876-11-191

Miles, Fischer, Joshi, and Niles. (2015). Ascorbic acid and ascorbate-2-phosphate decrease HIF activity and malignant properties of human melanoma cells. *BMC Cancer, 15*(1), 867. doi:10.1186/s12885-015-1878-5

Mills, Joshi, and Niles. (2009). Expression and function of hypoxia inducible factor-1 alpha in human melanoma under non-hypoxic conditions. *Mol Cancer, 8*, 104. doi:10.1186/1476-4598-8-104

Minor, Court, Young, and Wang. (2013). Ascorbate induces ten-eleven translocation (TET) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. *J Biol Chem, 288*(19), 13669-13674. doi:10.1074/jbc.C113.464800

Monfort, and Wutz. (2013). Breathing-in epigenetic change with vitamin C. *EMBO Rep, 14*(4), 337-346. doi:10.1038/embor.2013.29

Munoz-Montesino, Roa, Pena, Gonzalez, Sotomayor, Inostroza, Munoz, Gonzalez, Maldonado, Soliz, Reyes, Vera, and Rivas. (2014). Mitochondrial ascorbic acid transport is mediated by a low-affinity form of the sodium-coupled ascorbic acid transporter-2. *Free Radic Biol Med, 70*, 241-254. doi:10.1016/j.freeradbiomed.2014.02.021

Nagao, Nakayama, Etoh, Saiki, and Miwa. (2000). Tumor invasion is inhibited by phosphorylated ascorbate via enrichment of intracellular vitamin C and decreasing of oxidative stress. *J Cancer Res Clin Oncol, 126*(9), 511-518.

Nam, Ko, Jung, Yoon, Kim, Choi, Park, Chang, Kim, and Lee. (2011). A hypoxia-dependent upregulation of hypoxia-inducible factor-1 by nuclear factor-kappa b promotes gastric tumour growth and angiogenesis. *Br J Cancer, 104*(1), 166-174. doi:10.1038/sj.bjc.6606020

Ogiri, Sun, Hayami, Fujimura, Yamamoto, Yaita, and Kojo. (2002). Very low vitamin C activity of orally administered L-dehydroascorbic acid. *Journal of Agricultural and Food Chemistry, 50*(1), 227-229. doi:10.1021/jf010910f

Ohno, Ohno, Suzuki, Soma, and Inoue. (2009). High-dose vitamin c (ascorbic acid) therapy in the treatment of patients with advanced cancer. *Anticancer Research, 29*(3), 809-815.

Ooi, O'donnell, and Bestor. (2009). Mammalian cytosine methylation at a glance. *J Cell Sci, 122*(Pt 16), 2787-2791. doi:10.1242/jcs.015123

Otsuka, Kurata, and Arakawa. (1986). Antiscorbutic effect of dehydro-L-ascorbic acid in vitamin C-deficient guinea pigs. *Journal of Nutritional Science and Vitaminology, 32*(2), 183-190. doi:10.3177/jnsv.32.183

Ozer, and Bruick. (2007). Non-heme dioxygenases: Cellular sensors and regulators jelly rolled into one? *Nat Chem Biol, 3*(3), 144-153. doi:10.1038/nchembio863

Postovit, Seftor, Seftor, and Hendrix. (2006). Influence of the microenvironment on melanoma cell fate determination and phenotype. *Cancer Res, 66*(16), 7833-7836. doi:10.1158/0008-5472.can-06-0731

Prasad, Allard, and Green. (2008). Confirming the phylogeny of mammals by use of large comparative sequence data sets. *Mol Biol Evol, 25*(9), 1795-1808. doi:10.1093/molbev/msn104

Raimundo, Baysal, and Shadel. (2011). Revisiting the TCA cycle: Signaling to tumor formation. *Trends Mol Med, 17*(11), 641-649. doi:10.1016/j.molmed.2011.06.001

Rasmussen, and Helin. (2016). Role of TET enzymes in DNA methylation, development, and cancer. *Genes & Development, 30*(7), 733-750. doi:10.1101/gad.276568.115

Reidling, Subramanian, Dahhan, Sadat, and Said. (2008). Mechanisms and regulation of vitamin C uptake: Studies of the hSVCT systems in human liver epithelial cells. *Am J Physiol Gastrointest Liver Physiol, 295*(6), G1217-1227. doi:10.1152/ajpgi.90399.2008

Rumsey, Kwon, Xu, Burant, Simpson, and Levine. (1997). Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J Biol Chem, 272*(30), 18982-18989.

Sasaki, Majamaa, and Uitto. (1987). Reduction of collagen production in keloid fibroblast cultures by ethyl-3,4-dihydroxybenzoate. Inhibition of prolyl hydroxylase activity as a mechanism of action. *J Biol Chem, 262*(19), 9397-9403.

Satyamoorthy, and Herlyn. (2002). Cellular and molecular biology of human melanoma. *Cancer Biol Ther, 1*(1), 14-17.

Schleich, Rodemeister, Venturelli, Sinnberg, Garbe, and Busch. (2013). Decreased plasma ascorbate levels in stage IV melanoma patients. *Metabolism and Nutrition in Oncology*(EFirst).

Schofield, and Ratcliffe. (2005). Signalling hypoxia by HIF hydroxylases. *Biochemical and Biophysical Research Communications, 338*(1), 617-626. doi:http://dx.doi.org/10.1016/j.bbrc.2005.08.111

Semenza. (2002). Involvement of hypoxia-inducible factor 1 in human cancer. *Intern Med, 41*(2), 79-83.

Semenza. (2007). Evaluation of HIF-1 inhibitors as anticancer agents. *Drug Discov Today, 12*(19-20), 853-859. doi:10.1016/j.drudis.2007.08.006

Semenza. (2010). Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene, 29*(5), 625-634. doi:10.1038/onc.2009.441

Serrone, Zeuli, Sega, and Cognetti. (2000). Dacarbazine-based chemotherapy for metastatic melanoma: Thirty-year experience overview. *J Exp Clin Cancer Res, 19*(1), 21-34.

Siegel, Miller, and Jemal. (2016). Cancer statistics, 2016. *CA Cancer J Clin, 66*(1), 7-30. doi:10.3322/caac.21332

Siegel, Miller, and Jemal. (2017). Cancer statistics, 2017. *CA Cancer J Clin, 67*(1), 7-30. doi:10.3322/caac.21387

Skrzydlewska, Sulkowski, Koda, Zalewski, Kanczuga-Koda, and Sulkowska. (2005). Lipid peroxidation and antioxidant status in colorectal cancer. *World J Gastroenterol, 11*(3), 403-406.

Slipicevic, and Herlyn. (2012). Narrowing the knowledge gaps for melanoma. *Ups J Med Sci, 117*(2), 237-243. doi:10.3109/03009734.2012.658977

Song, Poliseno, Song, Ala, Webster, Ng, Beringer, Brikbak, Yuan, Cantley, Richardson, and Pandolfi. (2013). Microrna-antagonism regulates breast cancer stemness and metastasis via TET-family-dependent chromatin remodeling. *Cell, 154*(2), 311-324. doi:10.1016/j.cell.2013.06.026

Spielholz, Golde, Houghton, Nualart, and Vera. (1997). Increased facilitated transport of dehydroascorbic acid without changes in sodium-dependent ascorbate transport in human melanoma cells. *Cancer Res, 57*(12), 2529-2537.

Stephenson, Levin, Spector, and Lis. (2013). Phase I clinical trial to evaluate the safety, tolerability, and pharmacokinetics of high-dose intravenous ascorbic acid in patients with advanced cancer. *Cancer Chemother Pharmacol, 72*(1), 139-146. doi:10.1007/s00280-013-2179-9

Strickland, Pal, Elmets, and Afaq. (2015). Targeting drivers of melanoma with synthetic small molecules and phytochemicals. *Cancer Letters, 359*(1), 20-35. doi:http://dx.doi.org/10.1016/j.canlet.2015.01.016

Suzuki, and Bird. (2008). DNA methylation landscapes: Provocative insights from epigenomics. *Nat Rev Genet, 9*(6), 465-476. doi:10.1038/nrg2341

Tahiliani, Koh, Shen, Pastor, Bandukwala, Brudno, Agarwal, Iyer, Liu, Aravind, and Rao. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by mll partner TET1. *Science, 324*(5929), 930-935. doi:10.1126/science.1170116

Takeda, Aguila, Parikh, Li, Lamothe, Duan, Takeda, Lee, and Fong. (2008). Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. *Blood, 111*(6), 3229-3235. doi:10.1182/blood-2007-09-114561

Thienpont, Steinbacher, Zhao, D'anna, Kuchnio, Ploumakis, Ghesquiere, Van Dyck, Boeckx, Schoonjans, Hermans, Amant, Kristensen, Koh, Mazzone, Coleman, Carell, Carmeliet, and Lambrechts. (2016). Tumour hypoxia causes DNA hypermethylation by reducing TET activity. *Nature, 537*(7618), 63-68. doi:10.1038/nature19081

Todhunter, Mc, and Ehmke. (1950). Utilization of dehydroascorbic acid by human subjects. *J Nutr, 42*(2), 297-308.

Tsao, Atkins, and Sober. (2004). Management of cutaneous melanoma. *N Engl J Med, 351*(10), 998-1012. doi:10.1056/NEJMra041245

Tsukaguchi, Tokui, Mackenzie, Berger, Chen, Wang, Brubaker, and Hediger. (1999). A family of mammalian Na+-dependent L-ascorbic acid transporters. *Nature, 399*(6731), 70-75. doi:10.1038/19986

Uchiyama, Uhara, Uchiyama, Ogawa, Takazawa, Ashida, Koga, Hayashi, Kiniwa, and Okuyama. (2014). 5-hydroxymethylcytosine as a useful marker to differentiate between malignant melanomas and benign melanocytic nevi. *J Dermatol Sci, 73*(2), 161-163. doi:10.1016/j.jdermsci.2013.09.008

Valegard, Van Scheltinga, Lloyd, Hara, Ramaswamy, Perrakis, Thompson, Lee, Baldwin, Schofield, Hajdu, and Andersson. (1998). Structure of a cephalosporin synthase. *Nature, 394*(6695), 805-809. doi:10.1038/29575

Valencak, Kittler, Schmid, Schreiber, Raderer, Gonzalez-Inchaurraga, Birner, and Pehamberger. (2009). Prognostic relevance of hypoxia inducible factor-1alpha expression in patients with melanoma. *Clin Exp Dermatol, 34*(8), e962-964. doi:10.1111/j.1365-2230.2009.03706.x

Van Laarhoven, Schilderman, Bleijenberg, Donders, Vissers, Verhagen, and Prins. (2011). Coping, quality of life, depression, and hopelessness in cancer patients in a curative and palliative, end-of-life care setting. *Cancer Nurs, 34*(4), 302-314. doi:10.1097/NCC.0b013e3181f9a040

Vinci, Box, and Eccles. (2015). Three-dimensional (3D) tumor spheroid invasion assay. *J Vis Exp*(99), e52686. doi:10.3791/52686

Vislisel, Schafer, and Buettner. (2007). A simple and sensitive assay for ascorbate using a plate reader. *Anal Biochem, 365*(1), 31-39. doi:10.1016/j.ab.2007.03.002

Vissers, Gunningham, Morrison, Dachs, and Currie. (2007). Modulation of hypoxia-inducible factor-1 alpha in cultured primary cells by intracellular ascorbate. *Free Radic Biol Med, 42*(6), 765-772. doi:10.1016/j.freeradbiomed.2006.11.023

Wagner, Markosian, Ajili, Dolan, Kim, Alexandrescu, Dasanu, Minev, Koropatnick, Marincola, and Riordan. (2014). Intravenous ascorbic acid as an adjuvant to interleukin-2 immunotherapy. *J Transl Med, 12*, 127. doi:10.1186/1479-5876-12-127

Wang, Buss, Chen, Ponka, and Pantopoulos. (2002). The prolyl 4-hydroxylase inhibitor ethyl-3,4-dihydroxybenzoate generates effective iron deficiency in cultured cells. *FEBS Lett, 529*(2-3), 309-312.

Wilson. (2002). The physiological role of dehydroascorbic acid. *FEBS Letters, 527*(1–3), 5-9. doi:http://dx.doi.org/10.1016/S0014-5793(02)03167-8

Wohlrab, Phillips, and Dachs. (2017). Vitamin C transporters in cancer: Current understanding and gaps in knowledge. *Frontiers in Oncology, 7*(74). doi:10.3389/fonc.2017.00074

Yamamoto, Yamashita, Fujisawa, Kokura, and Yoshikawa. (1998). Oxidative stress in patients with hepatitis, cirrhosis, and hepatoma evaluated by plasma antioxidants. *Biochem Biophys Res Commun, 247*(1), 166-170. doi:10.1006/bbrc.1998.8752

Yin, Mao, Zhao, Chong, Yang, Zhao, Zhang, Huang, Gao, Li, Jiao, Li, Liu, Wu, Gu, Yang, Xu, and Wang. (2013). Ascorbic acid enhances TET-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *J Am Chem Soc, 135*(28), 10396-10403. doi:10.1021/ja4028346

Zbytek, Peacock, Seagroves, and Slominski. (2013). Putative role of HIF transcriptional activity in melanocytes and melanoma biology. *Dermatoendocrinol, 5*(2), 239-251. doi:10.4161/derm.22678

Zhang, Ren, Stammers, Baldwin, Harlos, and Schofield. (2000). Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. *Nat Struct Biol, 7*(2), 127-133. doi:10.1038/72398

Zhong, De Marzo, Laughner, Lim, Hilton, Zagzag, Buechler, Isaacs, Semenza, and Simons. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res, 59*(22), 5830-5835.

Zimna, and Kurpisz. (2015). Hypoxia-inducible factor-1 in physiological and pathophysiological angiogenesis: Applications and therapies. *Biomed Res Int, 2015*, 549412. doi:10.1155/2015/549412

APPENDIX A:
LETTER TO IRB

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

May 16, 2017

Adam P. Fischer
Department of Biomedical Sciences
JCESOM, Marshall University

Dear Mr. Fischer:

This letter is in response to the submitted dissertation abstract entitled *"Employing the enzyme cofactor function of ascorbic acid to affect oncogenic pathways in human melanoma: modulating Hypoxia Inducible Factor-1 and the DNA demethylation pathways to reduce malignant potential."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B:
LIST OF ABBREVIATIONS

2-OG:	2-oxoglutarate
5caC:	5-carboxylcytosine
5fC:	5-formylcytosine
5hmC:	5-hydroxymethylcytosine
5mC:	5-methylcytosine
A2P:	Ascorbate 2-phosphate
AA:	Ascorbic acid
AML:	Acute myeloid leukemia
BER:	Base excision repair
BNIP3:	BCL2 Interacting Protein 3
BRAF:	Murine sarcoma viral (v-raf) oncogene homolog B1
BSA:	Bovine Serum albumin
$CaCl_2$:	Calcium chloride
CBP:	CREB binding protein
Ch:	Choline
ChCl:	Choline chloride
Clt:	Control
CMML:	Chronic myelomonocytic leukemia
$CoCl_2$:	Cobalt chloride
CO_2:	Carbon dioxide
Cont:	Control
CpG:	Cytosine-phosphate-guanine
C-TAD:	C-terminal transactivating domain

DHA: Dehydroascorbic acid

DKG: Diketogulonic acid

DNA: Deoxyribonucleic acid

DNMT: DNA methyltransferases

DSBH: Double-stranded β-helix

ECL: Enhanced chemiluminescence

EDHB: Ethyl 3, 4-dihydroxbenozoate

EDTA: Ethylenediaminetetraacetic acid

EGLN1: Prolyl hydroxylase 2

EGLN2: Prolyl hydroxylase 1

EGLN3: Prolyl hydroxylase 3

EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FBS: Fetal Bovine Serum

Fe: Iron

FH: Fumarate hydratase

Fig: Figure

FIH: Factor Inhibiting HIF

Glu: Glucose

GLUT: Glucose transporter

GULO: L-gulonolactone oxidase

HEMnLP: Human Epidermal Melanocytes, neonatal, lightly pigmented

HMGS: Human melanocyte growth supplement

HIF: Hypoxia Inducible Factor

H_2O_2: Hydrogen peroxide

HRE: Hypoxia response element

Hsp: Heat shock protein

IFN: Interferon

IL: Interleukin

IRB: Institutional review board

I.V.: Intravenous

KCl: Potassium chloride

Km: The Michaelis constant

MAPK: Mitogen-activated protein kinase

MET: Metastatic phase

$MgCl_2$: Magnesium chloride

NAC: N-acetyl cysteine

NaCl: Sodium Chloride

NADPH: Nicotinamide adenine dinucleotide

ND: Not detectable

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

NRAS: Neuroblastoma RAS viral oncogene homolog

ns: Not significant

O_2: Molecular oxygen

ODD: Oxygen dependent death domain

OPDA: o-Phenylenediamine dihydrochloride

PAGE: Polyacrylamide gels

PBS: Phosphate buffer saline

PBS-T: Phosphate buffer saline-Tween 20

PCR: Polymerase chain reaction

PHD: Prolyl hydroxylase

PMT:	Photo-multiplier tube
pVHL:	von Hippel-Lindau tumor suppressor protein
qPCR:	Quantitative PCR
RGP:	Radial growth phase
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RPMI:	Roswell park memorial institute medium
RT:	Room temperature
RT-PCR:	Reverse transcriptase-PCR
SDH:	Succinate dehydrogenase
SDS:	Sodium dodecyl sulfate
SEM:	Standard error of mean
siRNA:	Small interfering RNA
SSC:	Saline sodium citrate
SVCT:	Sodium dependent vitamin C transporter
TCA:	Tricarboxylic acid
TDG:	Thymine-DNA glycosylase
TEMPOL:	4-Hydroxy-2,2,6,6-tetramethyl-piperidinooxy
TET:	Ten-eleven translocation enzyme
Tris:	Tris(hydroxymethyl)aminomethane
UHRF1:	Ubiquitin-like PHD and RING finger domain-containing protein 1
UV:	Ultraviolet
VEGF:	Vascular endothelial growth factor
VGP:	Vertical growth phase
WGA:	Wheat germ agglutinin

β-gal: β-galactosidase

CURRICULUM VITAE

Adam P. Fischer, PhD

Current Position:

Department of Biomedical Sciences
Joan C Edwards School of Medicine
Marshall University

Contact Information:
JCESOM, Marshall University
Department of Biomedical Sciences
One John Marshall Drive
Huntington, WV 25755
Email: fischer35@marshall.edu
Phone: (304) 871-6050

Education:

2007	Gilmer County High School
2011	Glenville State College, BS, Biology, BA, Chemistry
2013	Certification in Radiation Safety, Marshall University
2014	Certification in Cell Culture Basics, Gibco
July 2017	Marshall University, PhD, Biomedical Sciences **Advisors:** Dr. Sarah L. Miles, Dr. W. Elaine Hardman

Professional/Honorary Memberships:

2014-pres	Member of the West Virginia Academy of Sciences
2014-pres	Member of The Society for Melanoma Research
2013-pres	Member of the Golden Key International Honour Society
2010-pres	Member of the Alpha Iota chapter of Chi Beta Phi, National Scientific Honorary
2006	National Honor Society
2005	National Junior Honor Society
2004	National Honor Role
2002	United States Achievement Academy

Awards/Honors:

2016	American Institute for Cancer Research (AICR) conference scholarship
2016	1st Place winner in Cancer poster presentation at Appalachian Regional Cell Conference
2015	1st Place winner in General Biology graduate oral presentation, 90th WVAS conference
2015	1st Place winner for Basic Science poster, 27th Research Day, Marshall University
2013-2014	Student member of curriculum committee, Biomedical Sciences, Marshall University
2013	Best Academic Performance by a first-year research student, Marshall University
2011	Graduated with Honors (Cum Laude), Glenville State College

2011 Excellence in Biology Award, Glenville State College
2011 1st Place winner for poster presentation at Pioneer Showcase, Glenville State College

Publications:

Fischer, A.P. and S.L. Miles, *Silencing HIF-1α induces TET2 expression and augments ascorbic acid induced 5-hydroxymethylation of DNA in human metastatic melanoma cells.* Biochemical and Biophysical Research Communications, 2017. **490**(2): p. 176-181.

Fischer, A.P. and S.L. Miles, *Ascorbic acid, but not dehydroascorbic acid increases intracellular vitamin C content to decrease Hypoxia Inducible Factor -1 alpha activity and reduce malignant potential in human melanoma.* Biomedicine & Pharmacotherapy, 2017. **86**: p. 502-513.

Miles, S.L., **Fischer, A.P.**, Joshi, S.J., Niles, R.M., *Ascorbic acid and ascorbate-2-phosphate decrease HIF activity and malignant properties of human melanoma cells.* BMC Cancer, 2015. **15**(1): p. 867.

Publications in Preparation:

Fischer, A.P., Herdman, M, and Miles, S.L., *Cofactor function of ascorbic acid as a viable anti-cancer therapy* (working title; Review Article)

Reviewed manuscripts for Journal of Biomedical Sciences

Teaching Experience:

Spring 2016 & 2017 Teaching Practicum Course, Marshall University
Fall 2011 Adjunct Professor, Department of Science and Mathematics, Glenville State
Spring 2011 Teaching Assistant, Department of Science and Mathematics, Glenville State

Experimental Techniques:

1. Cell Culture
2. Transient transfections
3. siRNA knockdown
4. Immunoblotting
5. DNA dot blots
6. Isolation of mitochondria
7. Isolation of DNA/RNA
8. RT-PCR and qPCR

9. Agarose gel electrophoresis
10. Multiple absorbance/fluorescence/luminescence assays
11. Invasion assays
12. Tumor spheroid invasion assays
13. Anchorage independent growth assays (soft agar)
14. Proficient in Microsoft Office

National meetings:

1. **Adam P. Fischer** and Sarah L. Miles. 2016. Ascorbic acid, and not dehydroascorbic acid, reduces expression of HIF-1 alpha in human melanoma; Implications for clinical use. Poster Presentation at American Institute for Cancer Research (AICR), Bethesda, MD.

Regional/Local meetings:

1. **Adam P. Fischer** and Sarah L. Miles. 2016. Use of ascorbic acid or dehydroascorbic acid as a potential adjuvant therapy to reduce HIF-1α in human melanoma. Poster Presentation (**Winner**) at Appalachian Regional Cell Conference, Charleston, WV.
2. **Adam P. Fischer** and Sarah L. Miles. 2016. Comparison of the use of ascorbic acid vs. dehydroascorbic acid to reduce HIF-1α stabilization in human melanoma. Poster Presentation, 28th Annual Research Day, March 2016, Marshall University Medical Center, Huntington, WV.
3. **Adam P. Fischer** and Sarah L. Miles. 2015. Vitamin C…not just for sailors: Examining ascorbate-mediated down regulation of hypoxia-inducible factor-1 in metastatic melanoma. Selected for **Oral Presentation** (**Winner**) at West Virginia Academy of Sciences, April 2015, West Liberty, WV.
4. **Adam P. Fischer** and Sarah L. Miles. 2015. Normoxic accumulation and activity of HIF-1 is associated with ascorbic acid transporter expression and localization in human melanoma. Poster Presentation (**Winner**), 27th Annual Research Day, March 2015, Marshall Medical Center, Huntington, WV.
5. **Adam P. Fischer**, Richard M. Niles, and Sarah L. Miles. 2014. Inhibition of human melanoma hypoxia-inducible factor-1 stability and activity by ascorbic acid and derivatives. Poster Presentation, Appalachian Regional Cell Conference, November 2014, Huntington, WV.
6. **Adam P. Fischer**, Richard M. Niles, and Sarah L. Miles. 2014. Ascorbate mediated reduction of HIF-1α in WM9 melanoma cells. Poster Presentation, August 2014, Marshall University, Huntington, WV.
7. **Adam P. Fischer** and Emine C. Koc. 2014. Tetracycline antibiotics and tamoxifen induce apoptosis in MCF7 by disrupting mitochondrial translation. Selected for **Oral Presentation** at the 26th Annual Research Day, March 2014, Marshall Medical Center, Huntington, WV.
8. **Adam P. Fischer**, Min-Joon Han, Hasan Koc, and Emine C. Koc. 2013. DAP3 and MRPS30-Mediated Induction of Apoptosis in Breast Cancer. Poster Presentation, Appalachian Regional Cell Conference, November 2013, Charleston, WV.

9. **Adam P. Fischer**, Westley Mullins, Joe Evans, and Gary Z. Morris. 2011. Zymurgy…Not Just for Drunks Using Beer to Stimulate Interest in the Science Laboratory. Selected for **Oral Presentation** at West Virginia Academy of Sciences, Montgomery, WV. Presented at 1st Annual Pioneer Showcase, Glenville, WV.

References:

1. Sarah L. Miles, PhD, Research Assistant Professor, Department of Biomedical Sciences, Marshall University
   Phone: (304) 633-2371 Email: kittlaus1@marshall.edu

2. W. Elaine Hardman, PhD, Professor, Department of Biomedical Sciences, Marshall University
   Phone: (304) 696-7339 Email: hardmanw@marshall.edu

3. Gary Z. Morris, PhD, Chair, Department of Science and Mathematics, Glenville State College
   Phone: (304) 462-6305 Email: Gary.Morris@glenville.edu

4. Beverly Delidow, PhD, Associate Professor, Department of Biomedical Sciences, Marshall University
   Phone: (304) 696-7266 Email: delidow@marshall.edu

5. Richard M. Niles, PhD, Emeritus Professor, Chair, Department of Biochemistry and Microbiology, Marshall University
   Email: niles@marshall.edu