TARGETING THE CANCER METABOLIC PHENOTYPE USING HIGH DOSE VITAMIN B_1 THERAPY

by

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(Under the Direction of Dr. Jason Zastre)

ABSTRACT

The dichotomous effect of thiamine supplementation on cancer cell growth is characterized by growth stimulation at low doses and growth suppression at high doses. Unfortunately, how thiamine affects cancer cell proliferation is currently unknown. Recent focuses on metabolic targets for cancer therapy have exploited the altered regulation of the thiamine-dependent enzyme pyruvate dehydrogenase (PDH). Cancer cells inactivate PDH through phosphorylation by overexpression of pyruvate dehydrogenase kinases (PDKs). Inhibition of PDKs exhibits a growth suppressive effect in many cancers. Recently it has been shown that the thiamine co-enzyme, thiamine pyrophosphate, can regulate the phosphorylation of PDH. Therefore, we hypothesize that high dose thiamine can normalize glycolysis in cancer cells leading to a cellular apoptosis. We have determined that high dose vitamin B_1 reduces cell proliferation in cancer cell lines by a mechanism involving a reduction in PDH phosphorylation. Additionally we have established that thiamine homeostasis is altered in breast cancer cells and in hypoxic microenvironments. Together these findings suggest that using high dose thiamine may provide an important targeting advantage directed towards altering the cancer metabolic phenotype.

INDEX WORDS: Thiamine, TPK1, Pyruvate Dehydrogenase, Hypoxia, Cell Metabolism, Transport, Chemotherapy

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DEDICATION

This dissertation is dedicated to my teacher and friend, Nancy Gordeuk. You instilled in me a love of science that continues to this day. Your positive attitude throughout your personal battle with breast cancer has been an inspiration to me. Thank you for showing me how fun science is!

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ABBREVIATIONS

α-KG: α-Ketoglutarate

α-KGDH: α-Ketoglutarate Dehydrogenase

AKT: Serine-threonine-specific protein kinase

AThTP: Adenosine Thiamine Triphosphate

ATP: Adenosine triphosphate

DCA: Dichloroacetate

DHF: Dihydrofolate

DHFR: Dihydrofolate Reductase

ECM: Extra-cellular Matrix

ER: Estrogen receptor

F-6-P: Fructose-6-phosphate

FAD: Flavin Adenine Dinucleotide

FMN: Flavin Mononucleotide

FR: Folate Receptor

G-3-P: Glyceraldehyde-3-phosphate

G-6-P: Glucose-6-phosphate

GLUT: Glucose Trasporter

GTPase: Guanosine triphosphatase

HF: High fat

HIF: Hypoxia inducible factor

HK2: Hexokinase 2

HMECs: Primary human mammilary epithelial cells

HT: High thiamine

LDHA: Lactate Dehydrogenase A

LT: Low thiamine

M2-PK: M2 isoform of Pyruvate Kinase

MAPK: Mitogen-Activated Protein Kinases

MCT: Proton-coupled Monocarboxylate Transporters

mtDNA: mitochondrial DNA

mTOR: Mechanistic Target of Rapamycin

MTX: Methotrexate

NAD: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide (reduced)

NADP: Nicotinamide Adenine Dinucleotide Phosphate

NF: Normal fat

NT: Normal thimaine

OAA: Oxaloacetate

OXPHOS: Oxidative Phosphorylation

PAGE: Polyacrylamide gel electrophoresis

PDC: Pyruvate Dehydrogenase Complex

PDH: Pyruvate Dehydrogenase

PDK: Pyruvate Dehydrogenase Kinase

PDP: Pyruvate Dehydrogenase Phosphatases

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PK: Pyruvate Kinase

PPP: Pentose Phosphate Pathway

qRT-PCR: Reverse transcriptase polymerase chain reaction

R-5-P: Ribose-5-phosphate

Ras: Rat sarcoma

RDA: Recommended Daily Allowance

RDI: Recommended Daily Intake

RFC1: Reduced Folate Carrier 1

ROS: Reactive Oxygen Species

SD: Standard deviation

SLC: Solute Carrier

SMVT: Sodium dependent Multivitamin Transporter

Src: Proto-oncogene tyrosine-protein kinase

TBS-T: Tris buffered saline-tween 20

TCA: Tricarboxylic Acid Cycle

TDP: Thiamine Diphosphate

THF: Tetrahydrofolate

TKT: Transketolase

TKTL: Transketolase-like protein

TMP: Thiamine Monophosphate

TMPase: Thiamine Monophosphatase

TPK1: Thiamine Pyrophosphokinase 1

TPP: Thiamine Pyrophosphate

TPPase: Thiamine Pyrophosphatase

TRMA: Thiamine-responsive Megaloblastic Anemia

TTP: Thiamine Triphosphate

VEGF: Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. MALIGNANT ALTERATIONS

Malignancy is defined by atypical cellular growth that results in the invasion of surrounding tissues (Bertram 2000). These diseases are characterized by alterations in growth signals, limitless replication, altered metabolism, sustained angiogenesis, and activation of metastasis (Hanahan and Weinberg 2000, Cantor and Sabatini 2012, Hainaut and Plymoth 2013). The change from a normal cell to malignant cell involves multiple cellular alterations in what is now termed malignant progression (Hanahan and Weinberg 2011, Hainaut and Plymoth 2013). This progression is initially asymptomatic but eventually results in a myriad of effects both locally and systemically (Holland-Frei 2003). Although it is difficult to establish that any single event results in the formation of a malignant cell, it is commonly accepted that a number of effectors induce this transformation (Tolar and Neglia 2003). It is currently thought that 90-95% of malignancies are the result of environmental factors while 5-10% are a result of genetic factors (Anand, Kunnumakkara *et al.* 2008). Whatever the cause of its initiation, malignant progression is characterized not by the cause of transformation but by the changes that are induced to support newly immortalized cells. There are three key requirements for rapidly dividing cells; maintenance of energy through ATP generation, biosynthesis of macromolecules, and maintenance of redox status (Bertram 2000, Cairns, Harris *et al.* 2011). Each of these are supported by alterations in the expression of tumor suppressor genes, oncogenes, and through the

transcriptional regulation of genes that augment cell growth and survival (Cairns, Harris *et al*. 2011).

1.1.1. Glycolysis

Within normal cells the constant supply of energy is produced through an organized series of catabolic reactions that are necessary to maintain cellular function. ATP is the most common form of chemical energy and is generated as a product of three main metabolic pathways; glycolysis, the tricarboxylic acid cycle (TCA), and oxidative phosphorylation (OXPHOS) (Michael and Schomburg 2013). Within all cells, byproducts of these glycolytic reactions can also be shuttled into pathways that serve specific roles in maintaining cellular function. However, rapid proliferation requires metabolic reprogramming to meet the bioenergetic and biomass requirements of rapidly proliferating cells.

The metabolic changes that occur within malignant cells were first identified over 80 years ago by Otto Warburg, who observed that ascites tumor cells consume glucose at a much higher rate when compared to normal cells (Warburg 1923). Warburg noted that malignant cells secrete most of the carbons obtained from glycolytic reactions as lactate instead of utilizing these carbons within oxidative metabolic reactions (Warburg 1923). Although rapid metabolism of glucose seems counterproductive, given the high-energy yield through OXPHOS, cancer cells preferentially utilize this pathway to support both bioenergetics and biosynthesis of macromolecules. These series of observations, now termed the Warburg effect, were the first to indicate that cancer cells primarily utilize glycolysis for energy production. Although Warburg believed that this phenomenon was a result of impaired or defective oxidative metabolism in

cancer cells, research has shown that many cancer types have fully functioning mitochondria (Moreno-Sanchez, Rodriguez-Enriquez *et al.* 2007).

Glycolysis utilizes the most abundant extracellular nutrient, glucose, for the production of energy. The rate of ATP produced through glycolysis alone exceeds that of OXPHOS if the flux of carbons into glycolysis is high enough (Guppy, Greiner *et al.* 1993). In cancer cells, the rate of glucose metabolism exceeds that of oxidative phosphorylation and therefore allows for rapid generation of ATP using this pathway exclusively (Pfeiffer, Schuster *et al.* 2001). However, a key regulatory and rate-limiting step in glycolysis is the ATP-independent transport of glucose across the cell membrane (Medina and Owen 2002, Macheda, Rogers *et al.* 2005). The facilitative uptake of glucose takes place through members of the Solute carrier 2A (SLC2A) family of genes also known as glucose transporters (GLUTs).

Smith *et al.* reviewed 30 studies that examined changes in SLC2A expression across a variety of cancers and found that GLUT1 (SLC2A1) is the most frequently over-expressed glucose transporter in malignant cells. GLUT1 overexpression is observed in a number of malignancies including colorectal, laryngeal, thyroid, prostate, pancreatic, and lung cancers (Sakashita, Aoyama *et al.* 2001, Luo, Zhou *et al.* 2010, Basturk, Singh *et al.* 2011, Jun, Jang *et al.* 2011, Grabellus, Nagarajah *et al.* 2012, Reinicke, Sotomayor *et al.* 2012). In addition to these alterations, the level of GLUT1 is indicative of proliferative index and may be beneficial as a prognostic factor in lung cancer (Smith 1999). Although GLUT 1 is the most widely studied, an increase in the expression of GLUT3 is also observed in many cancers including small intestine, kidney, and breast (Medina and Owen 2002). The increase in the expression of glucose transporters supports a shift in metabolism and plays an important role in the adaptive response to physiological stress. Alterations in the expression and function of glucose transporters are

driven by the activation of oncogenes such as c-myc, ras, and src (Rodriguez-Enriquez, Marin-Hernandez *et al.* 2009). Overexpression of GLUTs is also driven by transcription factors such as Hif1α (Marin-Hernandez, Rodriguez-Enriquez *et al.* 2006). Multiple groups have shown a hypoxia related increase in the mRNA expression of GLUT1 and GLUT3 in many malignant cells including thyroid lesions and hepatocellular carcinomas (Gleadle and Ratcliffe 1997, Chen, Pore *et al.* 2001, Jozwiak, Krzeslak *et al.* 2012).

Rapid glycolysis is also supported by alterations in the expression and function of glycolytic enzymes. Once glucose is transported into the cell it is phosphorylated to glucose-6-phosphate (G-6-P) by the enzyme Hexokinase-2 (HK2). An increase in HK2 activity supports malignant metabolism and is required for the initiation of tumors in many murine cancer models (Mathupala, Ko *et al.* 2006, Patra, Wang *et al.* 2013). Mathupala *et al.* also found a hypoxia induced increase in the expression and function of HK2 (Mathupala, Rempel *et al.* 2001). Alterations in the expression and function of other glycolytic enzymes are also observed during malignant progression. An increase in the expression of aldolase, an enzyme which catalyzes the conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, is implicated in supporting malignant progression through an increase in glycolytic metabolism (Pelicano, Martin *et al.* 2006). Overexpression of this enzyme is observed in lung squamous cell, pancreatic, and prostate carcinomas (Zhong, Chiles *et al.* 2000, Akakura, Kobayashi *et al.* 2001, Li, Xiao *et al.* 2006). Although alterations in the expression and function of glycolytic enzymes support cellular bioenergetics, an increase in the production of glycolytic intermediates is necessary to support biomass generation.

Influx of carbons into additional metabolic pathways, such as the pentose phosphate pathway (PPP), glycogen metabolism, serine biosynthesis, and triglyceride synthesis, is highly dependent

upon a buildup of glycolytic intermediates (DeBerardinis, Lum *et al.* 2008). Diverting the flow of glucose carbons away from glycolysis and into different pathways requires a truncation in glycolytic metabolism to increase the intracellular supply of glycolytic intermediates. This buildup can be achieved through the truncation of metabolism at key regulatory points within glycolysis. The M2 isoform of pyruvate kinase (M2-PK) is one example of a regulator of glycolysis within cancer cells. M2-PK, the dominant isoform in rapidly proliferating cells, oscillates between an active tetramer and an inactive dimer depending of the cells metabolic needs (Christofk, Vander Heiden *et al.* 2008, Mazurek 2011). Dimeric M2-PK is highly expressed in cancer cells and has been suggested as a malignant biomarker. PK catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP resulting in the formation of pyruvate and ATP. A reduction in PK activity within cancer cells results in a buildup of phosphometabolites such as fructose-6-phosphate (F-6-P) and glyceraldehyde-3-phosphate (G-3-P). These intermediates can be shuttled in to the PPP and converted to ribose-5-phosphate (R-5-P) by transketolase (TKT). Imatinib resistance in chronic myeloid leukemia cells is associated with an increase in glucose carbon flux into RNA through the non-oxidative portion of the PPP due to an increase in M2-PK expression (Serkova and Boros 2005, Zhao, Mancuso *et al.* 2010).

1.1.2. Oncogenes

Oncogenes arise from mutations in normal cellular genes, called proto-oncogenes, that lead to the formation of tumors through the dysregulation of cell division (Pierce 2005). These genetic mutations result in the constitutive activation or suppression of genes whose expression is normally regulated. Although cellular defects in DNA replication normally result in a cell death response, oncogenic activation supports survival and proliferation under these conditions

(Croce 2008). In addition to serving specific roles in regulating tumor initiation, oncogenes affect a number of different pathways that support malignant progression. These effected pathways include those through which oncogenes support the transition to a malignant glycolytic phenotype through the activation of genes involved in glucose uptake and metabolism (Edinger and Thompson 2002, Wieman, Wofford *et al.* 2007).

Several oncogenes, such as regulatory GTPases, support rapid proliferation. These genes are molecular switches that, when turned on, produce proliferation-stimulating signals. There are three Ras genes (HRAS, KRAS, and NRAS) that represent some of the most common oncogenes found in human cancers. Permanently activated Ras is found in approximately 20% of all tumors and in as much as 90% of pancreatic cancers (Downward 2003). Ras oncogenes mediate the activation of multiple effector pathways including mitogen-activated protein kinases (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase/serine-threonine-specific protein kinase/mechanistic target of rapamycin (PI3K/AKT/mTOR). Activation of this effector pathway results in an increase in metabolism through alterations in the expression and function of glucose transporters, glycolytic enzymes, fatty acid synthase, and ATP citrate lyase (Barthel, Okino *et al.* 1999, Yang, Han *et al.* 2002, Alberola-Ila and Hernandez-Hoyos 2003, Rathmell, Fox *et al.* 2003, Robey and Hay 2006, Ramjaun and Downward 2007, Ahmad, Patel *et al.* 2011). Transfection of ras or src into rodent cells increases the mRNA and protein expression of glucose transporters (Flier, Mueckler *et al.* 1987). Ramanathan *et al.* also showed that cells transformed by hTERT, SV-40T/t, and H-ras exhibited an increase in glucose dependency during tumorigenesis (Ramanathan, Wang *et al.* 2005). The PI3K/Akt pathway, which supports malignant transformation, not only enhances glycolysis but also results in glucose dependence in leukemia and glioblastoma cells (Elstrom, Bauer *et al.* 2004).

Another group of oncogenes that support rapid cell proliferation and malignant metabolism are transcription factors, such as the MYC oncogene. Alterations in the expression of the MYC oncogene have been implicated in a number of cancers including ovarian, pancreatic, breast, gastric, colorectal, and uterine (Ross, Ali *et al.* 2013, Chen, McGee *et al.* 2014). MYC plays a key role in regulating transformation, cell cycle progression, and apoptosis (Dominguez-Sola, Ying *et al.* 2007). The collaboration of MYC and HIF result in the activation of glucose transporters, lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase 1 (PDK1) (Kim, Gao *et al.* 2007, Dang, Kim *et al.* 2008, Dang, Le *et al.* 2009). This oncogene also activates transcription of target genes that increase the metabolism of glutamine (Li, Wang *et al.* 2005).

1.1.3. Mitochondrial Dysfunction

Although Otto Warburg suggested that cancer cells preferentially utilize glycolysis as a consequence of dysfunctional mitochondria, it is now known that cancer cells utilize glycolysis for energy and biomass production even in the presence of adequate amounts of oxygen. On average, the rate of glycolysis in malignant cells is often as much as five times higher than normal cells (Denko 2008). Although mitochondrial dysfunction does not completely explain Warburg's hypothesis, an increase in mitochondrial respiration injury is hypothesized to be a factor in supporting an increase in malignant glycolysis (Chatterjee, Dasgupta *et al.* 2011).

Studies have revealed that malignant cells have a high rate of mitochondrial DNA (mtDNA) mutations (Carew and Huang 2002, Taylor and Turnbull 2005, Pelicano, Xu *et al.* 2006, Singh, Sullivan *et al.* 2006). For example, high rates of mtDNA mutation have been observed in breast cancer, gastric cancer, prostate cancer, and leukemia (Carew, Zhou *et al.* 2003, Chen and

Kadlubar 2004, Zhao, Yang *et al.* 2005, Zhu, Qin *et al.* 2005). Factors affecting these mutations include the close proximity of mtDNA to reactive oxygen generation sites within the mitochondria, weak DNA repair capacity, and decreased histone protection. Since oxidative phosphorylation is one of two primary ATP generating pathways, malfunctioning mitochondria would result in an increased reliance on glycolysis (Pelicano, Xu *et al.* 2006). Genomic mutations in subunit 1 of cytochrome oxidase or NADH dehydrogenase provide tumor-promoting effects due to an increase in the levels of both cytosolic and mitochondrial reactive oxygen species (ROS) (Petros, Baumann *et al.* 2005, Ishikawa, Takenaga *et al.* 2008, Ralph, Rodriguez-Enriquez *et al.* 2010).

Additional evidence suggests that electron transport chain activity, specifically through complex I, maintains a high level of NAD+/NADH and limits breast cancer growth and metastasis (Santidrian, Matsuno-Yagi *et al.* 2013). Malignant alterations in the expression and function of phosphoglycerate dehydrogenase, phosphoglycerate mutase 1, and pyruvate kinase M2 function to divert glucose carbons away from the mitochondria (Vander Heiden, Cantley *et al.* 2009). The buildup of glycolytic intermediates increases carbon flux to alternative biosynthetic pathways such the PPP and serine biosynthesis pathways (Locasale, Grassian *et al.* 2011, Possemato, Marks *et al.* 2011, Hitosugi, Zhou *et al.* 2012).

Contrary to the conventional belief that all malignant cells have dysfunctional mitochondria, it has been suggested that many tumor cells adaptively regulate mitochondrial metabolism to provide a proliferative advantage (Koppenol, Bounds *et al.* 2011, Cantor and Sabatini 2012). Mitochondrial play a central role in regulating cell differentiation, apoptosis, necrosis, and onco-suppression (Scatena 2012). Therefore, alterations in the cellular utilization and activity of mitochondria support malignant progression. Mitochondrial mutations, although common in

malignancy, do not inactivate mitochondrial metabolism, but rather result in a shift in the bioenergetics and biosynthetic state (Wallace 2012). However, many malignant cell types also continue to utilize mitochondrial OXPHOS to produce ATP (Weinhouse 1976, Gottlieb and Tomlinson 2005). Differences in mitochondrial function illustrate metabolic heterogeneity within malignant cells.

1.1.4. Cellular Heterogeneity

Cellular heterogeneity is observed in all cell systems and contributes to a cells ability to respond to changing extracellular conditions (Altschuler and Wu 2010). Tumor heterogeneity refers to the observation that tumor cells exhibit different and distinct phenotypic and morphological variations. These variations include differences in gene expression, metabolism, morphology, proliferation, and metastatic potential (Marusyk and Polyak 2010). Within a solid tumor, cellular heterogeneity results in differences between malignant cells that originated within the same tumor. Tumor heterogeneity occurs both within and between tumors from the same tissue of origin and results in treatment resistance (Marusyk and Polyak 2010). Although glycolytic metabolism is a characteristic feature of malignant cells, the heterogeneities of an individual cell dictate how metabolic reprogramming is accomplished. For this reason, a single metabolic alteration cannot globally define malignancy (Cantor and Sabatini 2012).

Using single cell–derived clones from human primary colorectal tumors, Kreso *et al* observed that malignant cells display both genetic homogeneity and biological diversity (Kreso, O'Brien *et al.* 2013). Tumor heterogeneity has been observed in breast, colon, prostate, bladder, brain, head and neck, esophageal, and gynecological cancers (Konishi, Hiasa *et al.* 1995, Sauter, Moch *et al.* 1995, Califano, van der Riet *et al.* 1996, Macintosh, Stower *et al.* 1998, Samowitz

and Slattery 1999, Fujii, Yoshida *et al.* 2000, Maley, Galipeau *et al.* 2006, Shipitsin, Campbell *et al.* 2007, Campbell, Pleasance *et al.* 2008). For example, the cell specific expression of glutamine synthase, the enzyme responsible for catalyzing the conversion of glutamate to glutamine, differs between breast tumor subtypes (Kung, Marks *et al.* 2011). Although glucose and glutamine are the preferred carbon source by a number of malignant cells, leukemia and lung cancer cells utilize fatty acid oxidation as a means of obtaining energy (Wieman, Wofford *et al.* 2007, Zaugg, Yao *et al.* 2011). These observations illustrate variability in metabolic heterogeneity not only within the same tumor type but also across different tumor tissues.

In addition to genetic and epigenetic alterations that contribute to tumor heterogeneity, microenvironment also leads to alterations in tumor characteristics. Tumor microenvironment is simply defined as the environment in which tumor cells proliferate and includes the extracellular matrix, blood vessels, and signaling molecules (Weber and Kuo 2012). As illustrated in figure 1.1, malignant cells proliferate at a rate faster than that at which oxygen and nutrients can be supplied. Therefore, cellular adaptations are necessary to overcome physiological stress that occurs as a result of rapid cell proliferation. Within solid tumors, an incomplete vascular network results in the formation of hypoxic microenvironments requiring cells to undergo adaptations in order to scavenge available nutrients and utilize these nutrients in the most effective way possible.

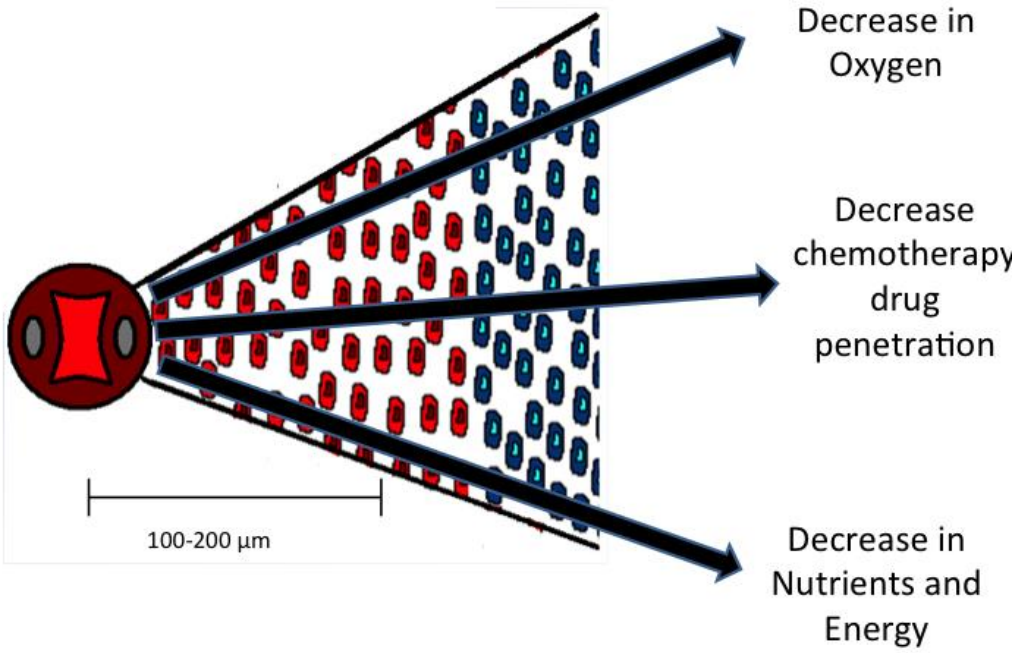


Figure 1.1 Diffusional gradient established during solid tumor formation. Within a solid tumor, malignant cells proliferate at a rate faster than that of which vasculature can be produced. As a result, limitations in the diffusion of oxygen and nutrients are observed in cells furthest away from blood vessels. An additional limitation and effect of malignant progression is the decrease in the penetration of chemotherapeutic drugs. Figure adapted from Threshold Pharmaceuticals.

Tumor hypoxia results in the stabilization of the transcription factor HIF1-α. HIF1 is a transcription factor complex that is responsible for gene expression changes under low oxygen conditions. The heterodimeric complex of the constitutively expressed HIF1β (aryl hydrocarbon receptor nuclear translocator) and HIF1α is stabilized following hypoxic exposure (Bertout, Patel *et al.* 2008, Cairns, Harris *et al.* 2011). Nuclear translocation of this complex results in the induction of genes involved in angiogenesis, glycolysis, lactate production, and mitochondrial activity (Firth, Ebert *et al.* 1995, Gleadle and Ratcliffe 1997, Chen, Pore *et al.* 2001, Blagosklonny 2004, Mathupala, Ko *et al.* 2006). Hypoxia-induced alterations in the extracellular environment also promote degradation of the extra-cellular matrix (ECM) and induce cell migration (van Sluis, Bhujwalla *et al.* 1999, Estrella, Chen *et al.* 2013). Tumor hypoxia also results in genetic instability through the down-regulating nuclear mismatch repair and nucleotide excision repair pathways (Bindra and Glazer 2005, Fuss and Cooper 2006, Iyer, Pluciennik *et al.* 2006, Li 2008). Stabilization of HIF1-α is also implicated in poor patient prognosis and increased metastatic potential (Blagosklonny 2004). Hypoxia has also been associated with both reduced radio-sensitivity and increased chemotherapeutic resistance. Overcoming therapeutic resistance presents a significant issue in the treatment of many cancers and therefore the inhibition of glycolysis presents a therapeutic alternative. Recent studies have shown that malignant cells have an increased sensitivity to oxamate, 2-deoxyglucose, and 3-bromopyruvate under hypoxic conditions (Liu, Savaraj *et al.* 2002, Maher, Krishan *et al.* 2004, Xu, Tong *et al.* 2005).

1.1.5. Autophagy

Cellular autophagy is necessary to support both metabolism and survival. Autophagy is defined as the process through which cellular proteins and organelles are broken-down by autophagosomes, digested by lysosomes, and then recycled to support cell survival and proliferation. In malignant cells, autophagy suppresses an intracellular accumulation of damaged proteins and promotes tumor growth. Self-cannibalism is a response to metabolic stress cues that results in the recycling of proteins and organelles to meet the metabolic demands associated with rapid proliferation (Mathew and White 2011, Yang, Chee *et al.* 2011). Autophagic degradation is a survival response in conditions where cells are growth-factor deprived or unable to uptake extracellular nutrients (DeBerardinis, Lum *et al.* 2008).

Mutated Ras results in an increase in cancer cell autophagy. Mutated H-rasV^{12} or K-rasV^{12} genes, introduced into immortal, non-tumorigenic baby mouse kidney epithelial (iBMK) cells, resulted in a 10-fold increase in autophagy when compared to control cells (Guo, Chen *et al.* 2011). In pancreatic cancer cells, Ras-driven autophagy functions to limit cells death under low nutrient stress conditions (Yang, Wang *et al.* 2011). In Ras-driven cells, autophagy supports oxidative metabolism through the maintenance of healthy mitochondria and maintenance of mitochondrial substrates through macromolecular degradation (Guo, Chen *et al.* 2011, Mathew and White 2011, Yang, Wang *et al.* 2011, Youle and Narendra 2011). An increase in malignant autophagy is also observed within hypoxic tumor microenvironments (Mazure and Pouyssegur 2010).

1.1.6. Metastasis

Metastasis is the process through which cancer spreads throughout the body. Metastatic tumors arise from a primary tumor and occur in almost all cancer types. A majority of cancer related deaths are a result of cancer spread from its tissue of origin throughout the body (Chaffer and Weinberg 2011). Malignant metastasis involves local invasion of surrounding tissues, intravasion into the blood of lymph nodes, extravasion into new tissue, proliferation within the tissue, and angiogenesis to support further survival and proliferation. Both the ability to metastasize and the site of metastasis are highly dependent on the cancer type. Malignant metastases are often found in the liver, lungs, bones, and brain (Bubendorf, Schopfer *et al.* 2000, Hess, Varadhachary *et al.* 2006, Aragon-Ching and Zujewski 2007, Coghlin and Murray 2010, Berman, Thukral *et al.* 2013).

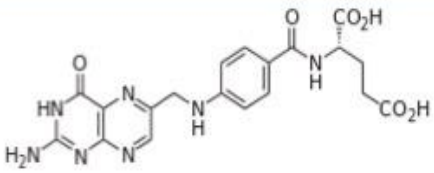
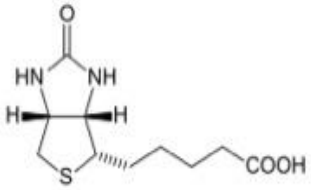
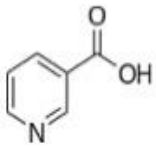
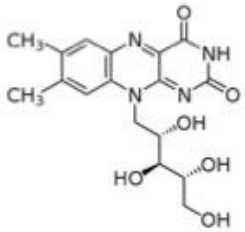
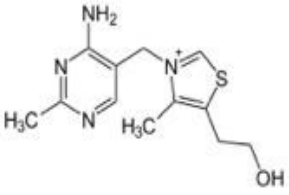
Metastasis occurs as a result of tumor cells breaking away from the primary tumor and breaking down proteins that surround the extracellular matrix (Nguyen and Massague 2007). Within malignant cells, alterations in cellular metabolism support the metastatic cascade of events. By diverting pyruvate away from the mitochondria and reducing it to lactate, malignant cells export a waste product that acidifies the ECM (Stasinopoulos, Mori *et al.* 2008). LDHA alleviates an intracellular buildup of pyruvate by converting it to lactate. Elevated LDHA levels are a hallmark of may tumors including squamous cell head and neck, colorectal, and non-small-cell lung cancers (Dawson, Goodfriend *et al.* 1964, Koukourakis, Giatromanolaki *et al.* 2003, Koukourakis, Giatromanolaki *et al.* 2005, Koukourakis, Giatromanolaki *et al.* 2009). LDHA is also a transcriptional target of Hif1α (Semenza, Roth *et al.* 1994, Firth, Ebert *et al.* 1995, Denko 2008). Hypoxia induced loss of p53 promotes an increase in the expression and function of proton-coupled monocarboxylate transporters (MCTs), which catalyze the transport of lactate

and pyruvate across the plasma membrane. An increase in the expression and function of MCT1 is observed in cervical, breast, and colon cancer cells (Boidot, Vegran *et al.* 2012). An increase in the expression of MCT4 is also observed in colorectal, breast, and pancreatic cancers (Ullah, Davies *et al.* 2006, Queiros, Preto *et al.* 2012, Gotanda, Akagi *et al.* 2013). The efflux of lactate results in an inflammatory response that attracts macrophages which then secrete cytokines and growth factors which support the growth of tumor cells and their metastasis (Shime, Yabu *et al.* 2008, Yabu, Shime *et al.* 2011).

1.2. THE ROLE OF COFACTORS IN CELLULAR METABOLISM

Vitamins play an essential role in mammalian cellular metabolism by serving as enzyme cofactors. An enzyme cofactor is defined as a non-protein component necessary for enzymatic activity. Vitamins such as niacin, folic acid, pantothenic acid, biotin, riboflavin and thiamine represent some of the most common enzyme cofactors (Table 1.1). These water-soluble vitamins serve a variety of metabolic functions including methyltransferase reactions (vitamin B12), fatty acid biosynthesis (biotin), and as precursor molecules utilized as activated electron carriers (riboflavin used in the generation of FAD and niacin for NAD) (Decker and Byerrum 1954, Wakil and Gibson 1960, Nishizuka and Hayaishi 1963, Weissbach and Taylor 1970). In addition to their role in cell metabolism, some vitamins are involved in regulating gene expression (Oommen, Griffin *et al.* 2005).

Table 1.1 Vitamins and their cofactor forms. All vitamins listed here are water-soluble vitamins that support metabolic function. Additionally, all require a transport system that is adaptively regulated during tumorigenesis.

Vitamin	Structure	Coenzyme
Folate		Tetra-hydrofolate (THF)
Biotin		D-(+)- Biotin
Niacin		NAD+
Riboflavin		Flavin adenine dinucleotide (FAD)
Thiamine		Thiamine-pyrophosphate (TPP)

1.2.1. Folate (Vitamin B_9)

Folate is an essential vitamin that is involved in DNA synthesis, repair, and methylation. Transport of folate and folic acid derivatives occurs through folate receptors (FR), organic anion transporters, a proton-coupled folate transporter, and the reduced folate carrier (RFC1) (Matherly and Goldman 2003). Following transport, folate is first converted to dihydrofolate (DHF) by the enzyme dihydrofolate reductase (DHFR). The biologically active derivative of folate is produced following the reduction of DHF, by DHFR, to tetrahydrofolate (THF) (Bailey and Ayling 2009). THF is involved in the metabolism of both amino acids and nucleic acids. This cofactor is important in the synthesis of nucleotides precursors such as serine and methionine through the transfer of enzymatically attached one-carbon units. Additionally, methylene groups attached to THF are used in the conversion of uracil-like pyrimidine bases to thymine, found in DNA. 5-methyltetrahydrofolate, another folate cofactor, is involved in the re-methylation of homocysteine during the methionine methylation cycle. Methionine can then be converted to S-adenosylmethionine, which acts as a methyl donor in many different cellular methyltransferase reactions. Low concentrations of folate can impair metabolic pathways and result in an accumulation of homocysteine, a reduction in methyl groups necessary for DNA methylation, and a decrease in precursors that are involved in DNA synthesis repair (Scott 1999, Weinstein, Hartman *et al.* 2003, Figueiredo, Grau *et al.* 2009). As a result of its role in both metabolism and proliferation, the intercellular homeostasis has been widely studied.

In 1945 Leuchtenberger *et al.* discovered that folic acid administration resulted in the regression of spontaneous breast cancer tumors in mice (Leuchtenberger, Leuchtenberger *et al.* 1945). This observation inspired Dr. Sidney Farber, who was working on treating acute leukemia in children, to utilize folic acid administration as a treatment strategy. However, Dr.

Farber discovered that administration of folic acid mimetics resulted in an acceleration of this disease. This observation suggested the potential of arresting the growth of leukemia cells if they were deprived of folic acid. In 1947, Dr. Farber administered aminopterin, a folic acid antagonist, to an ill child and observed a dramatic regression of this disease (Farber and Diamond 1948). Dr. Farber concluded that altering the maintenance of folate homeostasis, in malignant cells, represented a potential chemotherapeutic strategy.

An inverse relationship has been observed between folate intake and risk of developing pancreatic, lungs, esophageal, stomach, cervical, ovarian, breast, and colorectal cancers (Kim 1999). In these studies patients with a high level of folate intake showed a decrease in cancer incidence compared to patients with lower levels of circulating folate (Kim 1999, Choi and Mason 2000, Lucock 2000). Qin *et al.* also found that folate supplementation results in a significantly reduced risk in developing melanoma (Qin, Cui *et al.* 2013). Differential responses following folic acid supplementation suggest phenotypic differences in the utilization and uptake of this vitamin. An increase in the expression of hFR and RFC1 has been observed in nasopharyngeal epidermoid, leukemia, non-mucinous ovarian, lymphoma, cervical, uterine, metastatic endometrial, primary renal cell, and metastatic pancreatic carcinomas (Elwood, Nachmanoff *et al.* 1997, Kelley, Rowan *et al.* 2003, Pillai, Chacko *et al.* 2003, Elnakat and Ratnam 2004, Parker, Turk *et al.* 2005). Low expression is observed in primary endometrial, metastatic renal cell, lung, primary breast, primary bladder, primary pancreatic, colorectal, prostate brain, and liver carcinomas (Parker, Turk *et al.* 2005).

Chemotherapeutics, such as methotrexate (MTX), pemetrexed, and raltitrexed, target the folate-activating enzyme DHFR (Goldman and Matherly 1985, Vos, Ros *et al.* 1999, Goldman and Zhao 2002). These compounds are transported into the cell by RFC1 and reduce cell

proliferation through their effects on nucleotide synthesis (Goldman and Matherly 1985, Goldman and Zhao 2002, Matherly and Goldman 2003, Jansen 19991). MTX has also been used in the treatment of lymphoma, osteogenic sarcoma, breast, and pediatric cancers (Monahan and Allegra 2001). Impaired folate transport, observed in some breast cancers, results in MTX resistance (Pinard, Jolivet *et al.* 1996).

1.2.2. Biotin (Vitamin B_7)

Biotin is a coenzyme involved in the synthesis of fatty acids, gluconeogenesis, the synthesis of amino acids, and in branched-chain amino acid catabolism. Biotin also provides a necessary cofactor for enzymes that are responsible for the transfer of carbon dioxide in several carboxylase enzymatic reactions (Fiume and Cosmetic Ingredient Review Expert 2001). Transport of biotin occurs through a carrier mediated, Na+ dependent process (Said 1999). Sodium dependent multivitamin transporter (SLC5A6 or SMVT) is a transmembrane protein involved in the transport of biotin, pantothenic acid and lipoic acid. SMVT is expressed in a variety of human tissues including intestine, brain, liver, lung, and kidney. Conditional knockout of SLC5A6 in mice results in impaired biotin absorption in the intestine. Loss of gene expression resulted in the absence intestinal SMVT and premature death between 6 and 10 weeks (Ghosal, Lambrecht *et al.* 2013). Increased biotin uptake through SMVT has been observed in a number of cancers including prostate and breast (Patel, Vadlapatla *et al.* 2012, Vadlapudi, Vadlapatla *et al.* 2013). Minko *et al.* also found an increase in biotin-receptor expression in human ovarian carcinoma cells (Minko, Paranjpe *et al.* 2002). As a result of the broad substrate specificity, SMVT is an excellent candidate to enhance drug uptake (Vadlapudi, Vadlapatla *et al.* 2012).

Alterations in the expression of biotin transporters can be exploited through targeted drug delivery such as biotin conjugation of chemotherapeutics (Marek, Kaiser *et al.* 1997, Cannizzaro, Padera *et al.* 1998, Mishra and Jain 2002, Na, Bum Lee *et al.* 2003, Russell-Jones, McTavish *et al.* 2004). In cells with high biotin transporter expression, conjugation of a chemotherapeutic results in increased efficacy. Using a biotin-conjugated saquinavir, Luo *et al.* showed a significant inhibition of [^{3}H]-biotin in MDCK-MDR1 cells suggesting that this conjugate is recognized by SMVT (Luo, Kansara *et al.* 2006). Minko *et al.* hypothesized that a SMVT uptake of a biotin conjugated camptothecin–poly(ethylene glycol) increased the cellular permeability of camptothecin (Minko, Paranjpe *et al.* 2002).

Interestingly, extracellular supply of biotin alters gene expression. Using an *in vitro* model of small cell lung cancer, NCI-H69, Scheerger *et al.* observed an increase in the expression of oncogenes N-myc, c-myb, N-ras, and raf in cells cultured in pharmacologic biotin concentrations. When cells were cultured at borderline deficient biotin concentrations (0.025 nmol/L), expression of these oncogenes decreased by up to 47% (Scheerger and Zempleni 2003).

1.2.3. Niacin (Vitamin B_3)

Vitamin B3, niacin or nicotinic acid, is a necessary vitamin that is involved in the metabolism of lipids. Niacin uptake is facilitated by multiple membrane bound transporters, which include OATP2B1, MCT1, and SLC22A13 (Broer, Schneider *et al.* 1998, Bahn, Hagos *et al.* 2008, Hagenbuch and Gui 2008). Once inside the cell, niacin binds to G protein coupled receptors, niacin receptor 1 and niacin receptor 2, and inhibits cyclic adenosine monophosphate production (Soga, Kamohara *et al.* 2003, Wise, Foord *et al.* 2003). This inhibition results in an increase in both adipose tissue and free fatty acids that can be used in the production of

triglycerides and very-low-density lipoproteins (Gille, Bodor *et al.* 2008, Wanders and Judd 2011).

Within cells, niacin is converted to nicotinamide which is the precursor molecule for two cofactors; NAD+ (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate) (Jaconello 1992). These cofactors, which are involved in oxidation-reduction (redox) and other non-redox reactions, can be phosphorylated (NADP) and reduced (NADH and NADPH). More than 400 enzymes require either NAD+ or NADP as electron acceptors or donors in redox reactions (Panberthy and Kirkland 2012). In cells, NAD+ is involved in catabolic, energy-producing, reactions such as the breakdown of proteins, carbohydrates, fats, and alcohol. NADP is involved in anabolic reactions such as the biosynthesis of fatty acids and cholesterol (Brody 1999, Cervantes-Laurean, McElvaney *et al.* 1999).

Studies have also found that cellular maintenance of niacin homeostasis is essential in maintaining genomic stability. Poly ADP-ribosylations enzymes are NAD-dependent enzymes that facilitate DNA repair at strand breaks and are critical in the cellular response to DNA injury (Kirkland 2012). A loss of genomic stability results in a high rate of DNA damage and is now considered a hallmark of cancer (Negrini, Gorgoulis *et al.* 2010). Intracellular niacin and NAD deficiency leads to a decrease in the levels of tumor suppressor p53 in skin, breast, and lung cells (Jacobson, Shieh *et al.* 1999, Spronck, Nickerson *et al.* 2007). Interestingly, administration of nicotinamide increases the incidence of pancreatic islet-cell tumors after streptozotocin and heliotrine treatment. The development of kidney neoplasias was also promoted in rats that were pretreated with high doses of nicotinamide and then treated with diethylnitrosamine (Schoental 1977).

1.2.4. Riboflavin (Vitamin B_2)

Vitamin B2 (Riboflavin) is an essential component of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors. Riboflavin is transported by two membrane bound transporters, RFT1 and RFT2 (Yonezawa, Masuda *et al.* 2008, Yamamoto, Inoue *et al.* 2009). These cofactors are required for the enzymatic activity of flavoprotein enzymes such as those in the ETC, decarboxylation of pyruvate and ketoglutarate, and fatty acid oxidation. They are also necessary for the conversion of a number of vitamins to their active forms.

Riboflavin regulates the synthesis of 5-methyl THF and the conversion of retinol to retinoic acid (Higdon 2014). Co-administration of riboflavin and folate increase circulating levels of 5-methyl THF (Powers, Hill *et al.* 2007). As a direct result, homocystine metabolism is affected by riboflavin concentration through its role in mediating methylenetetrahydrofolate reductase (Yin, Ming *et al.* 2012, Gao, Ding *et al.* 2013, Shujuan, Jianxing *et al.* 2013, Wen, Yang *et al.* 2013). Riboflavin's implication in cellular metabolism and maintenance of vitamin homeostasis, alterations in the intracellular supply of riboflavin are implicated in malignant progression.

In 1943, Morris and Robertson found that riboflavin deficiency slowed the growth and spread of spontaneous mammary cancers in mice (Morris and Robertson 1943). Additionally, high doses of riboflavin enhance proliferation, invasion, and migration of lung cancer cell (Yang, Chao *et al.* 2013). Riboflavin has been used in conjunction with niacin and coenzyme Q_{10} to prevent oxidative stress associated with tamoxifen treatment (Yuvaraj, Premkumar *et al.* 2008).

1.2.5. Thiamine (Vitamin B_1)

Thiamine, vitamin B1, is classified as a water-soluble vitamin and is essential for cellular metabolism within all living organisms. Thiamine's structure is composed of a thiazole and pyrimidine ring joined together by a methylene bridge (See Figure 1.3). Both the presence of quaternary nitrogen and the overall hydrophilicity of this molecule result in the requirement for carrier-mediated transport to achieve both absorption and uptake.

1.2.5.1. Sources

Although thiamine biosynthesis occurs in bacteria, plants, and fungi, humans lack the genes necessary to produce this necessary vitamin (Webb, Marquet *et al.* 2007). As a result of this inability, humans must obtain this vitamin through dietary sources. Thiamine is found naturally in foods such as fish, meats, eggs, bread, legumes, pasta, watermelon and milk. A number of food products are also fortified with thiamine during processing. For example, refined grains have less thiamine when compared to whole grains. As a result, thiamine is often added to processed grains such as flour. Thiamine is also added to many soups to produce a meaty flavor (Jr. 2008).

The recommended daily intake (RDI) of thiamine varies by both age and gender. According to the National Institutes of Health (NIH) RDI of thiamine for men and women over the age of 18 is approximately 1 – 1.2 mg/day (Pitkin, Allen *et al.* 2000). A 2012 study administered thiamine to healthy patients at doses up to 1500 mg/day and found no adverse side effects (Smithline, Donnino *et al.* 2012). Additionally only nausea and indigestion have been observed in Alzheimer's patients who were administered up to 7500 mg/day (Meador, Loring *et*

al. 1993). Currently many over-the-counter vitamin supplements supply thiamine at values between 80 and 6,000% of the RDI (Pitkin, Allen *et al.* 2000).

Bacteria contain specialized genes that support the biosynthesis and utilization of thiamine. Within these organisms the thiazole and pyrimidine rings are synthesized independently and are combined by thiamine pyrophosphate synthase to form thiamine monophosphate (TMP). Microbiota in the gut also produce both thiamine and thiamine pyrophosphate (TPP) (Arumugam, Raes *et al.* 2011). Intracellular uptake of TPP, by mammalian cells, was previously though unlikely due to the size and charge of the molecule. Recently, however, Nabokina, *et al.* identified a TPP transporter present in human colon cells (Nabokina, Subramanian *et al.* 2013). This observation suggests that TPP, generated by microbiota, can be transported and utilized within human cells.

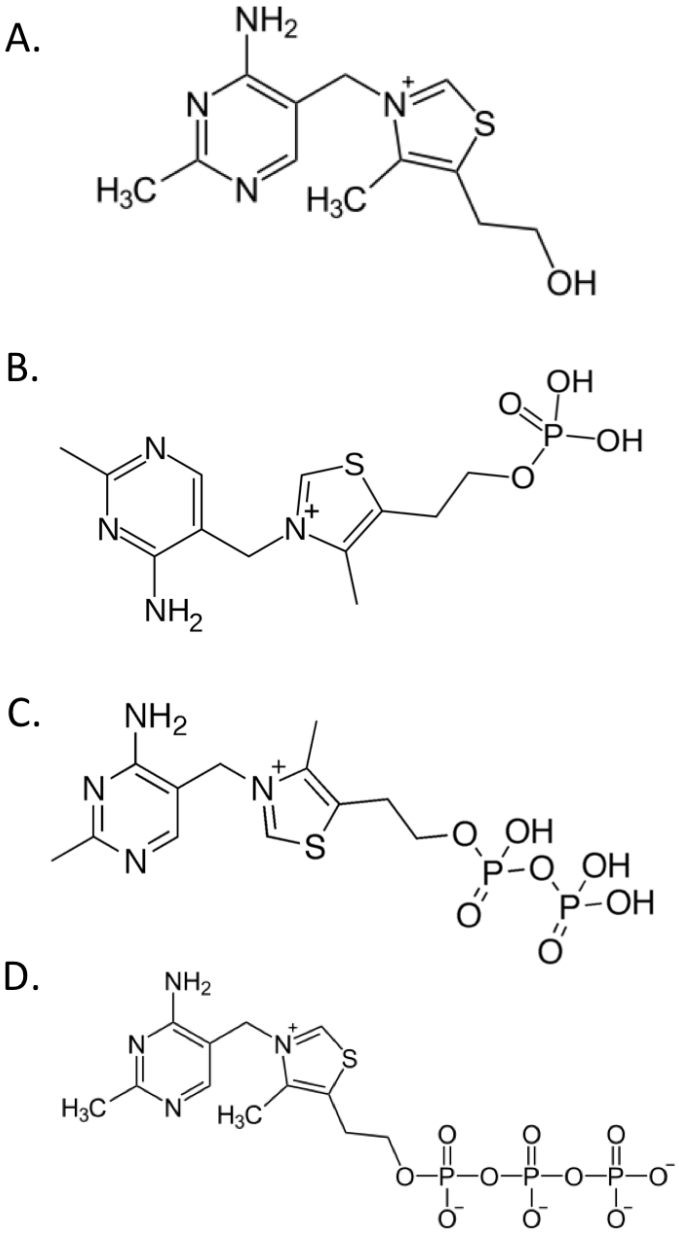


Figure 1.2. Thiamine and Thiamine Phosphate Derivatives. (A) Thiamine, (B) Thiamine Monophosphate, (C) Thiamine Diphosphate, and (D) Thiamine Triphosphate.

1.2.5.2. Absorption and Uptake

Thiamine is a hydrophilic organic cation and therefore requires carrier-mediated transport in order to cross the cell membrane. Although exogenous supply of thiamine is essential for cellular homeostasis, the intestinal absorption of thiamine plays a critical role in the body's maintenance of this essential vitamin. The facilitated transport of thiamine by members of the solute carrier family or transporters; THTR1 (SLC19A2) and THTR2 (SLC19A3), is necessary to sustain cellular homeostasis of this nutrient (Eudy, Spiegelstein *et al.* 2000, Fleming, Steinkamp *et al.* 2001). Once inside the cell, transport of TPP across the mitochondrial membrane occurs through SLC25A19 (Kang and Samuels 2008). Along with carrier-mediated transport of thiamine by SLC transporters, uptake by members of the cation family of transporters (OCT1) as well as passive diffusion at high concentrations has been observed (Hoyumpa 1980, Zielinska-Dawidziak, Grajek *et al.* 2008, Lemos, Faria *et al.* 2012, Nabokina, Subramanian *et al.* 2013).

1.2.5.2.1. SLC19A Family

There are currently three solute carrier transporters grouped into the SLC19 family. SLC19A1 (RFC1) is a folate transporter while SLC19A2 (THTR1) and SLC19A3 (THTR2) are both thiamine transporters (See Figure 1.4). RFC1 and THTR1 have a 39% sequence homology, RFC1 and THTR2 have a 42% sequence homology, and THTR1 and THTR2 have a 53% sequence homology (Ganapathy, Smith *et al.* 2004). Although the members of this family display a high degree of sequence homology they have high substrate specificity. RFC1 has not been shown to transport thiamine and THTR1/2 have not been shown to transport folate (Rajgopal, Edmondnson *et al.* 2001, Subramanian, Marchant *et al.* 2003).

At physiological pH the substrates for RFC1 exist as anions while the substrates for THTR1/2 exist as cations. Although each of the SLC19 family of transporters are pH responsive, RFC1 has a higher transport capacity at low pH (5.5) while THTR1/2 have higher transport capacities (pH 8 and 7.5 respectively) (Dutta, Huang *et al.* 1999, Rajgopal, Edmondnson *et al.* 2001, Rajgopal, Sierra *et al.* 2001). RFC1 transports folate through an inwardly directed H^+ gradient with a folate/H^+ symport and folate/OH^- antiport. In contrast, THTR1/2 transport thiamine by an outwardly directed H^+ gradient using a thiamine/ H^+ transport mechanism.

Each of the transporters in the SLC19 family is ubiquitously expressed, but the expression levels are dependent upon the specific tissue type. The expression of the SLC19 family of transporters is generally high in absorptive tissue such as the intestine, placenta, and kidneys. Additionally, while all SLC19 transporters are found only on the plasma membrane, RFC1 has also been shown to localize on the mitochondrial membrane (Said, Ortiz *et al.* 1999, Marchant, Subramanian *et al.* 2002, Boulware, Subramanian *et al.* 2003, Subramanian, Marchant *et al.* 2003, Said, Balamurugan *et al.* 2004).

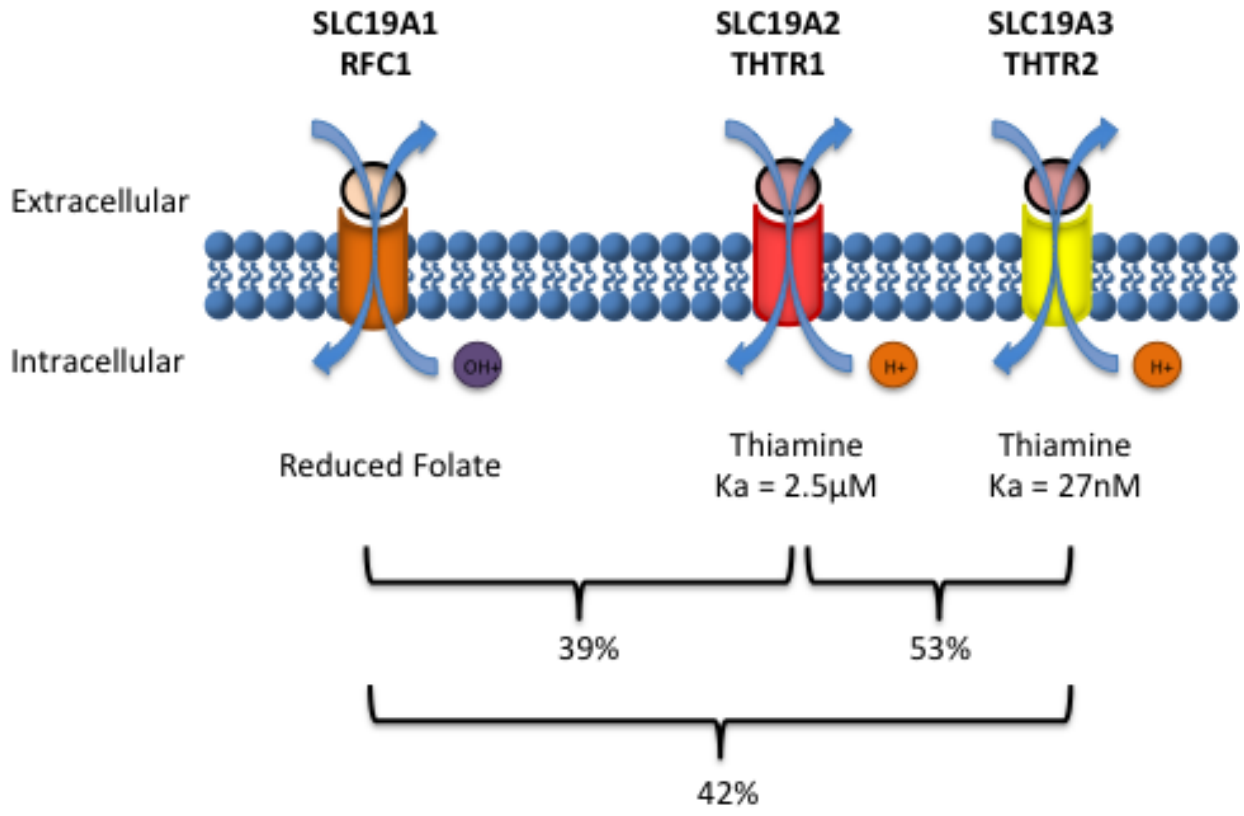


Figure 1.3. SLC19A Family. Percentages represent sequence homology between each of the transporters.

1.2.5.2.1.1. SLC19A1

Reduced Folate Carrier 1 (RFC1) is a 12 transmembrane domain protein with one N-glycosylation site (Moscow, Gong *et al.* 1995, Prasad, Ramamoorthy *et al.* 1995, Williams and Flintoff 1995, Wong, Proefke *et al.* 1995). RFC1 is expressed ubiquitously in the human body on both the plasma and mitochondrial membranes where it facilitates the cellular uptake of folate and its derivatives. Genetic knockout of the RFC1 protein is lethal in mouse embryos (Zhao, Russell *et al.* 2001). SLC19A1 expression is regulated in response to folate status in which increased availability results in a decrease in transporter expression (Said, Chatterjee *et al.* 2000). The expression of wild-type p53 also reduces the expression of RFC1 (Ding, Whetstine *et al.* 2001). Interestingly, although SLC19A1 does not transport free thiamine it plays a role in maintaining thiamine homeostasis through its ability to transport phosphate esters of thiamine. As a result, RFC1 may also provide an alternative route for thiamine uptake in diseases such as thiamine-responsive megaloblastic anemia (TRMA) (Zhao, Gao *et al.* 2002). RFC1 has received a substantial amount of attention as a chemotherapeutic target due to its uptake activity, widespread tissue distribution, and altered expression in cancer tissues. Chemotherapeutics such as MTX, pemetrexed, raltitrexed, and pralatrexate are all transported by RFC1 (Matherly, Hou *et al.* 2007, Desmoulin, Hou *et al.* 2012).

1.2.5.2.1.2. SLC19A2

SLC19A2 (THTR2) is a 497 amino acid protein that is ubiquitously expressed in human tissues. This high capacity, low affinity thiamine transporter (Km = 2.5μM) has 12 transmembrane domains with two N-glycosylation cites, which are not essential for transport function (Diaz, Banikazemi *et al.* 1999, Dutta, Huang *et al.* 1999, Fleming, Tartaglini *et al.* 1999,

Balamurugan and Said 2002). Within the cell, THTR1 is localized to the plasma membrane and relies on intact microtubules for trafficking to the cell membrane (Fleming, Steinkamp *et al.* 2001). This protein is present on both the apical and basolateral surfaces of epithelial cells (Subramanian, Marchant *et al.* 2003). Alterations in the expression of THTR1 are implicated in a number of disease states.

Mutations in SLC19A2 result in TRMA, which is characterized by megaloblastic anemia, diabetes, and hearing loss. Symptoms of TRMA are treatable following thiamine supplementation (Raz, Labay *et al.* 2000, Neufeld, Fleming *et al.* 2001). In 2003 Moscow *et al.* demonstrated that the RNA levels of SLC19A2 remain relatively unchanged when comparing normal breast tissue to breast cancer tissue (Liu, Huang *et al.* 2003). Recently, Zastre *et al.* showed an increase in the expression of SLC19A2 in human breast tissue samples when compared to normal breast tissues. This marked the first time that an increase in expression of this gene has been observed in cancer (Zastre, Hanberry *et al.* 2013). The conflicting data concerning SLC19A2 expression suggests that more work is necessary to understand alterations of this transporter during malignant progression.

1.2.5.2.1.3. SLC19A3

SLC19A3 (THTR2) is a 496 amino acid protein that is ubiquitously expressed in tissues throughout the human body. THTR2 is a high affinity, low capacity thiamine transporter (Km = 27nM) with 12 transmembrane domains and 2 N-glycosylation sites. THTR2 is localized primarily on the plasma membrane and is restricted to the luminal apical side of polarized epithelial cells (Boulware, Subramanian *et al.* 2003, Said, Balamurugan *et al.* 2004, Ashokkumar, Vaziri *et al.* 2006). Mutations in THTR2 result in biotin-responsive basal ganglia

disease for which the administration of thiamine is ineffective (Tabarki, Al-Shafi *et al.* 2013). Extensive evaluation has been conducted to elucidate the regulation and alteration of SLC19A3 expression in malignant cells.

Liu *et al.* demonstrated an approximately 14% reduction in the mRNA level of SLC19A3 when comparing normal breast tissue to breast cancer tissue (Liu, Huang *et al.* 2003). This study examined clinical samples of breast tissue and therefore represents a wide range of breast cancer subtypes. Zastre *et al.* confirmed the findings of this report in demonstrating a decrease in SLC19A3 using similar techniques (Zastre, Hanberry *et al.* 2013). Similarly, a down regulation has been observed in both colon and gastric cancers (Liu, Lam *et al.* 2009, Ikehata, Ueda *et al.* 2012). In 2010 Sweet *et al.* used a breast cancer cell line to show that the expression of SLC19A3 increases following chronic hypoxic exposure (Sweet, Paul *et al.* 2010). This study was the first to examine a microenvironment specific alteration in thiamine transport. Additional studies have elucidated the epigenetic regulation of SLC19A3. Liu *et al.* found that SLC19A3 expression decrease in gastric cancer due to promoter hypermethylation (Liu, Lam *et al.* 2009). Both hypermethylation and histone deacetylation of the SLC19A3 promoter are thought to be responsible for the epigenetic repression of this gene.

1.2.5.2.2. Mitochondrial Thiamine Transporter (SLC25A19)

SLC25A19 (DNC, TPC, MUP1, MCPHA, THMD3, THMD4) is a 320 amino acid mitochondrial thiamine pyrophosphate transporter located on chromosome 17q25.3. This transporter has three variants that encode the same protein. The minimal promoter region contains a nuclear factor Y binding site that controls basal activity (Nabokina, Valle *et al.* 2013). Although originally thought responsible for the transport of deoxynucleotides into the

mitochondria, comparisons to yeast TPP transporter lead to its identification as a human TPP transporter (Dolce, Fiermonte *et al.* 2001, Lindhurst, Fiermonte *et al.* 2006, Kang and Samuels 2008). The transport of TPP into the mitochondrial is essential for the enzymatic activity of both PDH and α-Ketoglutarate Dehydrogenase (α-KGDH). Mutations in SLC25A19 result in Amish lethal microcephaly, a metabolic disorder characterized by elevations in 2-ketoglutaric acid (Kelley, Robinson *et al.* 2002). Currently little is known about malignant alterations in the expression of this transporter. Zastre *et al.* were the first to show that the expression of SLC25A19 increases in breast cancer tissue when compared to normal breast tissue (Zastre, Hanberry *et al.* 2013).

1.2.5.2.3. Human Thiamine Pyrophosphate Transporter (SLC44A4)

SLC44A4 (hTTPT) encodes a recently characterized TPP transporter located on chromosome 6p21.3. This 710 amino acid transporter, recently described by Nabokina *et al.*, transports TPP within human colon cells (Nabokina, Valle *et al.* 2013). Transport of TPP through hTTPT is both temperature and energy dependent but NA^+ independent. hTTPT has a high specificity for TPP with an apparent Km of 0.17 μM (Nabokina, Inoue *et al.* 2014). The identification of this transporter suggests that bacterial-produced TPP produced may be transported and utilized by human cells. The recent nature of this finding has not allowed complete characterization and cellular localization of this transporter.

1.2.5.3. Physiological Function

1.2.5.3.1. Activation

Following its transport into the cell thiamine must first be phosphorylated to its active form, thiamine pyrophosphate (TPP), by the diphosphotransferase enzyme Thiamine Pyrophosphokinase (TPK1) (Liu, Timm *et al.* 2006). The conversion of thiamine to its cofactor form is hypothesized to be a driving force for thiamine uptake (Yoshioka 1984, Bettendorff 1995). TPP plays an essential role as an enzyme cofactor for three key enzymes within the glycolytic network; TKT, PDH, and α-KGDH. The continued presence of TPP is necessary to support enzymatic activity in subsequent daughter cells.

In humans, TPK1 is ubiquitously expressed with high levels found in the small intestine, kidneys, and testis. Within human cells, three other phosphorylated forms of thiamine have been observed; thiamine monphosphate (TMP), thiamine triphosphate (TTP), and adenosine thiamine triphosphate (AThTP) (Gangolf, Czerniecki *et al.* 2010). Currently, the physiological functions of TMP, TTP, and AThTP have not been established. Of the four phosphorylated forms, TPP is the only phosphorylated form known to function as an enzyme cofactor. The dephosphorylation of TPP to TMP occurs through the enzyme thiamine pyrophosphatase (See Figure 1.5). TMP can be subsequently recycled back to free thiamine through the enzyme thiamine monophosphatase (Rindi and Laforenza 2000, Zhao, Gao *et al.* 2002). Both TPP and TMP are transported outside of the cell by SLC19A1 (Zhao, Gao *et al.* 2001). TPP is transported across the mitochondrial membrane by the mitochondrial thiamine transporter, SLC25A19, where it serves as a necessary cofactor for PDH and α-KGDH (Lindhurst, Fiermonte *et al.* 2006). Additionally, TPK1 exists as a homodimer with two splice variants, a long from and short form (Nosaka, Onozuka *et al.* 2001).

Mutations to or deficiency of TPK1 results in developmental delays, encephalopathy, and hypotonia (Mayr, Freisinger *et al.* 2011, Fraser, Vanderver *et al.* 2014). Although the role of TPK1 in thiamine conversion to TPP is well established, little is known about alterations in the expression and function of this enzyme during malignant progression (Sanioto, Reinauer *et al.* 1977, Liu, Timm *et al.* 2006). Zastre *et al.* were the first to show an increase in TPK1 gene expression in breast cancer tissue compared to normal breast tissue (Zastre, Hanberry *et al.* 2013). The role and regulation of TPK1 in malignant progression is a current area of need in the field.

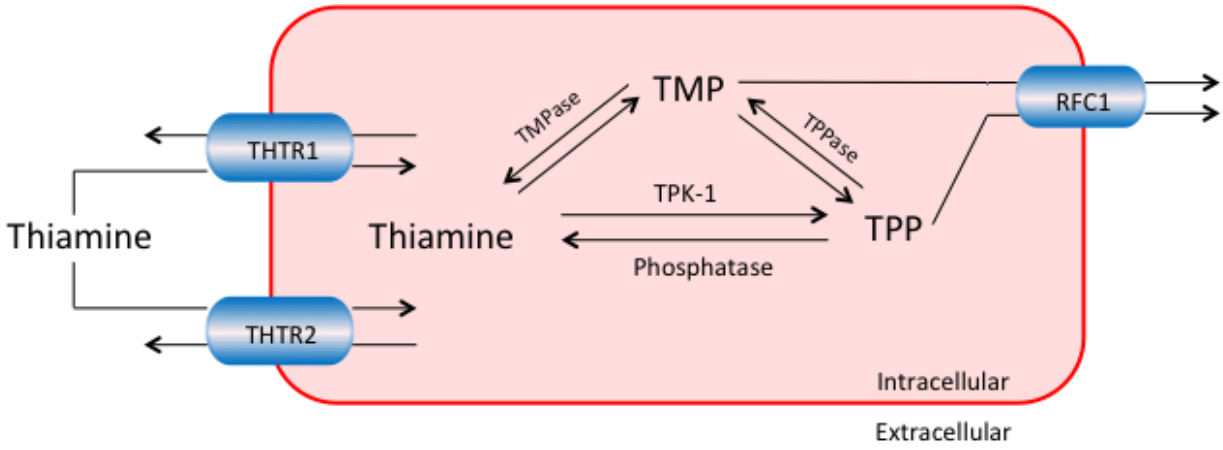


Figure 1.4. Thiamine Homeostasis. Once transported into the cell, by THTR1 or THTR2, thiamine is converted to the cofactor form (TPP) by the enzyme TPK1. TPP can then be converted to TMP by thiamine pyrophosphatase (TPPase), and can be effluxed through RFC1. TMP can also be converted to free thiamine through thiamine monophosphatase (TMPase).
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1.2.5.3.2. Distribution

Thiamine's inability to be stored by mammalian cells results in the requirement for constant dietary intake. Gangolf *et al.* hypothesized that as a result of this inability, humans are highly sensitive to thiamine deficiency (Gangolf, Czerniecki *et al.* 2010). External factors such as nutrition, disease, gender, age, and lifestyle all contribute to the inter-patient variability in relative tissue distribution. The greatest concentration of thiamine and its derivatives is found in skeletal muscle, brain, heart, kidneys, and liver (Gangolf, Czerniecki *et al.* 2010). Kimura *et al.* reported that the concentration of thiamine in human plasma ranges from 10 - 20nM. This report indicates that only thiamine and TMP are found in the plasma while TPP and TTP are found in erythrocytes (Kimura and Itokawa 1983).

1.2.5.3.3. Cofactor Role

Following transport into the cell, thiamine is phosphorylated to the active form TPP by the diphosphotransferase enzyme TPK1 (Yoshioka 1984, Bettendorff 1995, Liu, Timm *et al.* 2006). TPP functions as a cofactor for three key enzymes within the metabolic network; TKT, PDH, and α-KGDH (See Figure 1.6). TKT links glycolysis with the PPP and catalyzes a reversible conversion that produces R-5-P for use in *de novo* nucleotide synthesis. PDH links glycolysis to the TCA cycle by catalyzing the conversion of pyruvate to acetyl-CoA. α-KGDH plays an essential role in the TCA cycle and glutamine metabolism by catalyzing the conversion of alpha-ketoglutarate (α-KG) to succinyl-CoA.

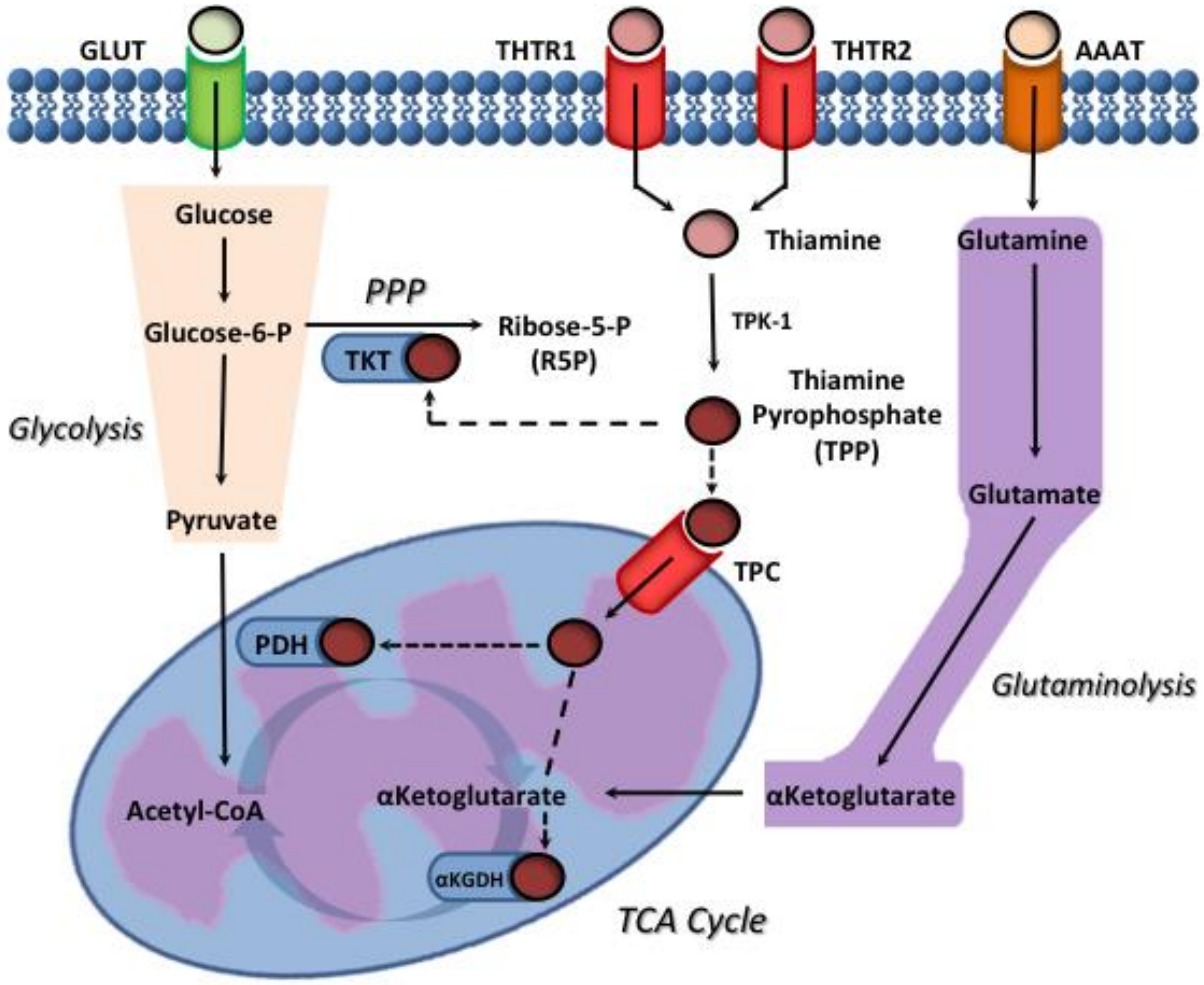

Figure 1.5 The role of TPP as an enzyme cofactor. Following intracellular transport and activation TPP serves as a cofactor for TKT, PDH, and αKGDH. TKT is found in the non-oxidative portion of the PPP. Following transport into the mitochondria, by the mitochondrial thiamine pyrophosphate carrier (TPC), TPP serves as a cofactor for PDH and αKGDH.

1.2.5.3.3.1. Transketolase (TKT)

The use of different metabolic pathways is regulated through an intricate balance of cellular mechanisms which regulate substrate availability (Berg, Tymocko *et al.* 2002). Intermediate products of metabolic pathways are used as precursors for small molecules and as a source of metabolic energy. The generation of precursor molecules and energy occurs through both catabolic and anabolic reactions. The PPP utilizes glycolytic intermediates to support cellular biosynthesis. The PPP has two pathways; the oxidative and non-oxidative (Wood 1985). The oxidative branch generates NADPH, which is used in reductive biosynthetic reactions such as the synthesis of fatty acids, and 5-carbon sugars such as ribose, which are used to synthesize nucleic acids. The production of R-5-P is also supported by reversible reactions within the non-oxidative portion (See Figure 1.7).

The reaction mediated by the enzyme TKT represent a rate-limiting step within the non-oxidative portion of the PPP. This enzyme serves a critical role at the intersection of glycolysis and the pentose shunt by linking glycolysis to the PPP. (Novello and McLean 1968, Kauffman 1972). G-6-P enters the PPP and is subsequently converted to R-5-P in the oxidative branch of this pathway. This diversion of glucose carbons away from glycolysis and into the oxidative or non-oxidative portion of the PPP is necessary in order to produce R-5-P (Deberardinis, Sayed *et al.* 2008). R-5-P can either be used for *de novo* nucleotide synthesis or converted into F-6-P and G-3-P by TKT. If not utilized for de novo nucleotide synthesis, F-6-P and G-3-P and can then enter the Embden-Meyerhoff pathway to be used in the production of ATP. Excess ribose, or any ribose obtained through diet can subsequently be converted into glycolytic intermediates and used in other reactions (Berg, Tymocko *et al.* 2002).

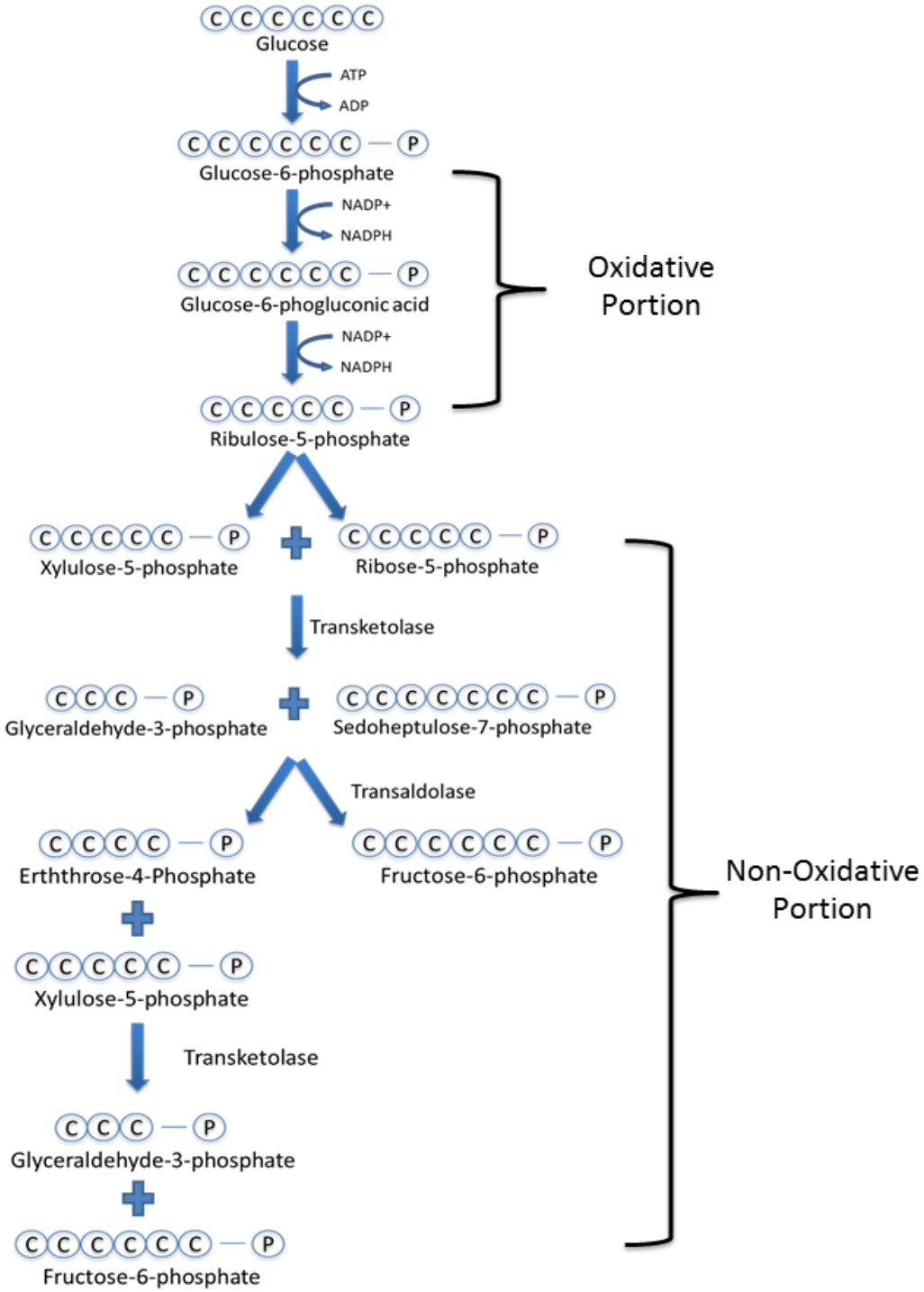

Figure 1.6. Pentose Phosphate Pathway. Glucose-6-phosphate enters the PPP in the oxidative portion, in which NADPH is produced. Reactions within the non-oxidative portion of the pathway are reversible. Transketolase and transaldolase produce intermediates (F-6-P and G-3-P) that are also substrates for glycolysis and thereby link the PPP back to glycolysis. Figure adapted from Missouri State-Mountain Grove.

In malignant cells, both the high rate of glycolysis and truncation of cellular metabolism support the shuttling of glycolytic intermediates into different metabolic pathways (DeBerardinis, Lum *et al.* 2008). The diversion of glucose carbons into the PPP to synthesize high amounts of nucleic acids is essential for rapid cellular proliferation in cancer cells. In H441 lung cancer cells, Boros *et al.* showed that 98% of ribose molecules is generated from the non-oxidative portion of the PPP (Boros, Torday *et al.* 2000). This group also showed that in pancreatic adenocarcinoma cells, 85% of ribose RNA came from the non-oxidative portion of the PPP (Boros, Puigjaner *et al.* 1997).

Utilizing alternative pathways such as the PPP allows cancer cells to limit ROS production in hypoxia when cells are under oxidative stress. Oxidative stress in cancer cells suppresses M2-PK activity leading to an increase carbon flux through the oxidative portion of the PPP and a decrease in reduced glutathione levels (Anastasiou, Poulogiannis *et al.* 2011). NADPH, generated in the PPP, is essential in the regeneration of glutathione for use in biosynthetic reactions. When human brain cells were exposed to hypoxia a reduction in Transketolase-like protein 1 (TKTL1) resulted in an increase in ROS generation and subsequent cell death (Wanka, Steinbach *et al.* 2012). In human HCT116 colon carcinoma cells, knockdown of TKTL1 resulted in a reduction in both NADPH and glutathione levels and an increase in apoptosis due to ROS production (Xu, Zur Hausen *et al.* 2009). It is currently unclear how TKTL1 activity effects NADPH production, although it has been suggested that TKTL1 activity maintains a continuous flux of glucose carbons into the PPP (Zastre, Sweet *et al.* 2013) .

Although the non-oxidative reactions carried out by TKT are thought to be the result of a single TKT gene there are two additional isoform of TKT; TKTL1 and TKTL2. TKTL1 has a 38 amino acid deletion within the cofactor and catalytic domains. This deletion suggests that this

protein is unable to bind to TPP and accomplish the TKT reaction (Mitschke, Parthier *et al.* 2010, Maslova, Meshalkina *et al.* 2012). A 38 amino acid deletion, resulting in a pseudo-TKTL1 was used to elucidate the functional role of this isoform. Schneider *et al.* used a spectrophotometric assay that monitored the conversion of known TKT substrates and showed that this TKTΔ38 mutant had TKT-like activity (Schneider, Ludtke *et al.* 2012). Both circular dichroism and proton nuclear magnetic resonance (1H-NMR) spectroscopy showed no association between the TKTΔ38 and TPP. Additional groups have used this TKTΔ38 mutant and confirmed the lack of TKT-like activity (Meshalkina, Drutsa *et al.* 2013). TKT exists as a homodimer and therefore it is unclear if the presence of the TKTΔ38 alters dimer formation. The expression of TKTL1 in mammals may influence the activity of TKT through its formation or heterodimers with other TKT isoforms. Although TPP binding to the TKTΔ38 was not observed it does not rule out the possibility of the binding of thiamine derivatives. TPP does not contribute to the catalytic activity of TKT but rather functions as to anchor the cofactor to the apo-enzyme. Gangolf *et al.* suggested that other derivatives might function in binding to the catalytic site of TKTL1 (Gangolf, Czerniecki *et al.* 2010). More work is needed in order to fully understand the complex regulation and activity of TKTL1 in mammals.

TKTL1 overexpression is observed in a number of malignant tissues including endometrial, breast, ovarian, lung, colonic, urothelial, gastric and laryngeal (Langbein, Zerilli *et al.* 2006, Staiger, Coy *et al.* 2006, Foldi, Stickeler *et al.* 2007, Krockenberger, Honig *et al.* 2007, Volker, Scheich *et al.* 2007, Schultz, Kahler *et al.* 2008, Krockenberger, Engel *et al.* 2010, Schmidt, Voelker *et al.* 2010, Diaz-Moralli, Tarrado-Castellarnau *et al.* 2011). In many of these studies a high expression of TKTL1 showed a direct correlation with both tumor progression and poor patient prognosis (Langbein, Zerilli *et al.* 2006, Volker, Scheich *et al.* 2007, Diaz-Moralli,

Tarrado-Castellarnau *et al.* 2011, Schwaab, Horisberger *et al.* 2011). When TKTL1 expression is silenced, a decrease in cancer cells proliferation and a decrease in the growth of xenograft tumors has been observed (Hu, Yang *et al.* 2007, Zhang, Yang *et al.* 2007, Zhang, Yue *et al.* 2008, Chen, Yue *et al.* 2009, Xu, Zur Hausen *et al.* 2009, Yuan, Wu *et al.* 2010). Overexpression of TKTL1 in head and neck squamous cell carcinoma cells resulted in an increase in cellular proliferation when compared to vector control (Sun, Liu *et al.* 2010). This data suggests that an increase in the usage of the PPP, and specifically TKT, may be an important pro-survival response in malignant cells. Although HIF is hypothesized to regulate the expression of TKTL2, little is known about the role this thiamine depended enzyme plays in supporting malignant progression (Semenza 2012).

1.2.5.3.3.2. Pyruvate Dehydrogenase (PDH)

The conversion of pyruvate to acetyl-CoA links glycolysis to the TCA cycle. Like glycolysis, the TCA cycle does not directly require molecular oxygen although it is required for NADH to generate NAD+. The oxygen dependent loss of electrons from NADH and $FADH_2$ allow the TCA cycle to continue by donating these electrons to the electron transport chain.

The irreversible conversion of pyruvate to acetyl-CoA takes place through a series of reactions that occur within the pyruvate dehydrogenase complex (PDC). This complex, which is located within the mitochondrial matrix, is composed of three enzymes that link glycolysis to the citric acid cycle (Zhou, McCarthy *et al.* 2001). The three components within the PDC are PDH (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3). As a result of its location at the junction of glycolysis and the citric acid cycle, the PDC is a key regulatory complex for both the metabolism of glucose and the oxidation of fatty acids (Rardin, Wiley *et al.*

2009). The first enzymatic reaction in the PDC involves the enzyme PDH. PDH is a thiamine dependent enzyme that functions to convert pyruvate to hydroxyethyl-TPP. Figure 1.8 illustrates how each of the two active sites in PDH binds TPP and interacts during the conversion of pyruvate (Ciszak, Korotchkina *et al.* 2003). As is the case with many other tightly regulated biochemical reactions, PDH activity is controlled through reversible phosphorylation reactions (Randle 1986, Patel and Roche 1990). Within the mitochondria both pyruvate dehydrogenase kinases (PDKs) and pyruvate dehydrogenase phosphatases (PDPs) regulate PDH activity.

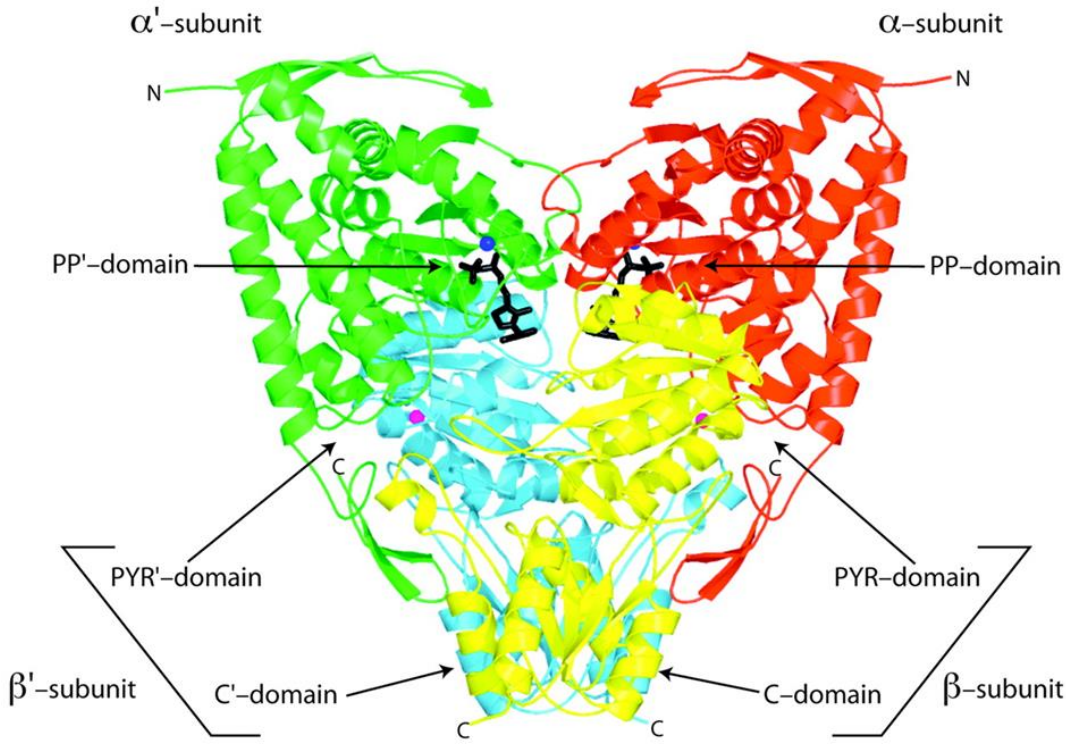

Figure 1.7. Structure of the tetrahedral subunits of the PDC E1 complex. α, red; α′, green; β, yellow; and β′, blue. PP and PP' domains indicate TPP binding sites. PYR and PYR' domains are binding sites for the aminopyrimidine ring of TPP. The two TPP cofactor molecules are shown in black. Mg^{2+} ions at the pyrophosphate terminus of TPP are shown as dark blue spheres. K^{+} ions are shown as magenta spheres. Ciszak *et al.* (2003)

PDK is a family of four isoenzymes (PDK1-4) that function to inhibit PDH activity through ATP-dependent phorphorylation (Yeaman, Hutcheson *et al.* 1978, Korotchkina and Patel 2001). Each of the isoenzymes differs in their ability to phosphorylate each of the three potential sites and is tissue specific. Phosphorylation by PDKs can occur at any of three serine residues; Ser-293, Ser-300, and Ser-232 often referred to as site 1, site2, and site 3 respectively (Korotchkina and Patel 1995, Kolobova, Tuganova *et al.* 2001). Phosphorylation at any of the three sites on PDH leads to inactivation of the complex (Kolobova, Tuganova *et al.* 2001). The four known PDKs differ in their ability to phosphorylate each of the three serine residues found on PDH. While PDK1 is able to phosphorylate PDH as any of the three phosphorylation sites, PDK 2-4 are only able to phosphorylate Ser-293 and Ser-300 (Korotchkina and Patel 2001). The PDKs also differ in the extent to which they phosphorylate PDH. PDK2 was shown to phosphorylate both sites 1 and 3 but to a lesser extent that PDK3 and PDK4. Interestingly they also reported that the binding of TPP to PDH altered the rate at which each of the serine sites could be phosphorylated. The rate at which each of the four PDKs phosphorylate site one decreased as a result of TPP binding. TPP also decreased the amount of phosphate incorporation at both sites 2 and 3 by PDK1. They also indicated that although TPP did not significantly affect phosphate incorporation in site 2 by PDK3 and 4 it leads to a decrease in the rate at which this site was phosphorylated. Therefore, TPP is a significant regulator of phosphorylation by PDKs (Kolobova, Tuganova *et al.* 2001).

Regulation of PDK activity is also mediated through the accumulation of metabolic products such as ATP, NADH and acetyl-CoA, which stimulate activity, while pyruvate and ADP inhibit when in excess [33]. PDK is inhibited by accumulation of the reactions substrates including; TPP, pyruvate, NAD+, and CoA. When bound to PDH, reduces the rate and extent of

PDK-mediated phosphorylation [25]. Thus increasing concentrations of TPP through thiamine supplementation may be pro-apoptotic through restoration of PDH activity in cancer cells. This may explain why a reduction in tumor growth was observed with high-dose thiamine supplementation (Comín-Anduix, Boren *et al.* 2001). Therefore, the potential of high-dose vitamin B_1 to reduce cancer cell growth would be of particular significance and warrants further study.

The expression of each of the PDKs differs in tissues throughout the body. PDK1 is expressed mainly in the heart but also in the skeletal muscle, pancreas, and liver. PDK2 is expressed in many tissues throughout the human body. PDK3 is expressed in the testis and also to some extent in the kidney, lung, spleen, heart, and brain. PDK4 is found in the skeletal muscle and heart and also in the kidney, lung, and liver. This inactivation of PDH is reversible through dephosphorylation by the enzyme PDP (Kolobova, Tuganova *et al.* 2001).

Two enzymes, Pyruvate Dehydrogenase Phosphatases (PDP 1-2) are involved in the dephosphorylation PDH (Teague, Pettit *et al.* 1982, Huang, Gudi *et al.* 1998). PDPs are able to dephosphorylate any of the phorphorylated sites on PDH (Karpova, Danchuk *et al.* 2003). The expression of the two PDPs differs as PDP1 is expressed in the brain, heart, and testis and PDP2 is expressed in the kidney, liver, and heart but not in the testis or skeletal muscle (Bowker-Kinley, Davis *et al.* 1998, Huang, Wu *et al.* 2003).

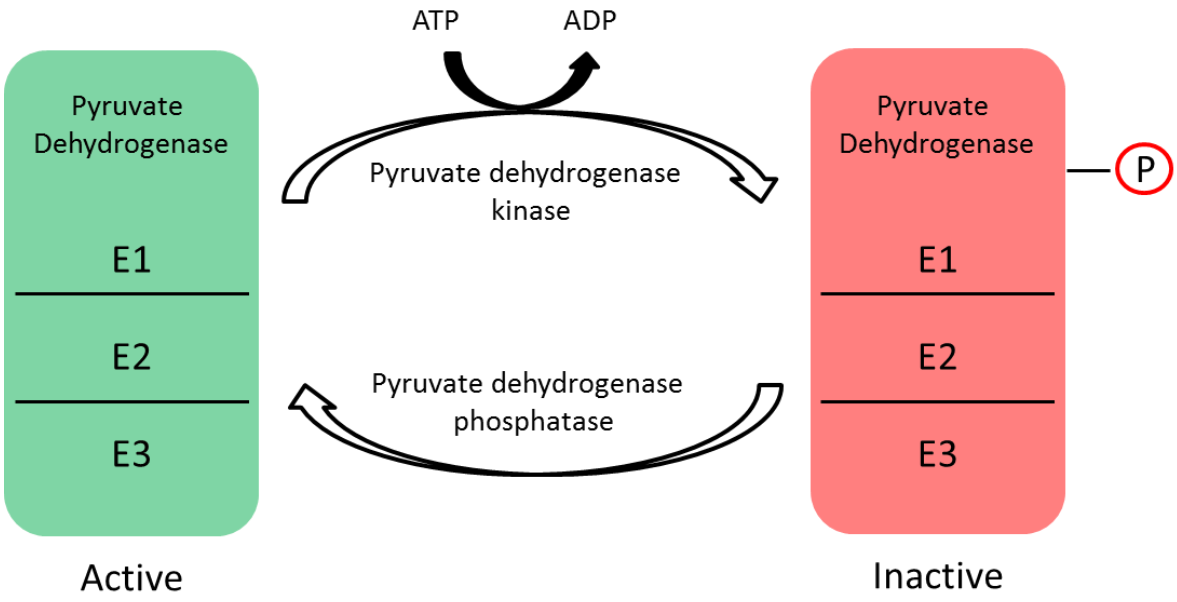

Figure 1.8. Regulation of the E1 alpha component of the pyruvate dehydrogenase complex. Pyruvate dehydrogenase kinases (PDK) utilize ATP to phosphorylate the E1 alpha subunit of PDH at any of three serine residues. Phosphorylation at any site leads to inactivation of the enzyme. Phosphorylation is removed by pyruvate dehydrogenase phosphatases (PDP). Figure adapted from Abcam.

In normal cells PDH is active, allowing cells to maintain oxidative metabolism to produce ATP and other components necessary for cell survival and proliferation. However, PDH activity is suppressed in cancer due to its down-regulation and the overexpression of PDK isoforms (Koukourakis, Giatromanolaki *et al.* 2005, Lu, Lin *et al.* 2011, Baumunk, Reichelt *et al.* 2013, Hur, Xuan *et al.* 2013). The truncation of glucose metabolism, highlighted by preferential conversion of pyruvate to lactate, provides cancer cells with a metabolic advantage to maintain rapid proliferation (McFate, Mohyeldin *et al.* 2008). Interestingly, in tumor-associated stromal tissue, such as fibroblasts and vascular endothelial cells, PDH expression is elevated with a reciprocal decrease in PDK isoform expression (Koukourakis, Giatromanolaki *et al.* 2005, Koukourakis, Giatromanolaki *et al.* 2006). The increased PDH activity in surrounding tissues is hypothesized to assist in the detoxification of extracellular lactate produced by cancer cells (Koukourakis, Giatromanolaki *et al.* 2006). This metabolic symbiosis between cancer cells and stromal tissue may be enhanced through increasing thiamine availability, promoting PDH activity in normal tissue surrounding tumors.

In hypoxic microenvironemnts, Hif-1α translocation affects the expression of genes such as PDK1. Kim *et al.* demonstrated a hypoxia induced increase in the expression of PDK1 in human colon carcinoma. This group suggested that an increase in PDK1 supports not only a cells adaptation to hypoxia but also helps maintain adequate ATP levels and attenuates ROS production in the mitochondria. Interestingly, blocking the Hif-1α mediated stabilization of PDK1 resulted in apoptosis. This study hypothesized that the cause of apoptosis, following PDC activation, was a result of oxygen depletion (Papandreou, Cairns *et al.* 2006). Additional studies have shown that Hif-1α can also induce the induction of PDK3. This suggests that treatment methods targeted at the PDK family of enzymes present a potential target for therapeutic

intervention (Kim, Tchernyshyov *et al.* 2006). Although hypoxia leads to the stabilization of Hif-1α, alterations in cellular metabolism can also lead to PDK induction.

Using a metastatic head and neck squamous carcinoma cells McFate *et al.* showed that Hif-1α stabilization occurs under both normoxic and hypoxic conditions. They found that the metastatic cell line showed a greater level of PDK1, which increased the phosphorylation of PDH at site 1. Although they found a concentration dependent decrease in PDH-P293 in primary tumor cells, no change was observed in metastatic cells. Additionally the PDK-1 shRNA transfected cells metabolized [14C] glucose to [^{14}C] CO2 at twice the rate of cells transfected with control shRNA (McFate, Mohyeldin *et al.* 2008). Since PDK induction provides a metabolic advantage to cancer cells it illustrates a window for potential therapeutic intervention.

Restoration of PDH activity in cancer cells has been shown to promote apoptosis and is actively being assessed as a potential therapeutic strategy. A new approach to increasing the activity of the PDC is through the use of Dichloroacetic acid (DCA) (See Figure 1.10). DCA inhibits PDK-mediated phosphorylation of PDH in several cancer types (Michelakis, Webster *et al.* 2008, Madhok, Yeluri *et al.* 2010). The re-establishment of glucose oxidation in cancer cells through restored PDH activity results in an increase in mitochondrial ROS and cytochrome C release, ultimately leading to apoptosis (Bonnet, Archer *et al.* 2007, Xie, Wang *et al.* 2011). An additional study showed how inhibition of PDK1 activity, by the drug DCA, leads to a decrease in lactate production and pseudohypoxic Hif-1α stabilization. In this study, DCA decreased the level of phosphorylated PDH in primary cells but not in metastatic cells. Their study concluded that there may be other methods for restoring normal metabolism to cancer cells which might include targeting the vitamins and cofactors required for the activity of PDH (McFate, Mohyeldin *et al.* 2008).

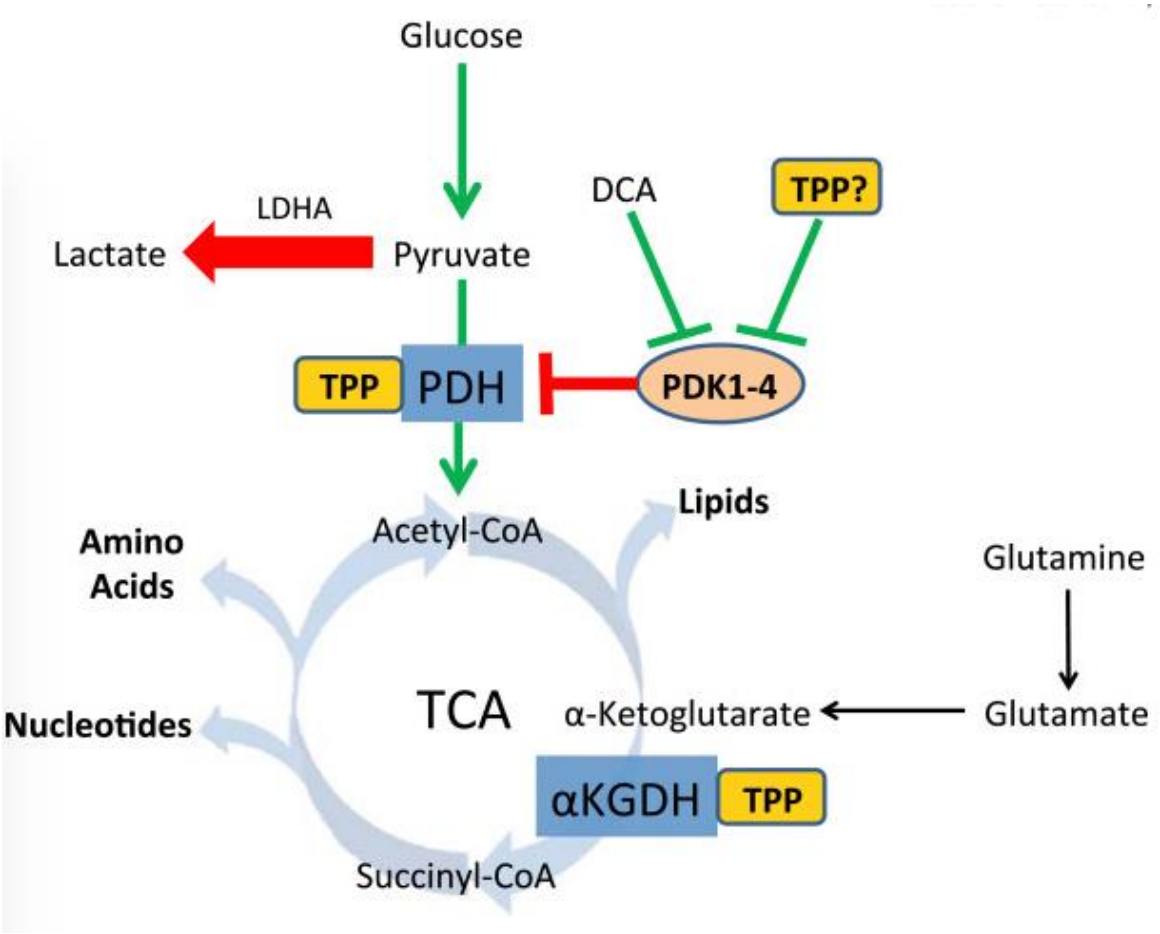

Figure 1.9. Malignant Metabolic Alterations. Malignant survival and proliferation is supported through the truncation of glycolytic metabolism at the thiamine-dependent pyruvate dehydrogenase axis. PDKs phosphorylate PDH and inhibit the conversion of pyruvate to acetyl-CoA. DCA is a pyruvate mimetic which inhibits the inactivation of PDH through its interaction with PDKs. TPP has also been shown to decrease the extent of phosphate incorporation into PDH by PDKS.

1.2.5.3.3.3. Alphaketoglutarate dehydrogenase (α-KGDH)

α-KGDH is located in the mitochondria and catalyzes the conversion of α-KG and NAD+ to succinyl-CoA and NADH. Like the PDC, the α-KGDH complex is composed of three subunits: a thiamine pyrophosphate-dependent dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) (Koike, Hamada *et al.* 1974, Koike and Koike 1976). The first two members of this complex, E1 and E2, are unique while the third is identical to the PDC (Perham 1991, Berg and de Kok 1997). The products of the PDC, NADH and CO_2 are also similar to the products of the α-KGDH reaction. As is the case with most enzymes, α-KGDH is regulated through feedback inhibition by end products of the reaction (Garland 1964, Smith, Bryla *et al.* 1974, Lawlis and Roche 1981). In addition to feedback inhibition, α-KGDH is regulated by calcium and ATP/ADP ratio. Denton *et al.* found that micromolar levels of calcium and ADP lower the Km of α-KGDH while ATP increases the Km (McCormack and Denton 1979, Denton and McCormack 1980).

Glutamate derived α-KG provides a carbon backbone structure that is used for the synthesis of nucleotides and amino acids (DeBerardinis, Mancuso *et al.* 2007). Additionally, α-KG can form citrate that can be used in lipid biosynthesis (Metallo, Gameiro *et al.* 2012, Mullen, Wheaton *et al.* 2012). A reverse in the flux of carbons into the TCA cycle, as observed in the reductive decarboxylation of α-KG, allows cells to bypass the use of α-KGDH. It has been suggested that hypoxic stress is a trigger in regulating α-KGDH and allowing α-KG to move in reverse (Wise, Ward *et al.* 2011, Metallo, Gameiro *et al.* 2012).

The TCA cycle plays a key role in supporting the synthesis of proteins, lipids, and nucleic acids, in proliferating cells. Intermediate molecules produced within this cycle support catapleurotic reactions that are necessary to support cell proliferation. For example, citrate can

be transported into the cytosol and converted to oxaloacetate (OAA) and acetyl-CoA. Within tumor cells an increase in ATP citrate lyase and fatty acid synthase function to support cell proliferation (Bauer, Hatzivassiliou *et al.* 2005, Hatzivassiliou, Zhao *et al.* 2005). Additionally the production of OAA and α -KG supply cells with nonessential amino acids that can be used in the production of both proteins and nucleotides (DeBerardinis, Lum *et al.* 2008). In the 1950's Eagle *et al.* showed that tumors consume a large amount of glutamine (Eagle, Oyama *et al.* 1956). Glutaminolysis, now considered to be a hallmark of tumor cell metabolism, allows cells to metabolize glutamine in a manner similar to the oxidation of glucose in aerobic metabolism. Metabolism of glutamine, the most abundant amino acids found in mammals, provides proliferating cells with intermediates necessary for bioenergetics and biosynthesis (Eagle, Oyama *et al.* 1956, Kovacevic and McGivan 1983). Forbes *et al.* also showed that estrogen stimulation of breast cancer cells induces glutaminolysis (Forbes, Meadows *et al.* 2006). The subsequent export of malate, a product of the TCA cycle, to the cytoplasm is a major source of NADPH production in tumor cells (DeBerardinis, Mancuso *et al.* 2007).

1.2.5.4. Thiamine related alterations observed in malignancy

1.2.5.4.1. The role of supplementation

In 1974 Basu *et al.* reported that patients with breast and bronchial carcinomas are often thiamine deficient (Basu, Dickerson *et al.* 1974). These deficiencies have also been reported in colorectal, uterine, and B-chronic lymphocytic leukemia (Ostrovskii Iu, Trebukhina *et al.* 1979, Seligmann, Levi *et al.* 2001, Bruce, Cirocco *et al.* 2005). It has been hypothesized that the thiamine deficiency associated with cancer is a result of the glycolytic sweet tooth that must be satisfied by the maintenance of enzyme cofactors. As a result of thiamine deficiency, medical

professionals often supplement thiamine to patients in order to overcome many of the deleterious side effects associated with this condition. Although no correlation has been shown between thiamine supplementation and incidence of cancer in humans, research has shown that higher doses of thiamine can support tumor growth (Bruce, Furrer *et al.* 2003).

Boros *et al.* also hypothesized that an excess of thiamine supplementation found in Western diet may correlate with an increase in cancer incidences (Boros 1999). In Western countries thiamine is often supplemented in food and as an over-the-counter vitamin. In direct contrast, African and Asian countries consume foods rich in thiaminase which naturally degrades thiamine and results in a reduced level of thiamine exposure (Boros 1999). Thiaiminase I, an enzyme that degrades thiamine, has been shown to inhibit the growth of breast cancer cells in vitro (Daily, Liu *et al.* 2011). Using MDA-MB-231 cells implanted into mice as a Xenograft, the same group showed a reduction in tumor proliferation when the animals were fed a thiamine free diet. Thiamine deficiency presents a unique problem in cancer treatment, as thiamine plays an important role in neurological function.

In 1987 Kaul *et al.* conducted a study in which they surveyed patients with prostate cancer and found that they consumed less thiamine when compared to individuals without cancer. This observation suggests a negative correlation between thiamine supplementation and cancer risk (Kaul, Heshmat *et al.* 1987). In 2006, Kabat *et al.* examined samples from breast, endometrial, ovarian, colorectal, and lung cancer and found no correlation between the intake of B vitamins and cancer risk (Kabat, Miller *et al.* 2008). Additional studies have suggested that thiamine deficiency plays a role in tumor development (Lee, Yanamandra *et al.* 2005). Taken together, this data suggests a differential role in malignant maintenance of thiamine homeostasis.

Over-the-counter vitamin supplementation is often viewed as harmless and potentially beneficial to one's health. Unfortunately, supplementation of thiamine to overcome deficiency-related complication is done with little understanding of thiamine's role in malignant progression. In 2001, Comin-Anduix *et al.* utilized an Ehrlich ascites tumor-mouse model to evaluate the effects of thiamine supplementation. They found a statistically significant increase in tumor growth in mice that were supplemented with thiamine at a level between 12.5 to 37.5 times the Recommended Daily Allowance (RDA). At 25 times the RDA the tumor volume in these mice grew to 250 times that of control mice administered thiamine at the RDA. Interestingly, at values above 75 times the RDA no change in tumor proliferation was observed. When these mice were supplemented with 2,500 times the RDA a decrease in proliferation was observed when compared to control animals (Comin-Anduix, Boren *et al.* 2001). These observations suggest that thiamine supplementation both supports and reduces malignant proliferation based solely upon dose.

A 2012 study examined the effects of a high fat diet on thiamine levels using a FVB/N-Tg(MMTVneu)202Mul/J transgenic breast cancer-tumor mouse model. In this study mice were fed either a diet that consisted of different fat and thiamine levels. Normal-fat (NF) diet, which contained 10% of the calories from fat, or a high-fat (HF) diet, which contained 60% of the calories from fat. Additionally, the low thiamine (LT) diet contained 2 mg of thiamine per 4,057 kcal while the normal thiamine (NT) diet contained 6 mg per 4,057 kcal. Mice fed a NF/LT diet displayed significantly longer (295 days) tumor latency when compared with animals fed a NF/NT diet (225 days). When NF/LT animals were give a HF diet this observation was eliminated the delay in tumor latency was eliminated (Daily, Liu *et al.* 2012). This data illustrates a cross-talk between diet and nutrient availability. These studies have established a

clear role of thiamine in malignant progression. In cases where patients require thiamine to overcome deficiency, thiamine should be carefully supplemented so as to avoid worsening this disease. As a direct result, the effects of thiamine supplementation on tumor progression require further study.

The role of thiamine in cancer progression is further illustrated through the evaluation of the effects of thiamine analogs on tumor proliferation. Oxythiamine, an anti-coenzyme of thiamine, reduces tumor proliferation both in vitro and in vivo (Boros, Puigjaner *et al.* 1997, Rais, Comin *et al.* 1999, Ramos-Montoya, Lee *et al.* 2006). This mimetic inhibits the thiamine dependent enzyme TKT thereby reducing R5P production and subsequent DNA and RNA synthesis. This alteration in nucleotide synthesis resulted in a G1 cell cycle arrest in Ehrlich's tumor cells (Rais, Comin *et al.* 1999). Chornyy *et al.* utilized rat PC-12 cells to show that oxythiamine also induced apoptosis through a caspase-3 signaling pathway (Chornyy, Parkhomenko *et al.* 2007). Liu *et al.* continued evaluating the effects of thiamine deficiency on tumor progression by adding thiaminase to cell culture media. This group found a significant decrease in breast cancer cell proliferation following culturing cells under these conditions (Liu, Monks *et al.* 2010). Thiamine's role in supporting bioenergetics with cancer cells was demonstrated by a reduction in ATP levels in lymphoid leukemia cells cultured in the presence of thiaminase (Daily, Liu *et al.* 2011). Together these findings suggest a strong connection between thiamine dependent pathways and malignant proliferation.

1.2.5.4.2. Thiamine status in cancer patients

Basu *et al.* utilized a whole blood TKT assay to quantify thiamine status in cancer patients (Basu, Dickerson *et al.* 1974, Basu and Dickerson 1976). This assay measures changes

in the activity of TKT after the exogenous addition of TPP. Samples that are thiamine deficient show an increase in TKT activity following the addition of TPP, termed the TPP effect (Chamberlain, Buttery *et al.* 1996). They found that patients with advanced stages of cancer exhibited a greater TPP effect when compared to control patients. This data suggests a decrease in thiamine status during malignancy. This group suggested that a decrease in thiamine status is a result of an inability to activate thiamine as opposed to an alteration in a patient's dietary intake. Other groups have gone on to quantify changes in thiamine and thiamine phosphorylated in whole blood. Tsao *et al.* found that patients with advanced stages of non-small cell lung cancer show a decrease in circulating TPP (Tsao, Yin *et al.* 2007). Additional studies have gone on to demonstrate the same phenomena in patients with acute myelomonocytic leukemia, Burkett's lymphoma, and B-chronic lymphocytic leukemia (van Zaanen and van der Lelie 1992, Seligmann, Levi *et al.* 2001). In each of these studies thiamine status was quantified in whole blood instead of tumor tissue. As a result the reduction in peripheral thiamine may be a consequence of increase uptake and utilization by cancer cells. Using an Ehrlich ascites tumor model, Trebukhina *et al.* demonstrated a correlation between tumor growth circulating thiamine depletion (Trebukhina, Ostrovsky *et al.* 1984). Biopsies of colon adenocarcinomas have shown a 2.5 fold increase in thiamine levels when compared to un-invaded tissues (Baker, Frank *et al.* 1981). Each of these studies suggests that a decrease in peripheral thiamine levels may be a direct result of an accumulation of thiamine in cancer cells and tissues.

1.3. RATIONALE AND GOAL

Within cancer cells, alterations in enzymatic activity support malignant progression through the maintenance of bioenergetic and biosysnthetic processes. However, an increase in metabolic

activity must be coupled with an adequate supply of nutrient cofactors to support enzyme activity. Thiamine, vitamin B1, is an essential vitamin that provides all cells with a necessary metabolic cofactor. Intracellular supply and activation of thiamine is mediated via thiamine transporters and the thiamine-activating enzyme, TPK. Once activated, TPP, serves as a necessary cofactor for three key enzymes within the glycolytic network. Interestingly, thiamine supplementation has a dichotomous effect on cancer cell growth. This effect is characterized by growth stimulation at low doses and growth suppression at high doses. Unfortunately, how thiamine reduces cancer cell proliferation is currently unknown. Therefore the overall goal of this research is to determine the effects of high dose thiamine on cancer cell proliferation and then evaluate how changes in thiamine homeostasis impact the effectiveness of high dose thiamine supplementation.

1.4. RESEARCH OBJECTIVES

1.4.1. Determine the impact of high dose thiamine supplementation on cancer cell proliferation

1.4.2. Establish the alterations in thiamine homeostasis that occur within breast cancer

1.4.3. Determine the influence of hypoxic tumor microenvironments on the expression and function of the thiamine activating enzyme, TPK1

CHAPTER 2

HIGH-DOSE VITAMIN B1 REDUCES PROLIFERATION IN CANCER CELL LINES ANALOGOUS TO DICHLOROACETATE[1]

[1] Bradley S Hanberry, Ryan Berger, and Jason A Zastre. (2014). *Cancer Chemother & Pharmacol. 73:585–594.* Reprinted here with permission of publisher.

2.1. ABSTRACT

Purpose: The dichotomous effect of thiamine supplementation on cancer cell growth is characterized by growth stimulation at low doses and growth suppression at high doses. Unfortunately, how thiamine reduces cancer cell proliferation is currently unknown. Recent focuses on metabolic targets for cancer therapy have exploited the altered regulation of the thiamine-dependent enzyme pyruvate dehydrogenase (PDH). Cancer cells inactivate PDH through phosphorylation by overexpression of pyruvate dehydrogenase kinases (PDKs). Inhibition of PDKs by dichloracetate (DCA) exhibits a growth suppressive effect in many cancers. Recently it has been shown that the thiamine co-enzyme, thiamine pyrophosphate reduces PDK mediated phosphorylation of PDH. Therefore, the objective of this study was to determine if high dose thiamine supplementation reduces cell proliferation through a DCA like mechanism.

Methods: Cytotoxicity of thiamine and DCA were assessed in SK-N-BE and Panc-1 cancer cell lines. Comparative effects of high dose thiamine and DCA on PDH phosphorylation were measured by Western blot. The metabolic impact of PDH reactivation was determined by glucose and lactate assays. Changes in the mitochondrial membrane potential, ROS production, and caspase-3 activation were assessed to characterize the mechanism of action.

Results: Thiamine exhibited a lower IC50 value in both cell lines compared to DCA. Both thiamine and DCA reduced the extent of PDH phosphorylation, reduced glucose consumption, lactate production, and mitochondrial membrane potential. High dose thiamine and DCA did not increase ROS but increased caspase-3 activity.

Conclusion: Our findings suggest that high dose thiamine reduces cancer cell proliferation by a mechanism similar to that described for dichloroacetate.

Key Words: Metabolism, Vitamin, Thiamine, Pyruvate Dehydrogenase, Dichloroacetate

2.2. INTRODUCTION

A high rate of glycolysis and an increase in lactate production have long been considered hallmarks of cancer. This oncogenically driven change in cellular metabolism supports an increase in biomass and ATP generation that is necessary to sustain rapid proliferation. A critical regulatory step supporting the enhanced glycolytic activity in cancer cells is the reduced activity of the vitamin B1 (thiamine) dependent enzyme pyruvate dehydrogenase (PDH), which limits the conversion of pyruvate to acetyl-CoA. The decrease in PDH activity reduces glucose carbon flux into the tricarboxylic acid (TCA) cycle and consequently excess glucose carbons are converted to lactate in order to regenerate NAD+ for continued ATP production. This truncation in glucose metabolism also facilitates the shunting of phospho-metabolites into additional metabolic pathways for biosynthetic reactions (Gatenby and Gillies 2007, Hsu and Sabatini 2008)

The activity of PDH is regulated through reversible phosphorylation reactions controlled by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase. PDK is a family of four isoenzymes (PDK1-4) that inactivate PDH through phosphorylation on three serine residues (Ser-293, Ser-300, and Ser-232) (Korotchkina and Patel 2001). PDK1 is the only isoform that has been shown to phosphorylate all three sites on PDH, while PDK2/3/4 primarily phosphorylate Ser-293 and Ser-300 (Kolobova, Tuganova *et al.* 2001). Although

phosphorylation at any of the three sites leads to inactivation, Ser-293 is the predominant phosphorylation site (Yeaman, Hutcheson *et al.* 1978, Sale and Randle 1981). Overexpression of PDK1 has been found in cancers such as breast, head and neck, and lung cancers (Koukourakis, Giatromanolaki *et al.* 2005, Lin, Hsieh *et al.* 2005). When PDK1 is knocked down, PDH activity and glucose oxidation are restored resulting in reduced lactate production and suppression of tumor growth (McFate, Mohyeldin *et al.* 2008). PDK isoforms have also been characterized as direct transcriptional targets for hypoxia inducible factor 1-α (Lu, Lin *et al.* 2008, Wigfield, Winter *et al.* 2008). Up-regulation of PDKs in hypoxic tumor microenvironments directly supports the increase in anaerobic glycolytic activity observed in hypoxic cancer cells (Kim and Dang 2006). Overexpression of PDKs is also associated with chemoresistance. Knockdown of PDK1 and PDK3 increased cytotoxicity of the anticancer drugs paclitaxel and cisplatin (Lu, Lin *et al.* 2008). Thus, inactivation of PDH activity in cancer cells through overexpression of PDKs is an important pro-survival pathway exploited by cancer cells.

The inhibition of PDK mediated phosphorylation of PDH has shown considerable promise as a targeted strategy to reduce cancer cell proliferation (Bonnet, Archer *et al.* 2007). One such compound, dichloroacetate (DCA) is effective at reducing cell growth in a number of malignancies including breast, endometrial, lung, and colorectal (Bonnet, Archer *et al.* 2007, Madhok, Yeluri *et al.* 2010, Sun, Fadia *et al.* 2010). Re-establishment of PDH function normalizes glycolytic activity in cancer cells, directing glucose carbons through mitochondrial oxidative phosphorylation pathways. Cancer cells with defective mitochondrial electron transport chain are unable to cope and as a result increase reactive oxygen species (ROS) generation, cytochrome C release, and ultimately apoptosis (Garber 2006, Papandreou, Goliasova *et al.* 2011). An advantage to targeting PDK activity is that overexpression of PDKs and extensive

phosphorylation of PDH is found in cancer cells and not in normal tissue (Madhok, Yeluri *et al.* 2010). This may provide for selective targeting towards malignant tissue and avoiding unwanted off target toxicity commonly observed with conventional chemotherapy.

PDH activity is also regulated by substrate and end product feedback. The products of the PDH reaction (NADH and acetyl-CoA) stimulate phosphorylation by PDKs, while an excess of substrates (pyruvate and ADP) or cofactor (thiamine pyrophosphate; TPP) inhibits phosphorylation (Roche and Hiromasa 2007). Kolobova *et al.* found that TPP decreased both the rate and extent of PDH phosphorylation by PDK isoforms (Kolobova, Tuganova *et al.* 2001). Thus increasing concentrations of TPP through thiamine supplementation may be pro-apoptotic through restoration of PDH activity in cancer cells similar to the mechanism observed with DCA. This may provide a mechanistic understanding as to why thiamine suppressed tumor growth at doses greater than 75 times the recommended daily intake (Comín-Anduix, Boren *et al.* 2001). Therefore, the objective of this study was to determine if high dose thiamine supplementation reduces cellular proliferation of cancer cells through a mechanism involving a reduction in PDH phosphorylation.

2.3. MATERIALS AND METHODS

Materials: All cell culture reagents including gentamicin and trypsin/EDTA were obtained from Mediatech (Manassas, VA). Custom formulated thiamine-deficient RPMI 1640 was also prepared by Mediatech to allow for the controlled addition of thiamine. Cell culture treated flasks, plates, and dishes were obtained from Greiner Bio-one (Monroe, NC). Fetal bovine serum (FBS), crystal violet, thiamine hydrochloride, and sodium dichloroacetate (DCA) were

purchased from Sigma Aldrich (St. Louis, MO). Actinomycin-D was obtained from. Cycloheximide was obtained from.

Cell Culture: The cancer cell lines Panc-1 (pancreatic) and SK-N-BE (neuroblastoma) were obtained from ATCC (Manassas, VA). All cell lines were maintained in thiamine-deficient RPMI 1640 supplemented with 30 nM thiamine, 10% FBS, and 0.2% gentamicin (referred to as T30 media). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, referred to as normoxia. All hypoxia treatments were conducted at 37°C with 5% CO_2 and 1% O_2 using an incubator outfitted with a ProOX oxygen controller (Biospherix, Lacona, NY) which supplies nitrogen gas. Oxygen levels and calibration were monitored daily using a Bacharach Fyrite Gas Analyzer (Bacharach, Inc., Pittsburgh, PA). All cell culture media for hypoxic treatments and subsequent media changes was pre-equilibrated in the hypoxic environment at least 24 h prior to use.

Cell Proliferation Assays: The effects of thiamine and DCA on cell proliferation were determined using the crystal violet assay as described previously (Journe, Laurent *et al.* 2008). Briefly, cells were seeded into 96 well plates at a density of 3,000 (Panc-1) or 9,000 (SK-N-BE) cells/cm^2 and allowed to attach overnight. Media was than replaced with increasing concentrations of either thiamine or DCA and cells allowed to grow for 5 days at 37°C. After which the media was aspirated and cells washed with phosphate buffered saline (PBS) than fixed with 10% neutral buffered formalin (EMD Millipore, Darmstadt, Germany) for 1 h at 4°C. The fixed cells were gently washed under running distilled water and stained with 0.1% crystal violet for 30 min, than washed again with distilled water and allowed to dry overnight. To quantify the

extent of crystal violet staining, cells were de-stained with 200 μL of 1% Triton X-100 and the absorbance measured at 550 nm using a Spectra Max M2e (Molecular Devices, Sunnyvale, CA) microplate reader. The proliferation was calculated as the percent viability comparing treated cells to control cells cultured in T30 media only. The IC50 values were determined using non-linear regression using GraphPad Prism 6 software.

Western Blotting: The effects of thiamine and DCA on the extent of PDH phosphorylation was assessed by Western blot. Cells were seeded into 60 mm cell culture dishes and upon reaching ~75% confluency were treated with either T30 media, T30 media + 25 mM thiamine, or T30 media + 25 mM DCA for 24 and 48 h at 37^0C. Following treatment, cells were washed with ice cold PBS and lysed using 50 mM Tris, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with protease inhibitor and phosphatase inhibitor cocktail (EMD Biosciences, La Jolla, CA). Lysates were then centrifuged at 14,000xg and 4°C for 20 min with the supernatant collected. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Whole cell lysates (50 μg) were electrophoresed on a 10% SDS-PAGE gel than transferred to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, Piscataway, NJ). The membrane was blocked with 3% bovine serum albumin (KSE Scientific, Durham, NC) in Tris-buffered saline tween 20 (TBS-T) for 1 h. The membrane was blotted for either total PDH-E1α (Genetex, Irvine, CA), PDH pSer-293, PDH pSer-300, PDH pSer232 (Calbiochem, La Jolla, CA), or β-Actin (Sigma Aldrich, St. Louis, MO) overnight at concentrations of 1:1000, 1:500 (for all PDH phosphorylation antibodies), and 1:1000, respectively in TBS-T. Blots were washed in TBS-T three times for 10 min each and blotted with either goat anti-mouse

horseradish peroxidase (HRP) secondary antibody (Millipore, Billerica, MA) for β-Actin at 1:20,000 or donkey anti-rabbit HRP (Bethyl, Montgomery, TX) for PDH-E1α and PDH pSer-293 at 1:15,000 in TBS-T for 1 h. Blots were again washed 3 times for 10 min with TBS-T, visualized using Supersignal West Pico (Thermo Scientific, Rockford, IL), and imaged using a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry was performed using Fluorchem SP software. The change in PDH phosphorylation is expressed as a ratio of control (Ser-293/PDHE1α) to treated (Ser-293/PDHE1α) cells.

Glucose and Lactate Assay: The effects of thiamine and DCA on glucose consumption and lactate production were assessed using commercially available glucose (Sigma Aldrich, St. Louis, MO) and L-Lactate assay (Eton Biosciences, San Diego, CA) kits. Cells seeded into 60 mm cell culture dishes were allowed to reach ~75% confluency than treated with either T30 media, T30 media + 25 mM thiamine, or T30 media + 25 mM DCA for 24 and 48 h at 37°C. Following treatment, media samples were collected and centrifuged at 14,000xg and 4°C for 5 min to pellet any cell debris. For the quantitation of glucose remaining in the media, 10 μL of media sample or standard was added to a 96-well plate followed by addition of the supplied assay reagent (90 μL). The reaction was allowed to proceed for 15 min at room temperature and the absorbance measured at 340 nm. For lactate, 50 μL of media sample or standard was placed into a 96-well plate followed by addition of 50 μL of the supplied assay reagent. The plate was set at 37°C for 30 min, after which the reaction was stopped by the addition of 50 μL of 0.5 M acetic acid (EMD Millipore, Darmstadt, Germany) and the absorption measured at 490 nm. Results were normalized to total cellular protein using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Mitochondrial Membrane Potential: The potentiometric dye tetramethylrhodamine methyl ester (TMRM) (Sigma Aldrich, St. Louis, MO) was used to assess changes in mitochondrial membrane potential (MMP). Cells were seeded into 48 well plates and cultured until ~75% confluency. Cells were treated with either T30 media, T30 media + 25 mM thiamine, or T30 media + 25 mM DCA for 48 h. The protonophore FCCP (Sigma Aldrich, St. Louis, MO) was added 3 h prior to TMRM addition to a group of T30 media control cells (25 μM) as a positive control for changes in membrane polarization. TMRM was than added to each well (25 nM) and cells incubated at 37°C for 30 min. The media was then aspirated and wells washed twice with PBS (37°C) to remove extracellular TMRM. An aliquot of PBS (500 μL) was then placed into each well and the fluorescence was measured at EX=550 nm and EM=575 nm using a Spectra Max M2e microplate reader. Data was normalized to total cellular protein, which was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Hydrogen Peroxide (H_2O_2): Production of H_2O_2 was determined using the commercially available hydrogen peroxide fluorometric detection kit (Enzo Life Sciences, Farmingdale, NY). Cells were treated with the oxidative phosphorylation inhibitor rotenone (5 μM) (Calbiochem, La Jolla, CA), 25 mM thiamine, or 25 mM DCA for 30 min, 1 h, and 2 h prior to assay. After treatment, the media was removed and wells washed twice with PBS (37°C). 200 μL of supplied reaction mixture (Detection Reagent, Horse Radish Peroxidase, and PBS) was added to each well and the cells were allowed to incubate for 10 min at room temperature. Fluorescence was then measured at EX=550 nm and EM=590 nm. Data was normalized to total cellular protein as determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Caspase-3: Activation of caspase-3 was measured using the commercially available caspase-3 fluorescence assay kit (Cayman Chemical Company, Ann Arbor, MI). Treatment of cells with thiamine and DCA was performed as described above for the TMRM assay. 24 h prior to caspase-3 assay a set of control cells were treated with the topoisomerase I inhibitor (1 μM) SN-38 (LKT Laboratories, St. Paul, MN) as a positive control for caspase-3 induction. Following 48 h of thiamine and DCA treatment, the cells were washed with supplied caspase-3 assay buffer. 100 μL of supplied lysis buffer was added to each well and incubated for 30 min at room temperature on an orbital plate shaker. Cell lysates were collected and centrifuged for 10 min at 800xg after which 90 μL of supernatant was transferred to a 96-well plate. 100 μL of the supplied caspase-3 substrate solution was then added to each well and the plates were incubated at 37°C for 30 min. The fluorescence was measured at EX=485 nm and EM=535 nm. Data was normalized to total cellular protein as determined using the Bio-Rad Protein Assay Kit (Hercules, CA) and expressed as the relative fluorescence per mg protein.

Statistical Analysis: All experiments were performed with a minimum of three independent experiments unless otherwise noted. Statistical significance was evaluated using unpaired Students t-test with a significance level of $p<0.05$ using Graph Pad Prism 6.

2.4. RESULTS

Thiamine reduces cancer cells proliferation. The crystal violet assay was used to assess the effects of thiamine and DCA on cell proliferation. Figure 2.1 displays a decrease in cell proliferation for SK-N-BE and Panc-1 cells with increasing concentration of thiamine and DCA

following 5 days of treatment. The IC50 values for DCA were 23.8 for SK-N-BE and 10.3 mM Panc-1. Comparatively, the IC50 of thiamine was lower than DCA for both cell lines with values of 4.9 for SK-N-BE and 5.4 mM for Panc-1.

High dose thiamine reduces the extent of PDH phosphorylation. Based on cell proliferation results, a concentration of 25 mM of either thiamine or DCA was chosen for short-term studies as this dose provided the greatest decrease in cell proliferation after 5 days (Fig. 2.1). In both SK-N-BE and Panc-1 cells, DCA decreased the extent of phosphorylated PDH at Ser-293 as early as 24 h of treatment (Fig. 2.2). The ratio of Ser-293 to PDHE1α was calculated for each treatment group and was used to assess the reduction of phosphorylation. The ratio of untreated SK-N-BE to cells treated with DCA was 0.21 and 0.09 following 24 and 48 h treatment respectively. SK-N-BE cells treated with thiamine resulted in a ratio of 0.17 and 0.01 following 24 and 48 h treatment respectively. Treatment of Panc-1 cells with DCA resulted in a 0.29 and 0.42 ratio following DCA treatment and a 0.33 and 0.11 ratio following thiamine treatment. No detectible phosphorylation at Ser-300 or Ser-232 was found in control or in treated cells (data not shown).

High dose thiamine reduces glucose consumption and lactate production. In both cell lines, thiamine and DCA significantly decreased the consumption of glucose after 24 and 48 h treatment (Fig. 2.3). Both DCA and thiamine significantly reduced the level of extracellular lactate secreted by Panc-1 cells (Fig. 2.3). However, DCA significantly reduced lactate secretion in SK-N-BE cells while thiamine had no effect after 24 and 48 h exposure (Fig. 2.3).

Effect of high dose thiamine on mitochondrial polarization. The potentiometric dye TMRM was employed to assess the impact of thiamine and DCA on altering mitochondria polarization. As a positive control, the protonophore FCCP significantly decreased mitochondrial membrane polarization in both cell lines evaluated (Fig. 2.4). Following 48 h of treatment both thiamine and DCA resulted in a statistically significant decrease in mitochondrial membrane potential. (Fig. 2.4).

Effect of high dose thiamine on ROS production. As a measure of intracellular ROS, cells treated with thiamine or DCA were assayed for peroxide following 30 min, 1 h, and 2 h of treatment. No significant change in ROS was observed over all time points with thiamine and DCA as well as with the oxidative phosphorylation inhibitor rotenone (Fig. 2.5).

High dose thiamine increases caspase-3 activity. Induction of caspase-3 activity was used to assess apoptosis in SK-N-BE and Panc-1 cells after thiamine and DCA treatment. Both thiamine and DCA significantly increased caspase-3 activity after 48 h of treatment (Fig. 2.6).

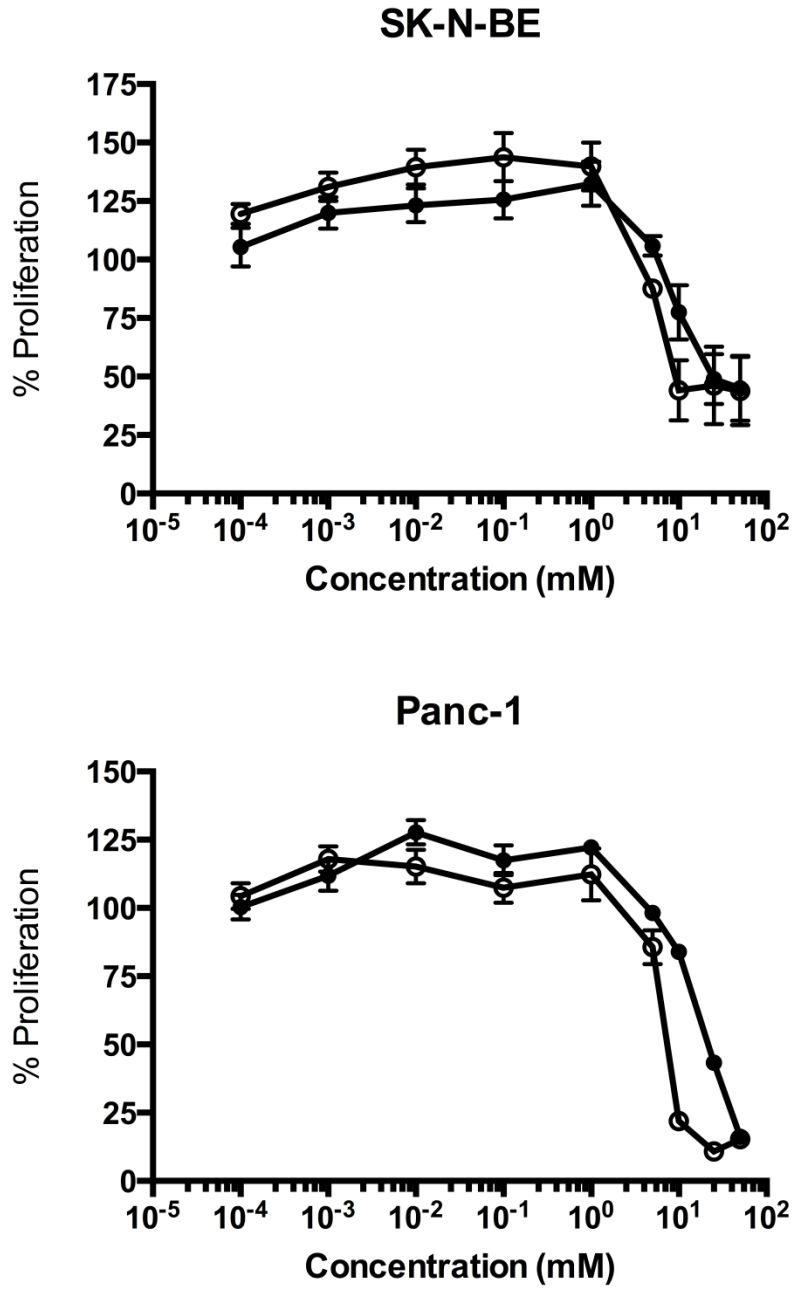

Figure 2.1 Cell proliferation of SK-N-BE and Panc-1 cells. Cells seeded into 96-well plates were treated with increasing concentrations of thiamine (○) or DCA (●) for 5 days. The extent of cell proliferation was measured using crystal violet. Results are presented as the percent proliferation comparing treated cells to untreated cells. Data represents mean ± SD on n=3 independent experiments.

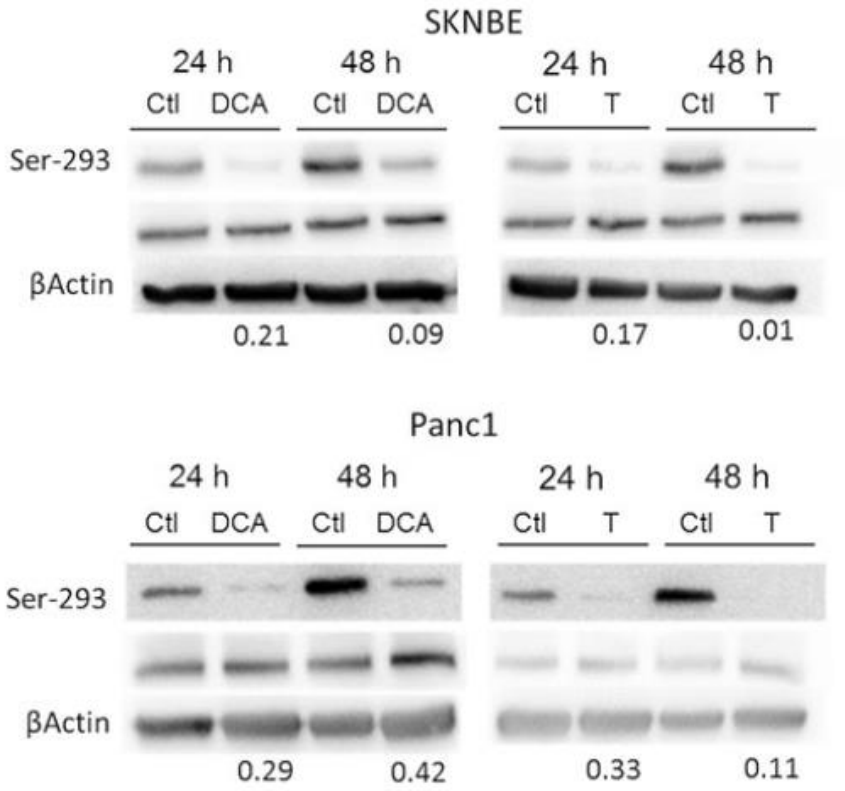

Figure 2.2 Representative Western blots of PDH phosphorylation at Ser-293. SK-N-BE and Panc-1 cells were treated with 25 mM of either thiamine (T) or DCA for 24 and 48 h. Whole cell lysates were immuno-fractionated on a 10% SDS-PAGE gel and probed for phosphorylation at Ser-293, total PDH (PDH-E1α subunit), and β-Actin as a loading control. Numbers below blots represent the ratio of Ser-293 to PDHE1α in control cells divided by the ratio of Ser-293 to PDHE1α in corresponding thiamine or DCA treated cells to indicate changes in the level of PDH phosphorylation.

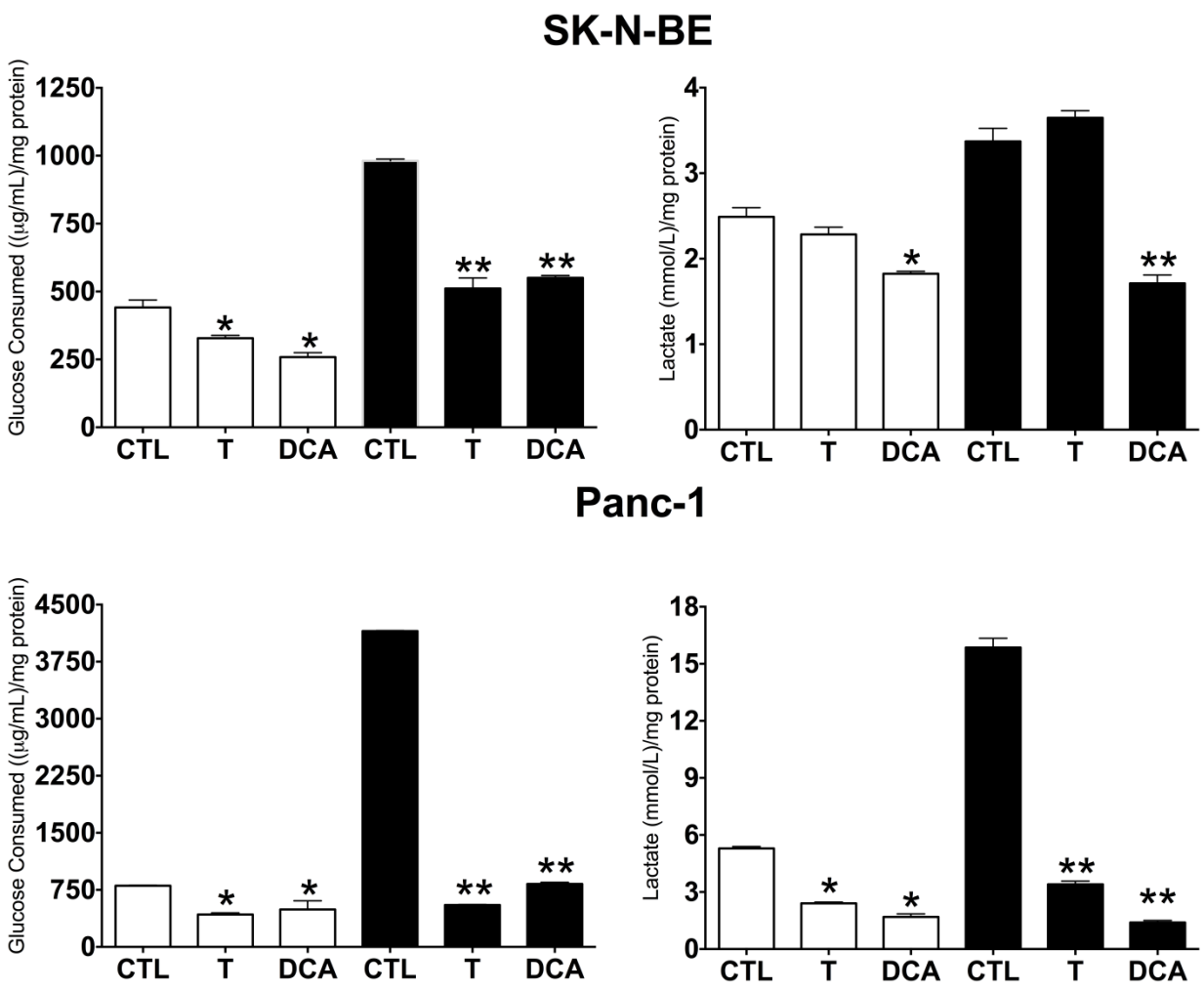

Figure 2.3 Glucose consumption in (A) SK-N-BE and (B) Panc-1 cells was assayed after 24 (open bars) and 48 h (solid bars) of treatment with either 25 mM thiamine (T), or 25 mM DCA. Lactate production in (C) SK-N-BE and (D) Panc-1 cells was assayed after 24 (open bars) and 48 h (solid bars) of treatment with either 25 mM thiamine (T), or 25 mM DCA. Data represents mean ± SD with n=3 independent experiments. Statistically significant ($p<0.05$) differences are expressed as comparisons between 24 h (✶) control media (T30) and corresponding thiamine or DCA treated cells. Statistically significant ($p<0.05$) differences are expressed as comparisons between 48 h (✶✶) control media (T30) and corresponding thiamine or DCA treated cells.

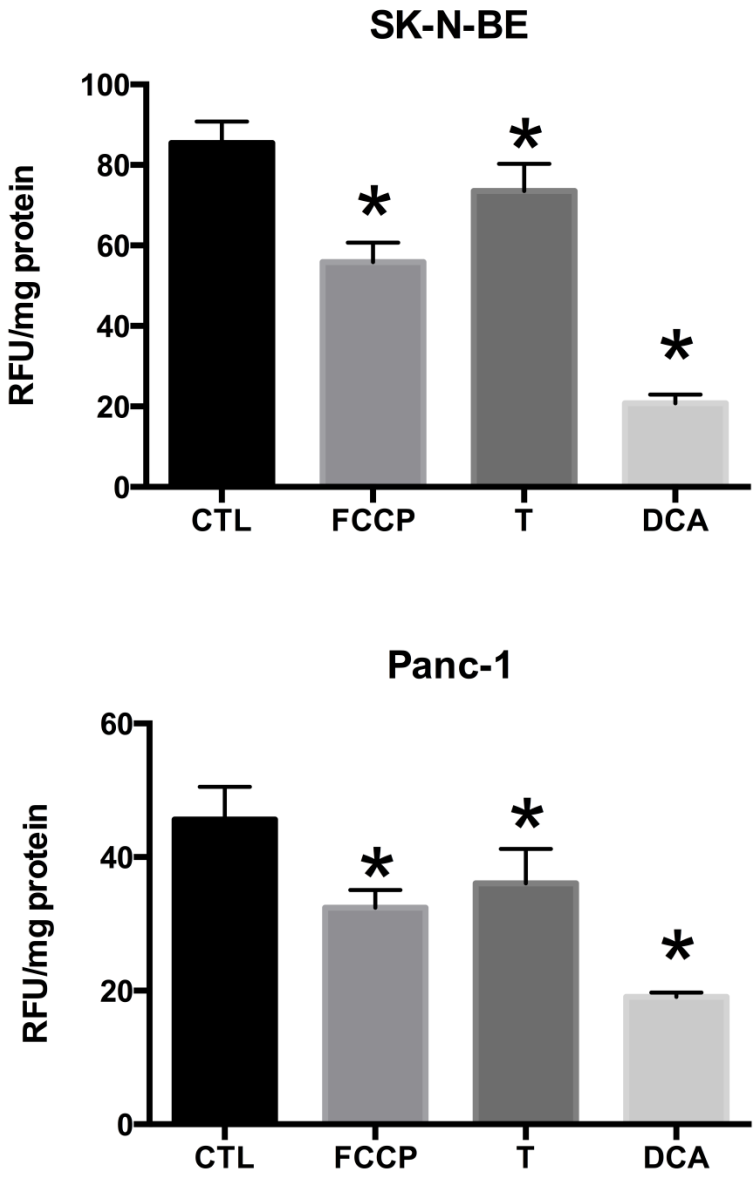

Figure 2.4 Changes in the mitochondrial membrane potential of SK-N-BE and Panc-1 cells after thiamine and DCA treatment. Cells treated with T30 media (CTL), 25 μM FCCP, 25 mM thiamine (T), or 25 mM DCA for 48 h prior to loading (30 min) with 25 nM TMRM and fluorescence measured at EX=540 and EM=575 nm. Data represents mean ± SD on n=3 independent experiments. (✶) Statistically significant ($p<0.05$) differences between control (T30 media) and treatments.

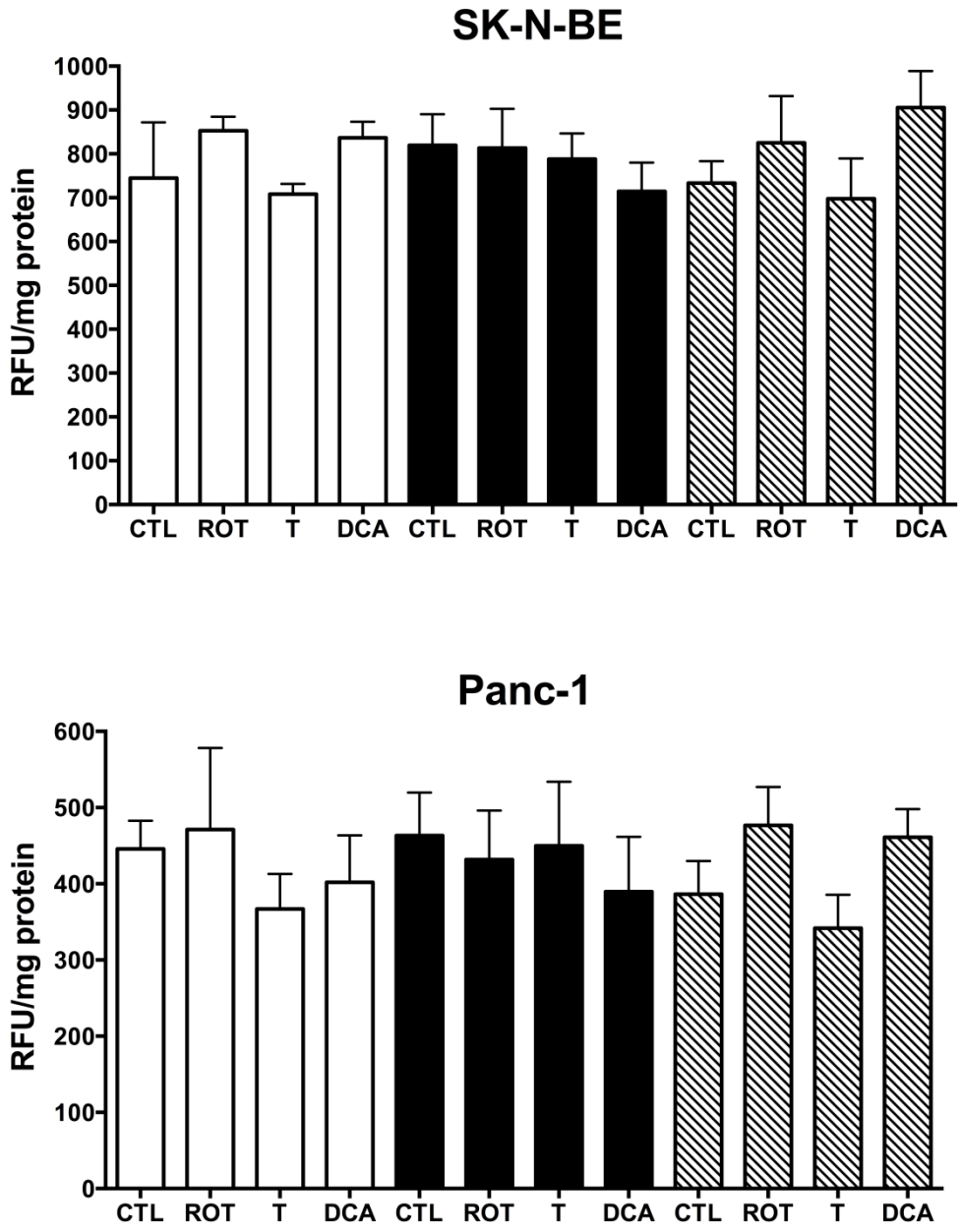

Figure 2.5 Production of H_2O_2 in SK-N-BE and Panc-1 cells. Cells were treated with T30 media (CTL), 25 mM thiamine (T), 25 mM DCA, or 5 μM rotenone for 30 min (open bars), 1 h (solid bars), and 2 h (hash bars). Data represents mean ± SD on n=3 independent experiments. (*) Statistically significant ($p<0.05$) differences between control (T30 media) and treatments.

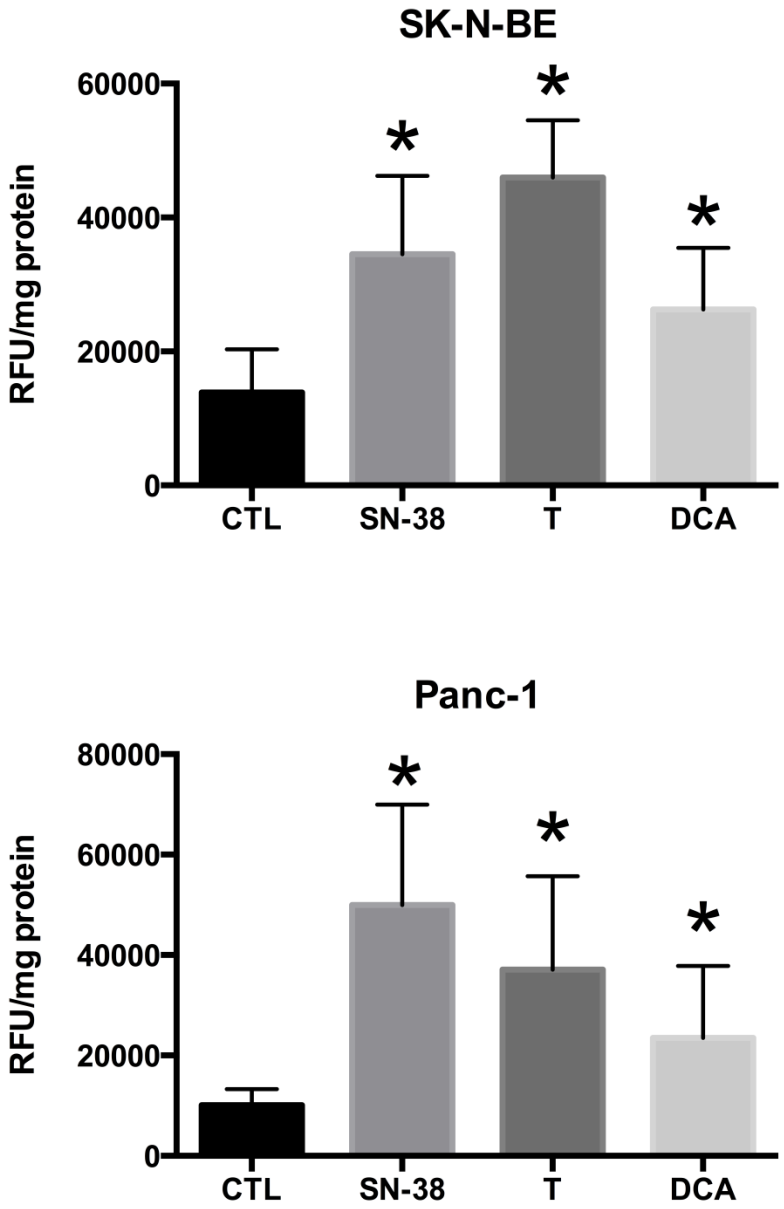

Figure 2.6 Activation of capase-3 in SK-N-BE and Panc-1 cells. Cells were treated with T30 media (CTL), 25 mM thiamine (T), 25 mM DCA, or 10 μM SN-38 for 48 h prior to assaying for caspase-3 activation. Data represents mean ± SD on n=3 independent experiments. (*) Statistically significant ($p<0.05$) differences between control (T30 media) and treatments.

2.5. DISCUSSION

Vitamin B1 supplementation has a duality of effects on cancer cell survival and proliferation. At low to moderate doses, thiamine has been shown to support cancer cell proliferation. Comín-Anduix *et al.* found an increase in tumor proliferation at values from 12.5 to 75 times the recommended daily allowance (RDA) in an Ehrlich ascites tumor model (Comín-Anduix, Boren *et al.* 2001). This stimulation of cell proliferation may be supported by alterations in expression and utilization of thiamine-dependent enzymes during malignancy. In particular, the thiamine-dependent enzyme transketolase has been shown to be up-regulated in a variety of cancers including colon, urothelial, breast, ovarian, and gastric and is essential for generation of nucleotide precursors to sustain rapid proliferation (Langbein, Zerilli *et al.* 2006, Staiger, Coy *et al.* 2006, Foldi, Stickeler *et al.* 2007, Krockenberger, Honig *et al.* 2007). However, at high doses (>75 times the RDA) no increase in tumor growth was found compared to control suggesting an anti-proliferative effect in cancer cells (Comín-Anduix, Boren *et al.* 2001). Using a pancreatic and neuroblastoma cell line, thiamine reduced cell proliferation with an IC50 of 4.9 and 5.4 mM, respectively. Although the IC50 with thiamine was lower than DCA, both reduced proliferation of SK-N-BE and Panc-1 cells at similar concentrations previously reported for DCA (Stockwin, Yu *et al.* 2010). These results indicate that thiamine has a similar dose response profile when compared to DCA.

Mechanistically, DCA stimulates PDH activity by acting as a pyruvate mimetic and blocking the action of PDK mediated phosphorylation (Knoechel, Tucker *et al.* 2006). The presence of TPP has also been shown to block the phosphorylation of PDH by PDKs (Kolobova, Tuganova *et al.* 2001). Figure 2.2 demonstrates that both DCA and thiamine reduced PDH

phosphorylation in both SK-N-BE and Panc-1 cells. Increasing PDH activity in cancer cells can lead to a shift in glycolytic metabolism that promotes reduced glycolytic flux and reduced conversion of pyruvate to lactate. DCA treatment reduces glucose consumption and lactate production in tumors as a consequence of increased PDH activity (Michelakis, Webster *et al.* 2008, Niewisch, Kuci *et al.* 2012). Similar to DCA, high dose thiamine decreased glucose consumption in both cell lines. However, high dose thiamine reduced lactate production only in Panc-1 cells, while DCA reduced lactate levels in both. It is unclear why lactate levels did not change following 48 h of treatment in the neuroblastoma cell line SK-N-BE. In a panel of neuroblastoma cell lines, DCA reduced lactate in some while having no effect on lactate production in others (Niewisch, Kuci *et al.* 2012). Additionally, the high dose of thiamine may have impacted the activity of other thiamine-dependent enzymes. The increase in PDH activity by high dose thiamine and subsequent increase in acetyl-CoA production may have increased the flux of glucose carbons through the TCA cycle. The thiamine-dependent enzyme α-ketoglutarate dehydrogenase found within the TCA cycle, converts α-ketoglutarate to succinyl-CoA that continues on to form maleate. The increased TCA flux may facilitate maleate conversion back to pyruvate and subsequently to lactate (Owen, Kalhan *et al.* 2002). High dose thiamine may have promoted increased lactate derived through the TCA cycle that counteracted the activity of PDH on reducing lactate production. Thus, the cellular metabolic phenotype may be an important dictator on the metabolic effects observed with high dose thiamine similar to the variability reported for DCA (Papandreou, Goliasova *et al.* 2011).

DCA mediated apoptosis is considered to be the result of mitochondria-dependent pathways initiated by the loss of MMP and subsequent release of pro-apoptotic factors (Grivicich, Regner *et al.* 2005, Madhok, Yeluri *et al.* 2010). Both DCA and thiamine

significantly reduced the MMP in SK-N-BE and Panc-1 cells. An increase in caspase-3 activation was also found for both DCA and thiamine consistent with an apoptotic mechanism. A consequence of the normalization of glycolysis through PDH activation is an increase in ROS production. DCA mediated ROS generation and reduction of MMP has been shown to induce the Kv1.5 potassium channel expression as a possible contributor for caspase activation (Bonnet, Archer *et al.* 2007). Using peroxide as a measure of ROS, neither DCA, thiamine, nor rotenone induced ROS after 2 h of treatment in SK-N-BE and Panc-1 cells. This may suggests that the activation of caspase 3 by DCA and thiamine in SK-N-BE and Panc-1 cells maybe unrelated to a burst in ROS. A similar lack of significant ROS generation by DCA in A549 cancer cells was associated with JNK1 activation (Ayyanathan, Kesaraju *et al.* 2012). Conversely, DCA treatment to several cancer cell lines reduced MMP and ROS generation with no observable apoptotic response. Interestingly, the same study demonstrated the lack of Kv1.5 induction even though DCA induced ROS generation and caspase activation (Stockwin, Yu *et al.* 2010). Thus the effects of PDH activation by either DCA or thiamine on initiating mitochondria mediated apoptosis needs to be further defined and may be highly dependent on cancer cell phenotypes.

A particular advantage of DCA treatment is the targeting of a metabolic phenotype found in cancer cells that is not observed in normal tissue (Michelakis, Webster *et al.* 2008). However, the high dose (mM) of DCA required to reduce cancer proliferation may limit the use *in vivo*. Doses as high as 25 mg/kg/day (~1.75 g/day for 70 kg patient) of DCA has been found to result in toxic neuropathy in patients with mitochondrial myopathy, encephalopathy, and stroke-like episodes (Kaufmann, Engelstad *et al.* 2006). Thiamine is an essential vitamin required for normal health and an endobiotic that the body has functional mechanisms to regulate homeostasis (Singleton and Martin 2001). Although our findings demonstrate that doses of

thiamine (mM) required to reduce cancer cell proliferation are similar to DCA, thiamine has few dose limiting toxicities. Smithline *et al.* reported no adverse effects in healthy patients who were given 1.5g/day of thiamine (Smithline, Donnino *et al.* 2012). Only minor side effects, such as nausea and indigestion were reported in patients given doses as high as 7.5 g/day (Meador, Loring *et al.* 1993). Thus high dose thiamine supplementation may be a safer and more tolerated treatment option.

The impact of thiamine homeostasis on the dose response may be an important component. Intracellular uptake of thiamine is dependent on the activity of the thiamine transporters THTR1 and THTR2 (Ganapathy, Smith *et al.* 2004). Once in the cells, thiamine is rapidly converted to the active cofactor TPP by thiamine pyrophosphokinase-1 (TPK-1). Transport of TPP across the mitochondrial membrane for PDH activity is mediated by the thiamine pyrophosphate carrier, which is encoded by the *SLC25A19* gene (Lindhurst, Fiermonte *et al.* 2006). Since only TPP has been reported to reduce PDH phosphorylation, the intracellular uptake and conversion of thiamine to TPP maybe an important contributor to the dose response. Overexpression of TPK1, *SLC19A2*, and *SLC25A19* in breast cancer tissue samples compared to normal breast tissue was recently described (Zastre, Hanberry *et al.* 2013). Thus, the cellular availability of thiamine may be augmented by the increase in thiamine homeostasis genes in cancer cells. Alternatively, a potential non-cofactor role of thiamine may contribute towards reducing cellular proliferation. Free thiamine has direct antioxidant properties as well as being essential for glutathione production (Lukienko, Mel'nichenko *et al.* 2000, Martin, Singleton *et al.* 2003, Schmid, Stopper *et al.* 2008). Antioxidant treatment of cancer cells has been described to have an anti-proliferative and pro-survival response through either reducing ROS status or protection against ROS stress (Trachootham, Alexandre *et al.* 2009). Thus, differences in

expression of thiamine homeostasis genes and the extent of intracellular thiamine and TPP levels may dictate the dose response and/or mechanism for reduced cell proliferation by high dose thiamine.

In conclusion, we have demonstrated that high dose thiamine decreased proliferation in a mechanism similar to that of DCA in SK-N-BE and Panc-1 cells. Both thiamine and DCA reduced PDH phosphorylation, decreased MMP and induced caspase-3 mediated apoptosis. These findings describe a potential mechanism by which high dose thiamine reduced *in vivo* tumor growth (Comín-Anduix, Boren *et al.* 2001). Treatment regimens with high dose thiamine may be a safer more tolerated alternative to DCA supplementation. Future work will need to establish the role of thiamine homeostasis in the dose response and if any non-cofactor roles of thiamine may contribute to the mechanism of toxicity.

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CHAPTER 3

HYPOXIA INDUCED UP-REGULATION AND FUNCTION OF THE THIAMINE ACTIVATING ENZYME THIMIANE PYROPHOSPHOKINASE 1 IN MDA-MB-231 BREAST CANCER CELLS[1]

[1] Submitted to Plos One. Bradley S Hanberry, Babak Basiri, Michael G Bartlett, and Jason A Zastre. (2014).

3.1. ABSTRACT

Hypoxia induces alterations in the expression and function of enzymes within the metabolic network. Many of these enzymes require an adequate supply of cofactors to support their metabolic activity. In particular, thiamine provides a cofactor for three enzymes within the metabolic network; transketolase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase. Within cells, thiamine must be converted to thiamine pyrophosphate (TPP) by thiamine pyrophosphokinase (TPK1) prior to functioning as an enzyme cofactor. Under hypoxic conditions a two-fold increase in the expression of TPK1 was observed independent of thiamine dose. A corresponding increase in intracellular thiamine and TPP levels was also found in cells cultured in hypoxia compared to those cultured in normoxia. Assessment of TPK1 regulation showed no change in gene transcription but a hypoxia induced increase in TPK1 protein translation. No change in the cell surface expression of the thiamine transporters THTR1 and THTR2 were found after hypoxic exposure suggesting that other transport processes may be involved to increase thiamine uptake. When TPK1 was exogenously overexpressed, an increase in intracellular TPP was found but no change in free thiamine suggesting that that increase in TPP during hypoxia was the result of an increase in TPK1 function and not due to increases in thiamine. Overall, the adaptive increase in thiamine homeostasis may be an essential component in the anaerobic metabolic shift associated with hypoxic tumor microenvironments.

3.2. INTRODUCTION

Within solid tumors, rapid cell proliferation results in a dysfunctional vascular network which limits the diffusion of oxygen (Denko 2008). The resulting hypoxic tumor

microenvironment contributes to chemoresistance, decreased radiosensitivity, and amplified metastatic potential (Churchill-Davidson, Sanger *et al.* 1955, Gray 1961, van den Brenk, Moore *et al.* 1972, Youngberg and Myers 1990). Hypoxia also induces alterations in the expression and function of metabolic enzymes such as Hexokinase-2, Aldolase, Glyceraldehyde 3-phosphate dehydrogenase, and Enolase (Semenza, Roth *et al.* 1994, Gleadle and Ratcliffe 1997, Iyer, Kotch *et al.* 1998). These metabolic adaptations necessitate a constant supply of nutrients and micronutrients to maintain biomass and energy production that supports cell survival and proliferation (Grander 1998, Bertram 2000, Wise, DeBerardinis *et al.* 2008). However, limitations in the penetration of nutrients throughout the solid tumor require adaptive changes in the expression and activity of transporters to facilitate intracellular uptake. For instance, an increase in the expression and function of glucose transporters (GLUT1 and 3) is commonly observed in hypoxic cancer cells to support the increase in glycolysis (Chen, Pore *et al.* 2001). However, cancer cells must also adaptively regulate the intracellular supply and activation of cofactors that are critical for enzymatic activity (Cairns, Harris *et al.* 2011).

In particular, Vitamin B1 (thiamine) is an essential micronutrient and important component of cellular growth and metabolism within all cells. The facilitated transport of thiamine by THTR1 (SLC19A2) and THTR2 (SLC19A3) is necessary to ensure intracellular availability. Following transport into the cell, thiamine is phosphorylated to the active form thiamine pyrophosphate (TPP) by the diphosphotransferase enzyme Thiamine Pyrophosphokinase-1 (TPK1) (Yoshioka 1984, Bettendorff 1995, Liu, Timm *et al.* 2006). TPP functions as a cofactor for three key enzymes within the metabolic network; transketolase (TKT), pyruvate dehydrogenase (PDH), and α-ketoglutarate dehydrogenase (α-KGDH). TKT links glycolysis with the pentose phosphate pathway (PPP) and catalyzes a reversible conversion that

produces ribose-5-phosphate for use in *de novo* nucleotide synthesis. Boros *et al.* found that 85% of ribose in malignant cells is generated through the non-oxidative portion of the PPP (Boros, Puigjaner *et al.* 1997). Hypoxia has also been shown to induce an increase in the expression of the thiamine-dependent enzyme TKTL1 (Transketolase-like Protein 1) in colorectal cancer cells (Bentz, Cee *et al.* 2013). PDH links glycolysis to the TCA cycle by catalyzing the conversion of pyruvate to acetyl-CoA. Although the Warburg hypothesis suggests that the rapid glycolysis observed in cancer cells is a result of mitochondrial dysfunction, some tumors maintain active active glucose oxidation through PDH (Marin-Valencia, Yang *et al.* 2012). α-KGDH plays an essential role in the TCA cycle and glutamine metabolism by catalyzing the conversion of alpha-ketoglutarate (α-KG) to succinyl-CoA. Utilization of glutaminolysis allows cells to metabolize the most abundant amino acid, glutamine, to produce energy, nucleic acids, and serine (Eagle, Oyama *et al.* 1956, Kovacevic and McGivan 1983). In human glioblastoma cells, hypoxia promotes glutamine metabolism through α- KGDH (Wise, Ward *et al.* 2011). Therefore, maintaining an adequate intracellular supply of thiamine and subsequent activation to the TPP cofactor may be necessary to support the activity of these metabolic enzymes within hypoxic tumor microenvironments.

Interestingly, patients with breast, bronchial, non-small cell lung cancer, leukemia, and lymphoma often exhibit a reduction in peripheral thiamine levels potentially through an increase in thiamine uptake by malignant cells (Basu and Dickerson 1976, van Zaanen and van der Lelie 1992, Seligmann, Levi *et al.* 2001). Zastre *et al.* recently demonstrated an increase in the expression of SLC19A2 and TPK1 in breast cancer tissue when compared to normal breast tissue (Zastre, Hanberry *et al.* 2013). Furthermore, an adaptive increase in SLC19A3 was found after exposing breast cancer cells and neural fibroblasts to hypoxia (Sweet, Paul *et al.* 2010, Schänzer,

Döring *et al.* 2014). Together these findings suggest that alterations in thiamine homeostasis potentially support cancer cell metabolism. However, thiamine must first be converted to its active form, TPP before it can serve as an enzyme cofactor. Unfortunately there is little information regarding the changes in thiamine phosphorylation in hypoxic cancer cells. Therefore, the present study was undertaken to determine the expression and function of TPK1 during hypoxic stress.

3.3. MATERIALS AND METHODS

Materials: All cell culture reagents including gentamicin and trypsin/EDTA were obtained from Mediatech (Manassas, VA). Custom formulated thiamine-deficient RPMI 1640 was also supplied by Mediatech to allow for the controlled addition of thiamine. Cell culture treated flasks, plates, and dishes were obtained from Greiner Bio-one (Monroe, NC). Fetal bovine serum (FBS) was purchased from Seradigm (Radnor, PA). Thiamine hydrochloride was purchased from Sigma Aldrich (St. Louis, MO). Actinomycin D (ActD) and cycloheximide (CHX) were purchased from Calbiochem/EMD Milipore (Billerica, Massachusetts).

Cell Culture: The triple negative breast cancer cell line MDA-MB-231 was obtained from ATCC (Manassas, VA). Cells were maintained in custom formulated thiamine-deficient RPMI 1640 supplemented with 10% FBS, and 0.2% gentamicin. HPLC analysis confirmed that the addition of FBS provided approximately 15nM thiamine, a physiologically relevant concentration (Gangolf, Czerniecki *et al.* 2010). No alteration in cell proliferation was observed during the culturing of the cells at 15nM thiamine. Cells cultured at 37°C in a humidified atmosphere of 5% CO_2 are referred to as normoxic conditions.

Cloning and over-expression of TPK1: TPK1 variant 1 was cloned from human testis cDNA (Clontech Laboratories Inc., Mountain View, CA) by PCR amplification**.** Although two isoforms of the TPK1 gene exist, only variant 1 (NM_022445.3) has been shown to catalyze the activation of thiamine (Nosaka, Onozuka *et al.* 2001). TPK1 splice variant 2 (NM_001042482.1) lacks a 49 amino acids portion of the full-length protein and therefore a functional role in thiamine phosphorylation is unlikely (Mayr, Freisinger *et al.* 2011). Cloning primers for TPK1 were constructed (F: 5'-TCC***GCTAGC***ATGGAGCATGCC-3' and R: 5'-TCC**GGTACC**TTAGCTTTTGACGGCC-3') to be flanked with ***NheI*** (Forward) and **KpnI** (Reverse) restriction sites. PCR was preformed at 57°C for 32 cycles and the resulting 734 bp product was excised and purified using a Gel Extraction Kit (Omega Bio-tek, Norcross, GA). The resulting fragment was then digested and ligated into pcDNA3.1(+) (Life Technologies, Grand Island, NY). The sequence was verified using the Georgia Genomics Facility (Athens, GA). The resulting TPK1 construct and vector control were then transfected into MDA-MB-231 cells using Fugene 6 (Promega, Madison, WI). Stable transfection was achieved through the use of G418 (KSE Scientific, Durham, NC) selection (0.5 mg/mL).

Hypoxic Exposure: All hypoxic treatments were conducted at 37°C in a humidified atmosphere of 5% CO_2 and 1% O_2 using an incubator outfitted with a ProOx oxygen controller (Biospherix, Lacona, NY) which regulated the supply of nitrogen gas. The ProOx oxygen controller was calibrated on a weekly basis. Oxygen levels were monitored daily using a Bacharach Fyrite Gas Analyzer (Bacharach, Inc, Pittsburgh, PA). Cell culture media used for hypoxic treatments was pre-equilibrated in the hypoxic chamber for a minimum of 24 h prior to use.

Prior to hypoxic treatment, MDA-MB-231 cells were cultured for 5 days in either 15nM, 1μM, or 10μM thiamine supplemented RPMI 1640 media until reaching 75% confluency. Media was then changed with the corresponding thiamine dose and cells were placed in either normoxic or hypoxic conditions for 24, 48, or 72 h. The media was changed every 24 h during the hypoxic treatment time to avoid nutrient deprivation.

Whole Cells Lysate Preparation and Cell Surface Biotinylation: For determination of TPK1 protein expression, cells were seeded into 60 mm cell culture dishes and exposed to hypoxia as described above. At the designated time-point, cells were washed with ice cold PBS and lysed using 50 mM Tris, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with protease and phosphatase inhibitor cocktail (EMD Biosciences, La Jolla, CA). Lysates were then centrifuged at 14,000xg at 4°C for 20 min and the resulting supernatant was collected. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

The isolation of cell surface proteins (Pierce Cell Surface Protein Isolation Kit, Thermo Scientific, Waltham, MA) was used for the determination of THTR1 and THTR2 expression. MDA-MB-231 cells were treated in hypoxic conditions as described above for 72 h. Cell surface protein was harvested following the kit protocol as previously described (Zastre, Hanberry *et al.* 2013). Total protein was quantified using the Reducing Agent Compatible Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Western Blotting: The protein expression of THTR1, THTR2, and TPK1 was assessed by Western blot. Cell surface protein fractions and whole cell lysates (50μg) were electrophoresed

on a 10% SDS-PAGE gel than transferred to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, Piscataway, NJ). The membrane was blocked with 5% non-fat milk in tris-buffered saline tween 20 (TBS-T) for 1 h. The membrane was blotted for TPK1 (Genetex, Irvine, CA), THTR1 (Alpha Diagnostics, San Antonio, Texas), or THTR2 (Proteintech Group, Chicago, IL) overnight, at 4°C with a dilution of 1:1000 in TBS-T. β-Actin (Sigma Aldrich, St. Louis, MO) was used as a loading control at a dilution of 1:2000 in TBS-T. Blots were washed in TBS-T three times for 10 min each and blotted with either donkey anti-rabbit HRP (Bethyl, Montgomery, TX) for TPK1, THTR1, and THTR2 or goat anti-mouse horseradish peroxidase (HRP) secondary antibody (Millipore, Billerica, MA) for β-Actin at 1:20,000 in TBS-T for 1 h. Blots were again washed 3 times for 10 min with TBS-T, visualized using Supersignal West Pico (Thermo Scientific, Rockford, IL), and imaged using a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry was performed by 1D-Multi Analysis using FluorChem SP Software (Alpha Innotech, San Leandro, CA). The change in expression is expressed as a ratio of hypoxic to normoxic treatments normalized to βActin.

Determination of thiamine and thiamine phosphate derivatives: The effect of hypoxia on intracellular thiamine status was assessed by High-performance liquid chromatography (HPLC). Cells cultured in 10cm dishes were treated in either normoxia or hypoxic for 72 h. After which the media in all treatment groups was changed with the appropriate concentration of thiamine and treated for an additional 24 h in normoxic and hypoxic conditions. Cells were then harvested using trypsin/EDTA and washed three times using ice-cold PBS. Total cell count was determined using a hemocytometer and aliquots of 2×10^6 cells were pelleted at 4°C for 5 min at 500xg. Media was collected before and after treatment, centrifuged at 1000xg for 5 min at 4°C,

and aliquots were taken for analysis. Extraction, derivatization, and HPLC analysis was then preformed as described previously (Zastre, Hanberry *et al.* 2013).

Polymerase Chain Reaction (PCR): RNA was harvested using EZNA Total RNA Kit I (Omega Bio-tek, Norcross, GA) from cells treated in normoxia and hypoxia as described above. cDNA was prepared from each RNA (1μg) sample using the qScript™ cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). PCR amplification of cDNA was preformed using EconoTaq Plus Green 2X Master Mix (Lucigen, Middleton, WI) in a DNA Thermal Cycler (Thermo Scientific, Rockford, IL) at 57°C for 32 cycles. PCR primers (5' →3') were; F' CCTGAATTCATCAATGGAGACTTTG and R' AGCAAGCACATCATTTGTGAGG resulting in the amplification of TPK1 variant 1 (438 bp) and variant 2 (291 bp). The PCR product was then subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The resulting fragments were imaged under UV light using a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA)

Assessment of RNA Stability and Protein Synthesis: MDA-MB-231 cells were treated in normoxia and hypoxia for 72 h in media containing 15nM thiamine with either Actinomycin D (ActD) or Cycloheximide (CHX) at a concentration of 5μg/mL. Control cells were treated with an equal volume of DMSO that corresponded to the volume from the ActD and CHX stocks in the treated groups. During treatment, cell culture media was changed every 24 h with replacement of ActD or CHX. RNA (ActD) and whole cell lysates (CHX) were harvested and analyzed using PCR or Western blots as described above.

Statistical Analysis: All experiments were performed with a minimum of three independent experiments unless otherwise noted. Statistical significance was evaluated through one-way ANOVA multiple analysis with a significance level of $p<0.05$ using Graph Pad Prism 6.

3.4. RESULTS

TPK1 protein increases following hypoxic exposure. To determine the expression changes in the thiamine-activating enzyme, TPK1, whole cell lysates were evaluated using Western blotting. The results in Figure 1A show a representative Western blot of cells cultured in normoxia and hypoxia with 15nM thiamine for 24, 48, and 72 h. At all time points, an approximately 2-fold increase in TPK1 variant 1 expression was observed when compared with normoxia (Figure 1B). In order to assess the effects of extracellular thiamine concentration on TPK1 expression we also cultured the cells in 15nM, 1μM, and 10μM thiamine. Figure 1C demonstrates that TPK1 protein expression remained constant in normoxia regardless of thiamine concentration. Following 72 h of hypoxic exposure, a similar increase in TPK1 expression was observed for all thiamine doses evaluated. No change in variant 2 expression was observed following thiamine supplementation and hypoxic exposure (data not shown).

Thiamine uptake and phosphorylation in normoxic versus hypoxic MDA-MB-231 cells. Cells cultured in normoxia and hypoxia demonstrated a dose dependent increase in intracellular thiamine following thiamine supplementation (Figure 2A). A statistically significant increase in intracellular thiamine within hypoxic cells was found compared to cells cultured in normoxia with a thiamine dose of 10μM (Figure 2A). Although no change in intracellular TPP was observed in normoxia, a statistically significant increase in intracellular TPP was observed in

hypoxic cells cultured in 1μM and 10μM thiamine (Figure 2B). No detectable levels of intracellular TMP or extracellular TMP and TPP in post-treatment media were observed (data not shown).

Functional assessment of TPK1 variant 1. In order to evaluate the effects of thiamine supplementation and TPK1 expression on intracellular TPP levels, MDA-MB-231 cells were transfected with a TPK1 variant 1 plasmid construct. Figure 3A shows a representative western blot of MDA-MB-231 cells transfected with vector control and TPK1 variant 1. In these cells, TPK1 variant 1 expression increased while TPK1 variant 2 remained unchanged. When compared to the level quantified in vector control cells, no change in intracellular thiamine was observed in TPK1 overexpressing cells cultured with 15nM and 1μM thiamine. However, TPK1 overexpressing cells cultured in 10μM thiamine showed a statistically significant reduction in free thiamine when compared to vector control cells (Figure 3B). No proportional change in intracellular TPP was observed with increasing thiamine dose in vector control cells (Figure 3C). However, culturing cells transfected with TPK1 variant 1 in 1μM and 10μM thiamine resulted in a statistically significant increase in TPP when compared to vector control cells (Figure 3C). No detectable levels of intracellular TMP or extracellular TMP and TPP in post-treatment media were observed (data not shown).

Cell Surface Expression of THTR1 and THTR2 following Hypoxic exposure. Figure 4 shows a representative Western blot for the thiamine transporters THTR1 (Figure 4A) and THTR2 (Figure 4B) in MDA-MB-231 cells cultured with 15nM, 1μM, or 10μM thiamine. When

compared to normoxia, the cell surface expression of both THTR1 and THTR2 remained unchanged following 72 h of hypoxic exposure over the three individual thiamine doses.

Regulation of TPK1 in hypoxic breast cancer cells. Transcriptional regulation of TPK1 expression following hypoxic exposure was evaluated using PCR. Figure 5A shows a representative gel in which the mRNA expression of TPK1 variant 1 and variant 2 did not change over 72 h of hypoxic exposure compared to normoxia. Culturing cells with 1μM or 10μM thiamine had no effect on the level of TPK1 variant 1 or variant 2 mRNA expression following 72 h of hypoxic exposure (Figure 5B). Figure 5C shows a representative PCR gel comparing cells cultured in the presence and absence of ActD for 72 h in normoxia and hypoxia. The expression of TPK1 variant 1 and variant 2 decreased in both normoxia and hypoxia when treated with ActD. Cycloheximide (CHX) treatment was used to assess TPK1 translation following hypoxic exposure. Figure 5D shows an increase in TPK1 expression after 72 h between hypoxic and normoxic cells without CHX. No change in protein expression was observed in cells exposed to normoxia with and without CHX treatment. However, the addition of CHX attenuated the enhancement of TPK1 expression in hypoxia treated cells.

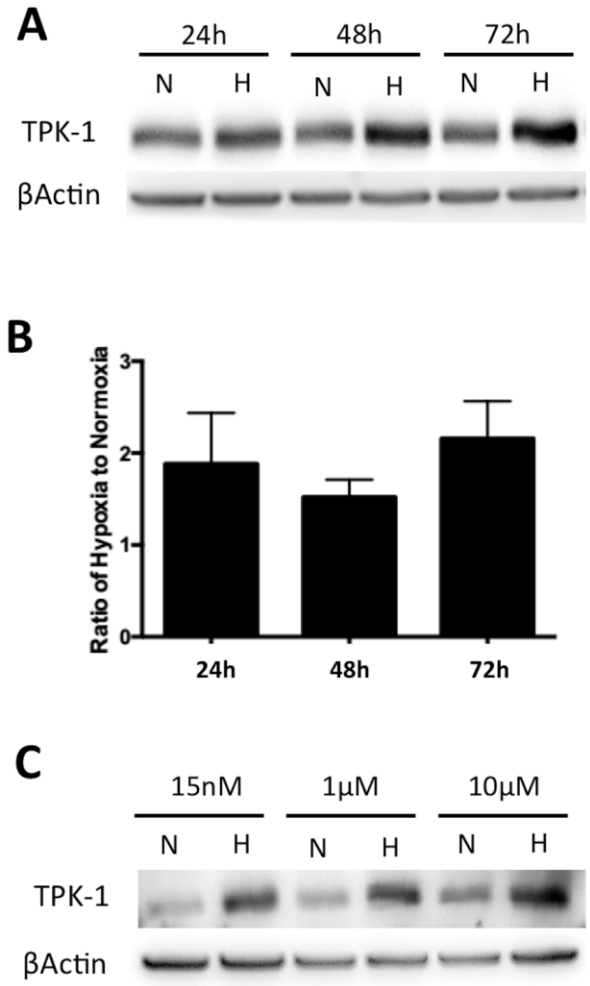

Figure 3.1. A. Representative Western blot of TPK1 expression in MDA-MB-231 cells cultured in 15nM thiamine for 24, 48, and 72h in normoxia (N) and hypoxia (H). βActin was used as a loading control. **B.** Densitometry showing fold change in TPK1 expression between normoxia and hypoxia (n=3). Ratio was normalized to βActin. **C.** Representative Western blot of TPK1 expression in MDA-MB-231 cultured at 15nM, 1μM or 10μM thiamine for 72h in both normoxia and hypoxia. βActin was used as a loading control.

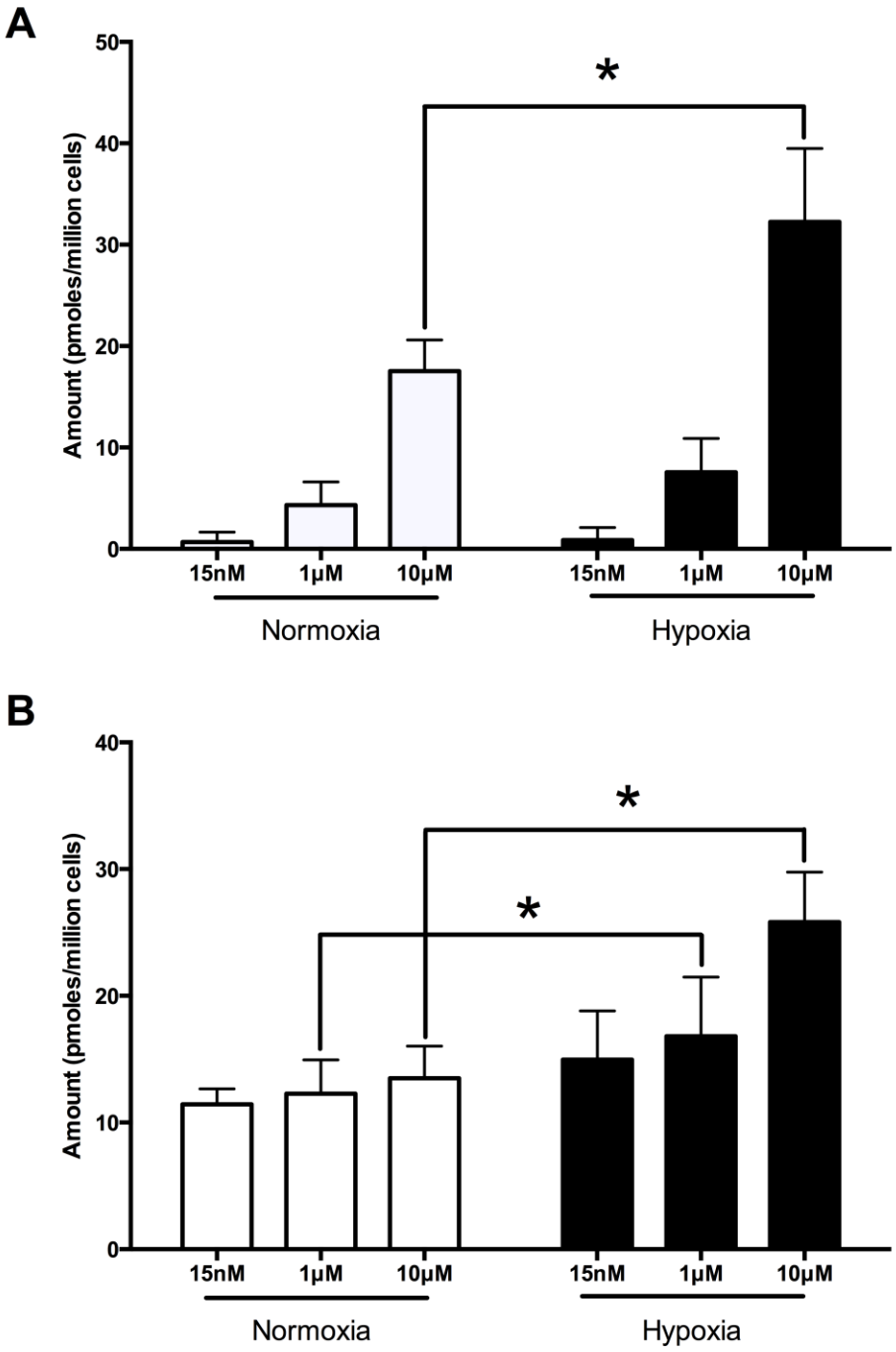

Figure 3.2. MDA-MB-231 cells were cultured 15nM, 1μM, or 10μM thiamine for 5 days prior to 96 hours of normoxic or hypoxic treatment. Changes in intracellular thiamine (**A)** and thiamine pyrophosphate (**B)** levels in MDA-MB-231 cells were quantified using HPLC. Statistically significant ($p<0.05$) differences are expressed as comparisons between normoxia (✶) treated cells hypoxia treated cells (n=10).

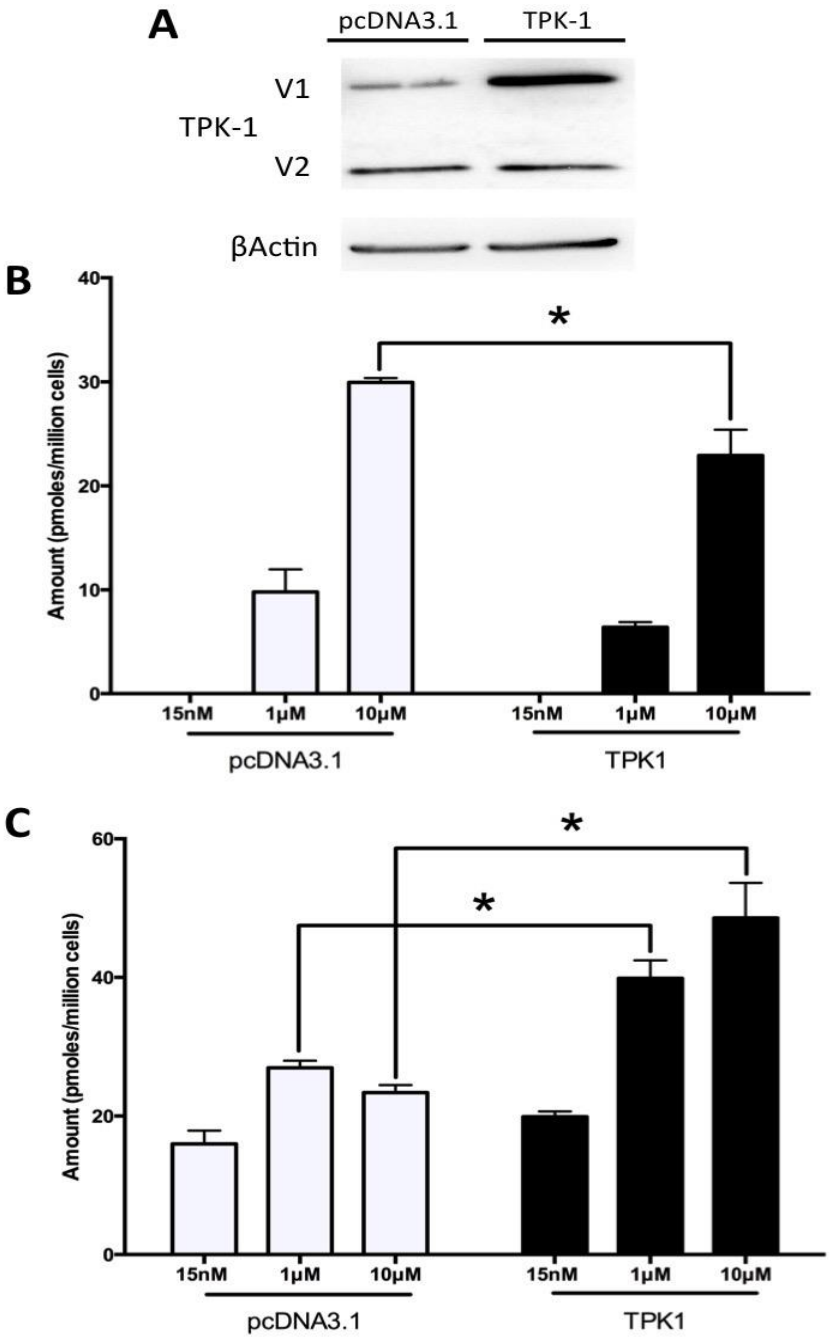

Figure 3.3. A. Representative Western blot of MDA-MB-231 cells transfected with TPK1 variant 1 or vector control. βActin was used as a loading control. Both vector control and TPK1 over-expressing cells were then cultured in 15nM, 1μM, or 10μM thiamine for 5 days prior to quantification of intracellular thiamine (**B**) and thiamine pyrophosphate (**C**) using an HPLC assay. Statistically significant ($p<0.05$) differences are expressed as comparisons between vector control and TPK1 transfected cells (✶) (n=3).

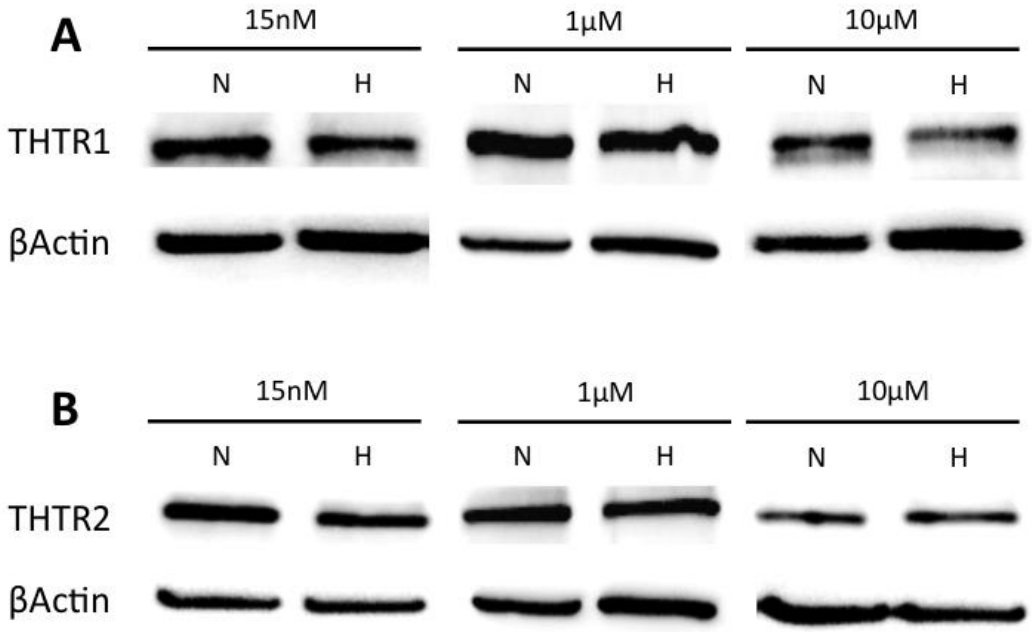

Figure 3.4. Representative Western blot of THTR1 and THTR2 in MDA-MB-231 cells treated with 15nM, 1μM, or 10μM thiamine for 5 days prior to 72 hours of hypoxic exposure. Comparison was between samples cultured under normoxic (N) and hypoxic (H) conditions. βActin was used as a loading control.

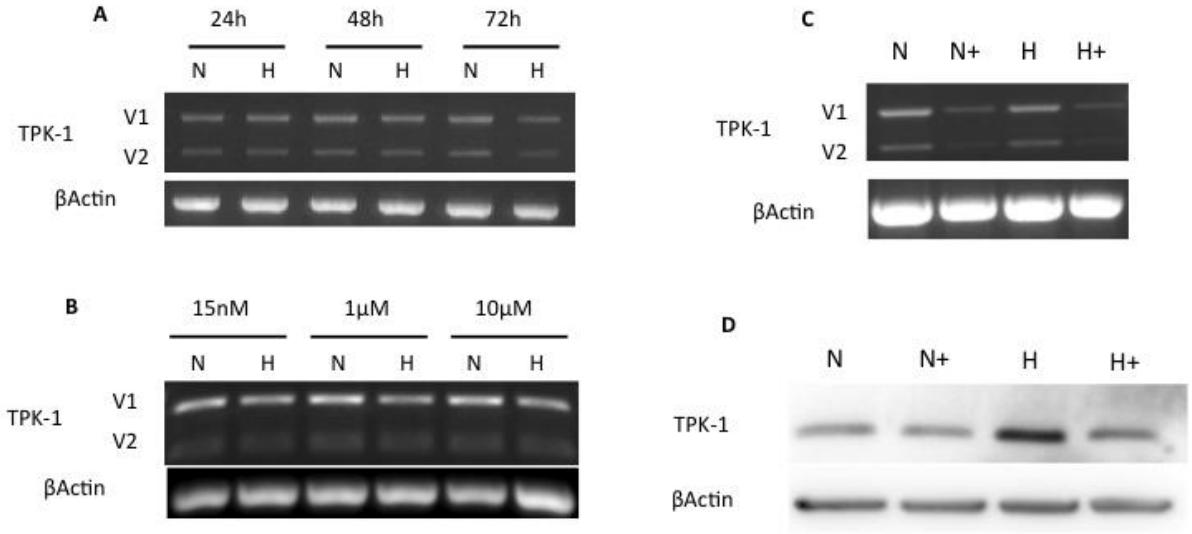

Figure 3.5. A. Representative PCR blot of TPK1 expression in MDA-MB-231 cells treated with 15nM thiamine for 24, 48, and 72h in both normoxia (N) and hypoxia (H). βActin was used as a loading control. **B.** Representative PCR blot of TPK1 expression in MDA-MB-231 cells cultured at 15nM, 1μM, or 10μM thiamine for 5 days prior to 72 hours of normoxic (N) or hypoxic (H) treatment. **C.** Representative PCR blot of TPK1 expression in MDA-MB-231 cells treated with Actinomycin D (+) for 72h in both normoxia (N) and hypoxia (H). βActin was used as a loading control. **D.** Representative Western blot of TPK1 expression in MDA-MB-231 cells treated with Cycloheximide (+) for 72h in both normoxia (N) and hypoxia (H). βActin was used as a loading control.

3.5. DISCUSSION

Recent evidence has demonstrated that thiamine supplementation supports malignant progression by increasing tumor proliferation. Using FVB/N-Tg(MMTVneu) mice, Daily *et al.* found that thiamine supplementation decreased tumor latency (Daily, Liu *et al.* 2012). An increase in Ehrlich ascites tumor proliferation was also observed in mice administered thiamine at 12.5 times the recommended daily allowance (Boros, Brandes *et al.* 1998). Our lab has previously shown that the effects of thiamine supplementation on tumor proliferation may be supported by alterations in thiamine homeostasis genes. An increase in the expression of the thiamine transporter, SLC19A2 was found in breast cancer tissue when compared to normal breast tissue. Correspondingly, the level of thiamine was higher in three out of four breast cancer cell lines compared to human mammary epithelial cells (Zastre, Hanberry *et al.* 2013). Furthermore, an increase in TPK1 gene expression was observed in breast tumor tissue when compared to normal breast tissue. In hypoxia an adaptive increase in the expression of the thiamine transporter, SLC19A3 and increase in thiamine transport was found in BT474 breast cancer cells (Sweet, Paul *et al.* 2010). Using MDA-MB-231 breast cancer cells we found a hypoxia induced increase in TPK1 protein expression. To our knowledge this is the first evidence demonstrating a hypoxia induced increase in TPK1 expression.

Although thiamine has demonstrated to increase tumor growth, an increase in intracellular TPP levels may not directly be achieved through thiamine supplementation. Smithline *et al.* found that thiamine administration increases levels of free thiamine in circulation but did not change levels of any phosphate derivatives of thiamine (Smithline, Donnino *et al.* 2012). Similarly, when MDA-MB-231 cells were cultured under normoxic conditions with increasing doses of thiamine, we found that the level of free thiamine increased with no

corresponding change in TPP. This further demonstrates that thiamine supplementation alone may not increase intracellular TPP levels. However, when TPK1 was exogenously over-expressed or up-regulated under hypoxic conditions, TPP levels increased with thiamine supplementation. This suggests that increasing intracellular TPP is independent of changes in intracellular thiamine levels but rather coupled with TPK1 expression.

Thiamine supplementation also resulted in a statistically significant increase in the levels of intracellular thiamine during hypoxia exposure. Gangolf *et al.* hypothesized that phosphorylation of thiamine to TPP may be a driving force for intracellular uptake of thiamine (Gangolf, Czerniecki *et al.* 2010). In our cell model system, exogenous over-expression of TPK1 did not change the level of intracellular thiamine when compared to vector control cells. Using an estrogen receptor positive breast cancer cell line, Sweet *et al.* found a hypoxia induced increase in SLC19A3 gene expression and corresponding increase in thiamine uptake (Sweet, Paul *et al.* 2010). However, in our estrogen receptor negative cell model system we did not detect changes in the membrane localization of either THTR1 or THTR2. Differential expression of thiamine transporters has been observed within breast, colon, and gastric cancers (Liu, Lam *et al.* 2009, Ikehata, Ueda *et al.* 2012). Both differences in gene regulation and disease phenotype could result in an alteration in transporter expression when comparing estrogen receptor positive and estrogen receptor negative breast cancer cells (Liu, Lam *et al.* 2009). Alternatively, other transport processes may be responsible for the increase in thiamine uptake in this cell model system. Chen *et al.* have recently identified OCT1 (SLC22A1) as a high-capacity thiamine transporter (Chen, Shu *et al.* 2014). The activity of a yet to be characterized or identified thiamine transporter may therefore be contributing to the increase in thiamine uptake during hypoxia in MDA-MB-231 cells.

Although the mechanism controlling the adaptive regulation of TPK1 under hypoxia is unclear, the increase in expression was not transcriptionally but rather translationally controlled. The regulation of protein expression through post-transcriptional modification and RNA-silencing could account for the hypoxia-induced increase in TPK1 protein expression. Micro-RNAs alter the ribosomal translation of a gene and have been shown to regulate approximately 60% of human genes (Ambros 2004, Calin, Sevignani *et al.* 2004). *In silico* sequence analysis of the TPK1 gene indicates binding sites for multiple microRNAs including mir-29b, let-7f, mir-18, and mir-20a. In MCF-7 breast cancer cells, the expression of mir-29b and let-7f were significantly down-regulated in hypoxia (Daily, Liu *et al.* 2012). Loss of let-7 results in the up-regulation of EZH2, which alters histone methylation and subsequently silences, the expression of many tumor suppressor genes (Suva, Riggi *et al.* 2009, Crea, Hurt *et al.* 2011, Kong, Heath *et al.* 2012). The level of mir-20a was also down-regulated following hypoxic exposure of acute myelogenous leukemia cells (He, Wang *et al.* 2013). Further assessment of the role of microRNAs in regulating TPK1 protein expression is warranted.

In conclusion, hypoxic exposure resulted in an increase in TPK1 protein expression that was coupled with an increase in the intracellular level of the active coenzyme, TPP. Culturing cells with increased doses of thiamine resulted in a corresponding increase in intracellular thiamine, but changes in cellular TPP were only observed in cells with increased expression of TPK1. The adaptive increase in TPK1 expression appears to be the result of hypoxia-mediated alterations in protein translation and not gene transcription. Although it is currently unclear what function increasing TPP levels has during hypoxic stress, the adaptive increase in thiamine homeostasis may be an essential component in the anaerobic metabolic shift associated with hypoxic tumor microenvironments.

CHAPTER 4

SUPPLEMENTAL DATA

4.1 High Dose Thiamine Supplementation and Analogs

4.1.1. Introduction

High dose thiamine supplementation represents a potentially safe alternative to current chemotherapeutic strategies. Meador *et al.* demonstrated that administration of thiamine to Alzheimer's patients at 7.5g/day resulted in minimal side effects including nausea (Meador, Loring *et al.* 1993). Although free thiamine can be taken up by the body through a non-saturable process, transport is often limited by a saturable uptake system in the small intestine (Thomson, Baker *et al.* 1970, Thomson and Leevy 1972, Laforenza, Patrini *et al.* 1997, Thomson 2000, Smithline, Donnino *et al.* 2012). The limitations presented by a facilitative uptake system could be overcome through the use of commercially available structural analogs that have been utilized to increase cell permeability of thiamine.

Benfotiamine (S-benzoylthiamine-O-Monophosphate), a synthetic derivative of thiamine, has been used in the treatment of diabetic neuropathies, alcoholism, type 1 and 2 diabetes mellitus, endothelial dysfunction, and diabetic retinopathy. This analog undergoes dephosphorylation in the intestinal tract producing a lipophilic molecule that can readily diffuse across the cell membrane. Following intracellular uptake, benfotiamine undergoes closure of the thiazole ring resulting in the formation of thiamine (Volvert, Seyen *et al.* 2008). Following

benfotiamine administrations, the plasma levels of thiamine are 5-fold higher and bioavailability is 3 to 4 times higher compared to administration of thiamine alone (Karpov, Rozanov *et al.* 1986, Bitsch, Wolf *et al.* 1991, Greb and Bitsch 1998, Frank, Bitsch *et al.* 2000, Geyer, Netzel *et al.* 2000). Therefore, benfotiamine represents a viable alternative to reducing the dose required to elicit an apoptotic response.

Additional thiamine mimetics, such as sulbutiamine, have been utilized to increase the intracellular supply of thiamine (Volvert, Seyen *et al.* 2008). Sulbutiamine is a lipophilic dimer of two thiamine molecules that is used to support long-term memory function and treat asthenia (Tiev, Cabane *et al.* 1999, Van Reeth 1999). Bettendorff *et al.* found an increase in tissue thiamine triphosphate levels following sulbutiamine administration (Bettendorff, Weekers *et al.* 1990). Sulbutiamine's lipophilicity also allows it to cross the blood brain barrier more easily when compared to thiamine alone (Bettendorff, Weekers *et al.* 1990, Bettendorff 1994). Utilization of lipophilic derivatives of thiamine, such as benfotiamine and sulbutiamine, increase the cellular uptake of free thiamine and maintain its ability to be utilized by normal cells. Therefore, the potential anti-proliferative effects of high dose thiamine derivatives warrant further evaluation.

4.1.2. Materials And Methods

Materials: Cell culture reagents were used as described previously (Chapter 2.3). Benfotiamine was purchased from Sigma Aldrich (St. Louis, MO). 4',6-diamidino-2-phenylindole (DAPI), Oxythiamine chloride hydrochloride and Sulbutiamine were purchased from BOC Sciences (Shirley, NY). Mannitol was purchased from EMD Millipore (Billerica, MA).

Cell Proliferation Assays: The effects of thiamine and DCA on cell proliferation were determined using the crystal violet assay as described previously (Chapter 2.3). Briefly, MDA-MB-231, SKOV-3, and PC-3 cells were seeded into 96 well plates at a density of 3,000 cells/cm^2 and allowed to attach overnight. Media was than replaced with increasing concentrations of either thiamine or DCA and cells allowed to grow for 5 days at 37°C.

To visualize the level of cell proliferation at the conclusion of experiments, bright-field images were taken. Panc-1 cells were seeded and treated as described in chapter 2.3. After 5 days cells were imaged using a Motic AE31 (British Columbia, CAN).

Osmotic Effects on Cell Proliferation: To assess the potential that high extracellular concentrations of thiamine perturb intracellular osmoregulation, the crystal violet assay was used (Chapter 2.3). Briefly, Panc-1 cells were seeded into 96 well plates at a density of 3,000 cells/cm^2 and allowed to attach overnight. Media was than replaced with increasing concentrations of the cell impermeable sugar, mannitol, and cells were cultured for 5 days at 37°C.

Anti-proliferative Effects of Thiamine and Thiamine Analogs: The effects of thiamine, benfotiamine, and sulbutiamine on cell proliferation were determined using the crystal violet assay as described previously (Chapter 2.3). Briefly, Panc-1 cells were seeded into 96 well plates at a density of 3,000 cells/cm^2 and allowed to attach overnight. Media was than replaced with increasing concentrations of either thiamine or DCA and cells allowed to grow for 5 days at 37°C.

Images of Panc-1 cells treated with thiamine, oxythiamine, benfotiamine and DCA were visualized using a Motic AE31 (British Columbia, CAN). Briefly, Panc-1 cells were seeded into 96 well plates at a density of 3,000 cells/cm^2 and allowed to attach overnight. Media was than replaced with increasing concentrations of either thiamine or DCA and cells allowed to grow for 5 days at 37°C.

DAPI Staining: To assess changes in nuclear morphology as a result of high dose thiamine and DCA supplementation 4',6-diamidino-2-phenylindole (DAPI) staining was used. Treatment of Panc-1 cells with thiamine and DCA was performed as described above (Chapter 2.3). Briefly, cells were cultured in 6-well chambered slides (BD Biosciences, San Jose, CA) until reaching 75% confluency. Media was then changed and cells were cultured for an additional 48 h. Following 48 h of thiamine and DCA treatment, cells were washed twice with 1 x PBS and then fixed with buffered formalin for 10 minutes. Cells were once again washed three times with 1 x PBS for three minutes each. PBS was then aspirated from each chamber and 100µl of a 100µM DAPI solution was added. Cells were incubated in a dark room at room temperature for 10 minutes on an orbital shaker. DAPI was then aspirated from each chamber and cells were washed twice with 1 x PBS for 10 minutes each. Cells were then mounted using fluoromount with a cover slip. Cells were imaged under red fluorescence using a Nikon AZ100 (Melville, NY).

4.1.3. Results

High dose thiamine reduces malignant proliferation. The crystal violet cell proliferation assay was used to assess the effects of thiamine and DCA on cell proliferation. Figure 4.1 displays a

decrease in cell proliferation for MDA-MB-231, Pc-3, and SKOV-3 cells with increasing concentration of thiamine and DCA following 5 days of treatment. The IC50 values for DCA were 10.1 for MDA-MB-231, 10.4 for Pc-3, and 9.9 mM for SKOV-3. Comparatively, the IC50 of thiamine was lower than DCA for all cell lines with values of 4.9 for MDA-MB-231, 5.6 for Pc-3, and 5.3 mM for SKOV-3.

In order to assess potential alterations in osmoregulation following high-dose supplementation, Panc-1 cells were cultured with mannitol. Figure 4.2 indicates that mannitol supplementation up to 10mM does not alter cell proliferation. This suggests that the anti-proliferative effects observed following high dose thiamine and DCA supplementation are a result of the intracellular activity of both compounds.

Thiamine Analogs decrease cell proliferation. To demonstrate that cell proliferation experiments were concluded prior to cells reaching full confluency, bright-field images were taken. Figure 4.3 shows Panc-1 cells seeded at the same density used for experiments assessing the anti-proliferative effects of high-dose thiamine supplementation. These cells were seeded at 3,000 cells/cm^2 and were cultured in 15nM thiamine for 5 days. At the conclusion of the treatment period cells had not reached maximum confluency.

The crystal violet cell proliferation assay was used to assess the effects of benfotiamine and sulbutiamine on cell proliferation. Figure 4.4 shows that both thiamine mimetics reduce cell proliferation in Panc-1 cells. This experiment shows that thiamine analogs reduce cancer cell proliferation at a much lower concentration when compared to treatment with thiamine hydrochloride alone.

In order to visualize the effects that thiamine and thiamine mimetics have on cell morphology and proliferation, bright-field images were taken. In this study DCA and Oxythiamine were used as methods of control as they have both been shown to reduce cancer cell proliferation and induce apoptosis (Boros, Puigjaner *et al.* 1997, Michelakis, Webster *et al.* 2008, Wang, Zhang *et al.* 2013). Figure 4.5 shows that high dose thiamine and benfotiamine reduce cell proliferation when compared to control cells. Benfotiamine resulted in a reduction in cell proliferation at a lower dose when compared to cells treated with thiamine.

High-dose thiamine alters nuclear morphology. DAPI staining was used to assess changes in nuclear morphology following high dose thiamine and DCA treatment of Panc-1 cells. Both thiamine and DCA altered nuclear morphology when compared to control cells (Figure 4.6). This observation supports the claim that high-dose thiamine supplementation results in a cascade of events that lead to cellular apoptosis.

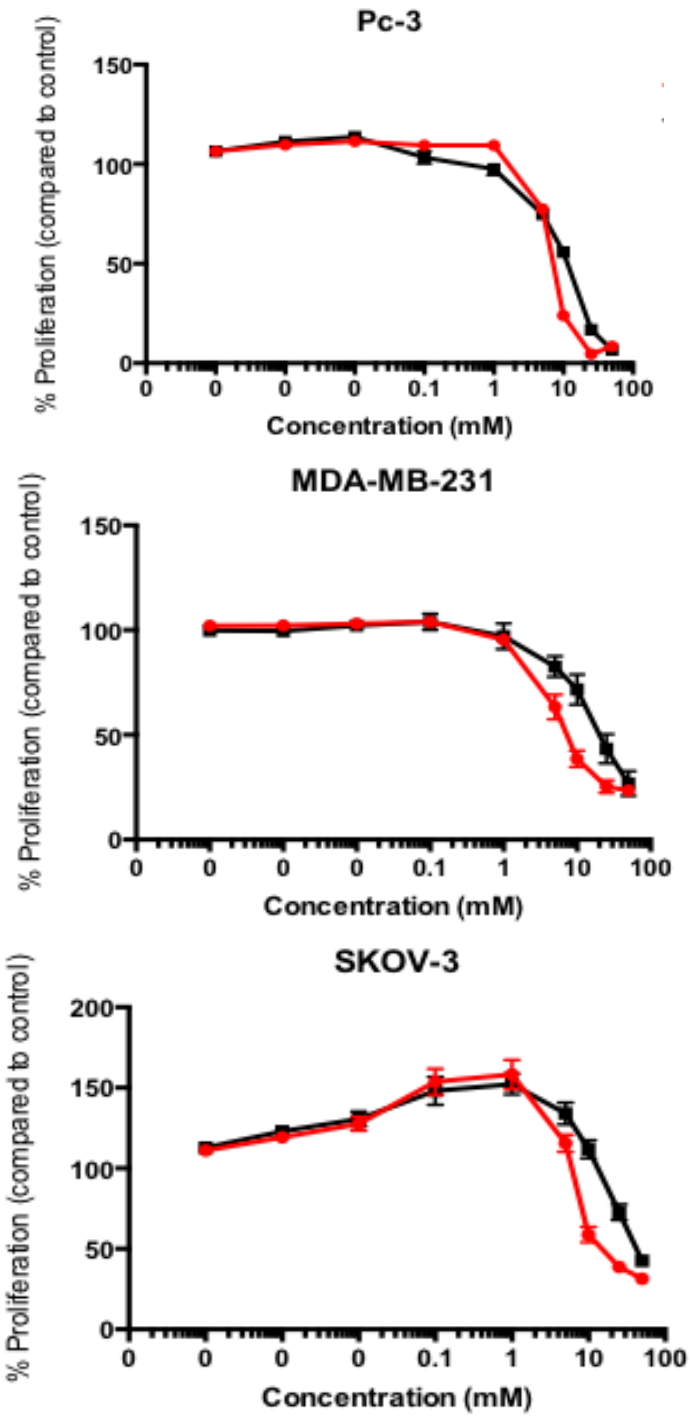

Figure 4.1. Cell proliferation of MDA-MB-231, Pc-3, and SKOV-3 cells. Cells seeded into 96-well plates were treated with increasing concentrations of thiamine (○) or DCA (●) for 5 days. The extent of cell proliferation was measured using crystal violet. Results are presented as the percent proliferation comparing treated cells to untreated cells. Data represents mean ± SD on n=3 independent experiments.

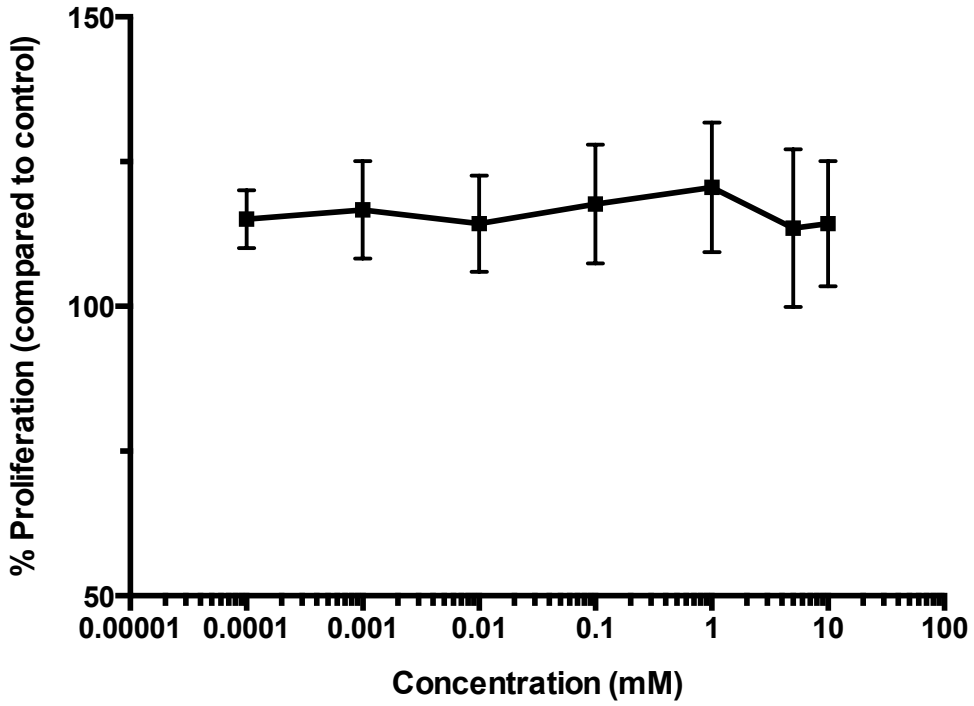

Figure 4.2. Cell proliferation of Panc-1 cells treated with Mannitol. Cells seeded into 96-well plates were treated with increasing mannitol (●) for 5 days. The extent of cell proliferation was measured using crystal violet. Results are presented as the percent proliferation comparing treated cells to untreated cells. Data represents mean ± SD on n=3 independent experiments.

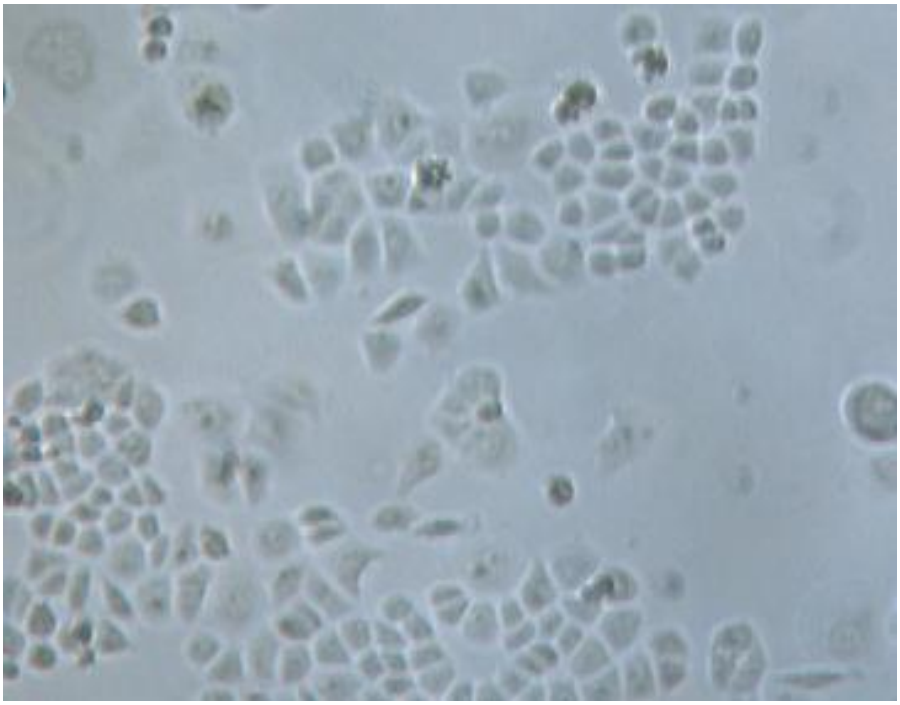

Figure 4.3. Cell proliferation of Panc-1 cells. Cells were seeded into 6-well plates and cultured in 15nM thiamine RPMI 1640 for 5 days.

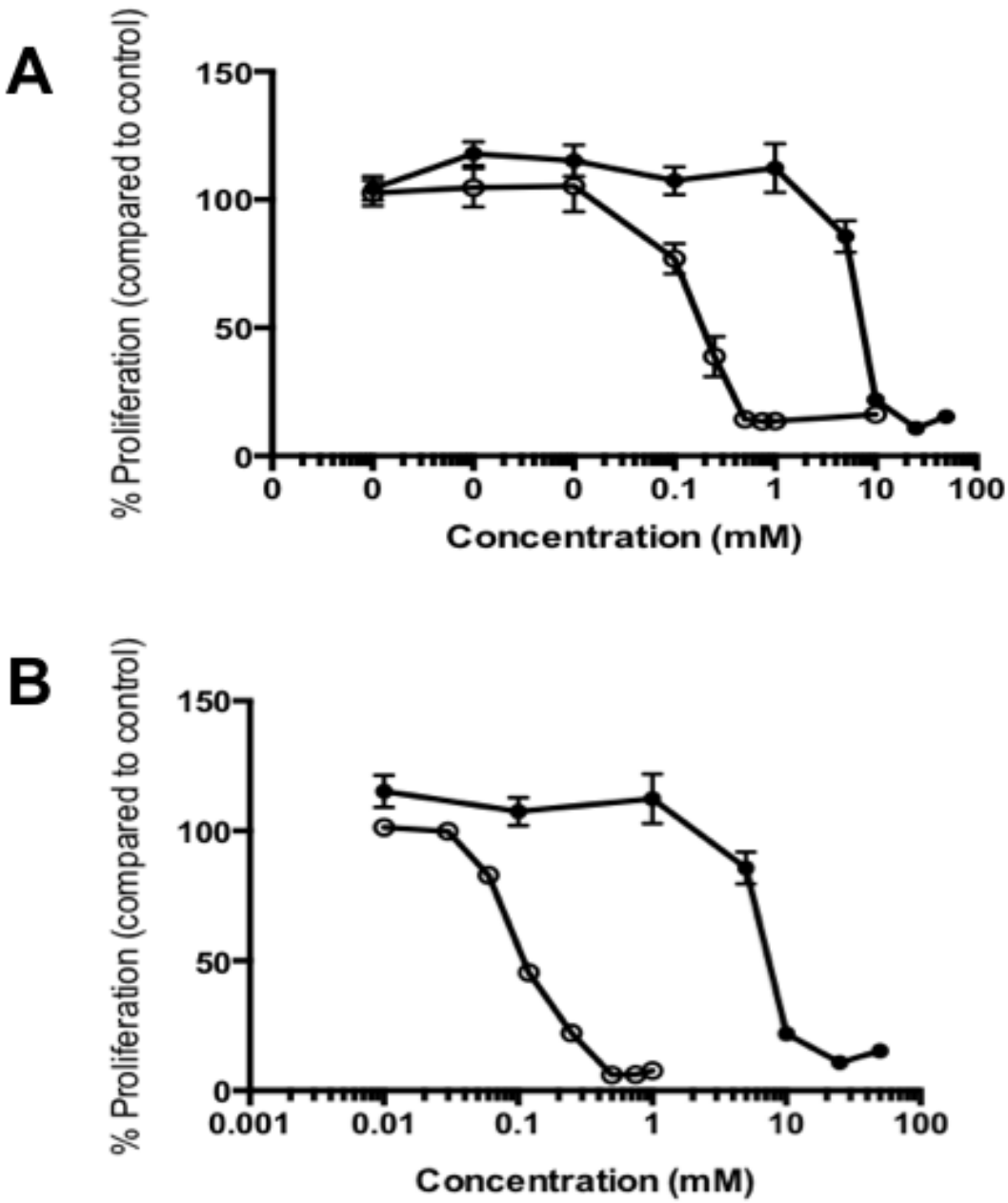

Figure 4.4. Cell proliferation of Panc-1 cells treated with Benfotiamine and Sulbutiamine. Cells seeded into 96-well plates were treated with increasing concentrations of A) benfotiamine (○), B) sulbutiamine (○), or thiamine (●) for 5 days. The extent of cell proliferation was measured using crystal violet. Results are presented as the percent proliferation comparing treated cells to untreated cells. Data represents mean ± SD on n=3 independent experiments.

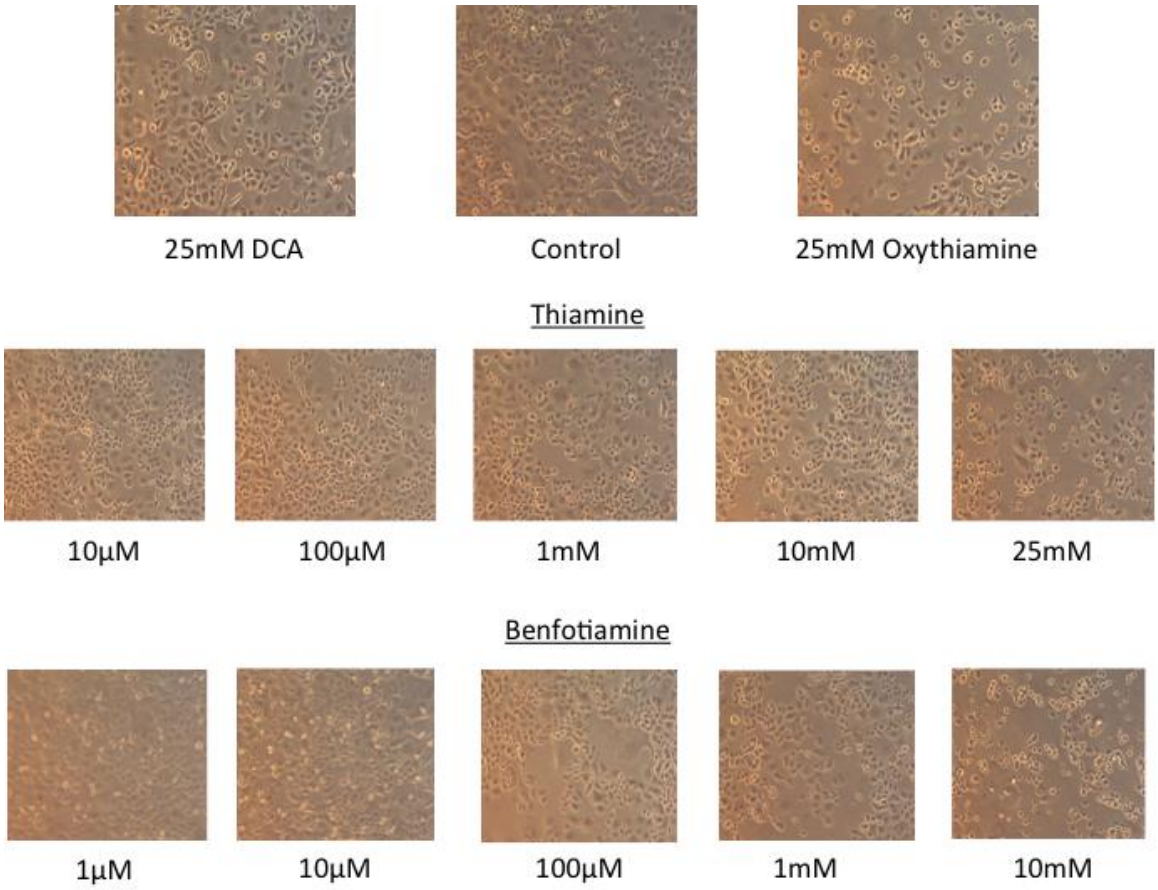

Figure 4.5. Cell proliferation of Panc-1 cells following 48 h of treatment. Cells seeded into 6-well plates were treated with 15nM thiamine RPMI 1640 (control), 25mM DCA, and oxythiamine. Additionally, cells were treated with increasing concentrations of thiamine and benfotiamine.

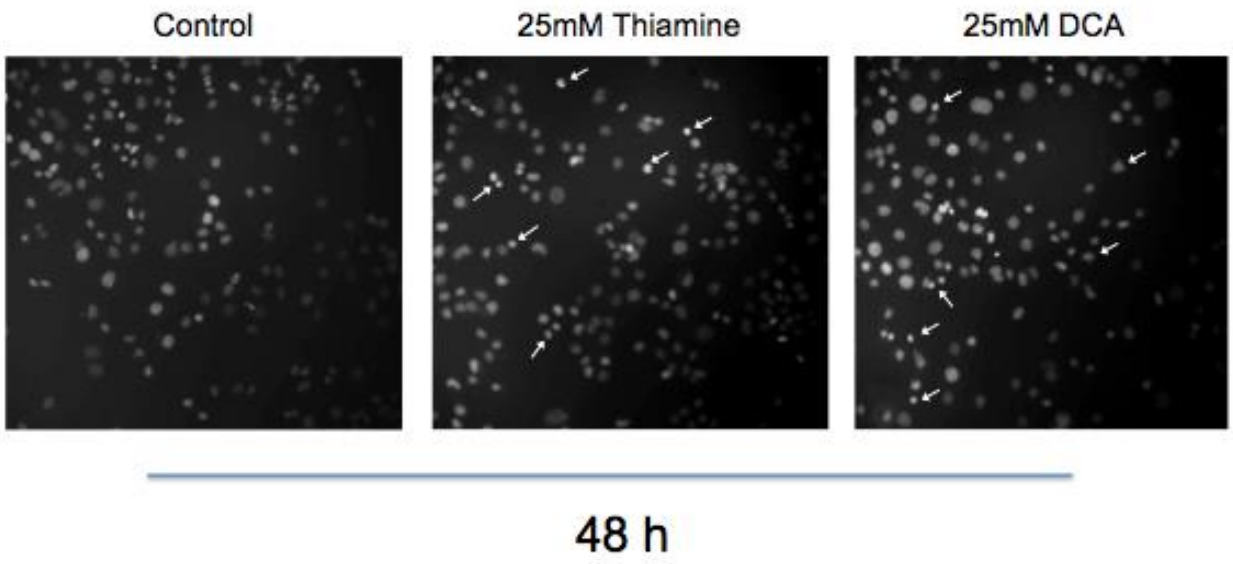

Figure 4.6. DAPI staining of Panc-1 cells. Cells were treated with 25mM thiamine or DCA and then stained with DAPI to assess nuclear morphology. Arrows indicate cells that are representative of changes in nuclear morphology and therefore apoptosis.

4.1.4. Discussion

High-dose thiamine supplementation reduces proliferation in pancreatic, neuroblastoma, breast, ovarian, and prostate cancer cells. The reduction in cell proliferation, observed following high dose thiamine supplementation, appears to be the result of changes in thiamine homeostasis within cells. Although this data provides a targeted approach at reducing cancer cells proliferation, the uptake of thiamine is limited by the activity of membrane bound transporters. Therefore, additional methods must be evaluated to increase in the intracellular concentration of thiamine at lower required doses.

The use of thiamine analogs decreases malignant proliferation at concentrations that are orders of magnitude lower when compared to high dose thiamine supplementation. Moreover, the use of thiamine mimetics represents a potentially safe alternative to current chemotherapeutic treatments as these mimetics are converted to free thiamine after cellular uptake (Bitsch, Wolf *et al.* 1991, Volvert, Seyen *et al.* 2008). High dose thiamine supplementation results in a cascade of intracellular events that lead to a reduction in proliferation through an apoptotic response. However, it is still necessary to further evaluate the mechanism of action and determine the active species involved in the reduction of malignant proliferation. Currently more work is required to determine how thiamine analogs alter the activity of thiamine dependent enzymes and evaluate how thiamine mimetics alter the intracellular supply of thiamine.

4.2 Targeting Homeostatic Regulation of Thiamine

4.2.1. Introduction

Alterations in the intracellular maintenance of nutrient cofactors are essential to support survival and proliferation. Within all cancer cells, the homeostatic regulation of nutrient cofactors represents a potential chemotherapeutic target. However, little in known about how malignant alterations in the maintenance and utilization of thiamine can be exploited through high-dose supplementation. Therefore, it is necessary to determine if alterations in the intracellular regulation of thiamine homeostasis genes support this targeted chemotherapeutic strategy.

4.2.2. Materials and Methods

Materials: See Chapter 3.3 for cell culture materials and treatment protocols.

Expression of TPK1 following hypoxic exposure: The protein expression of TPK1 was assessed by Western blot as described in Chapter 3.3. Briefly, BT-474 cells were cultured in normoxia for 72 h. Cells were additionally cultured in hypoxia for 24, 48, and 72 h. Whole cell lysates were collected and immunoblotted for TPK1 and βActin.

Over-expression of TPK1: Panc-1 cells were transfected with a TPK1 variant 1 plasmid to evaluate the effects of TPK1 overexpression on altering thiamine homeostasis. The protein expression of TPK1 was assessed by Western blot as described in Chapter 3.3. Cell proliferation was conducted using vector control cells and TPK1 variant 1 expressing cells as described

previously (Chapter 3.3). HPLC quantitation of thiamine and TPP was preformed as described previously (Chapter 3.3)

4.2.3. Results

Hypoxia-induced expression of TPK1 is observed in BT-474 cells. To further determine the expression changes in the thiamine-activating enzyme, TPK1, whole cell lysates were evaluated using Western blotting. The results in figure 4.7 show a representative Western blot of BT-474 cells cultured in normoxia for 72h and in hypoxia for 24, 48, and 72 h. At all time points, an approximately 2-fold increase in TPK1 variant 1 expression was observed when compared with normoxia.

TPK1 supports an increase in thiamine uptake and phosphorylation. Figure 4.8 shows a representative western blot of Panc-1 cells transfected with vector control and TPK1 variant 1. Intracellular supply of thiamine increases, as a function of dose, in both TPK1 over-expressing and vector control cells (chapter 3.4). Cells transfected with TPK1 variant 1 also demonstrated a concentration dependent increase in TPP when compared to vector control cells (Figure 4.9).

A crystal violet assay was used to assess the anti-proliferative effects of high dose thiamine supplementation in cells that over-express TPK1. Cells cultured in 10mM thiamine showed an approximately 50% decrease in cell proliferation when compared to vector control cells. This suggests that the conversion of thiamine to TPP is a key step in this process and that TPP may be the active species.

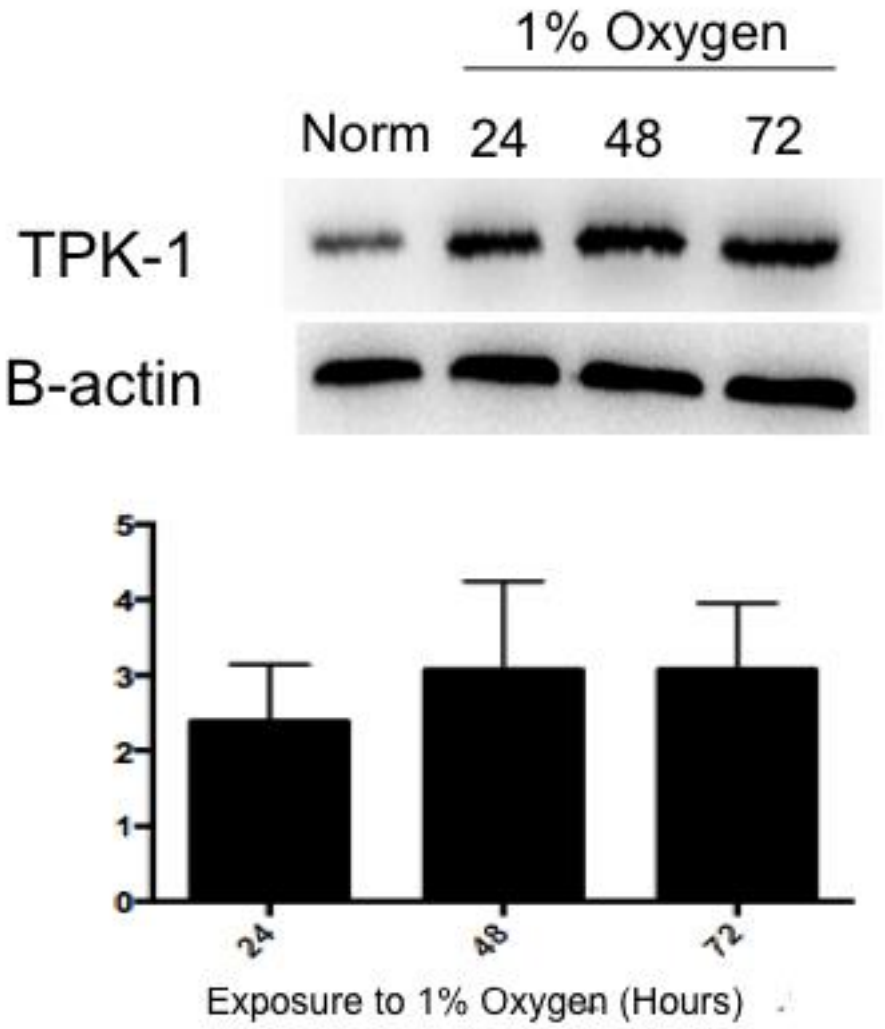

Figure 4.7. Representative Western blot of TPK1 expression in BT-474 cells cultured in 15nM thiamine in normoxia (Norm) and 1% oxygen for 24, 48, and 72 h. βActin was used as a loading control. Densitometry showing fold change in TPK1 expression between normoxia and hypoxia (n=3). Ratio was normalized to βActin.

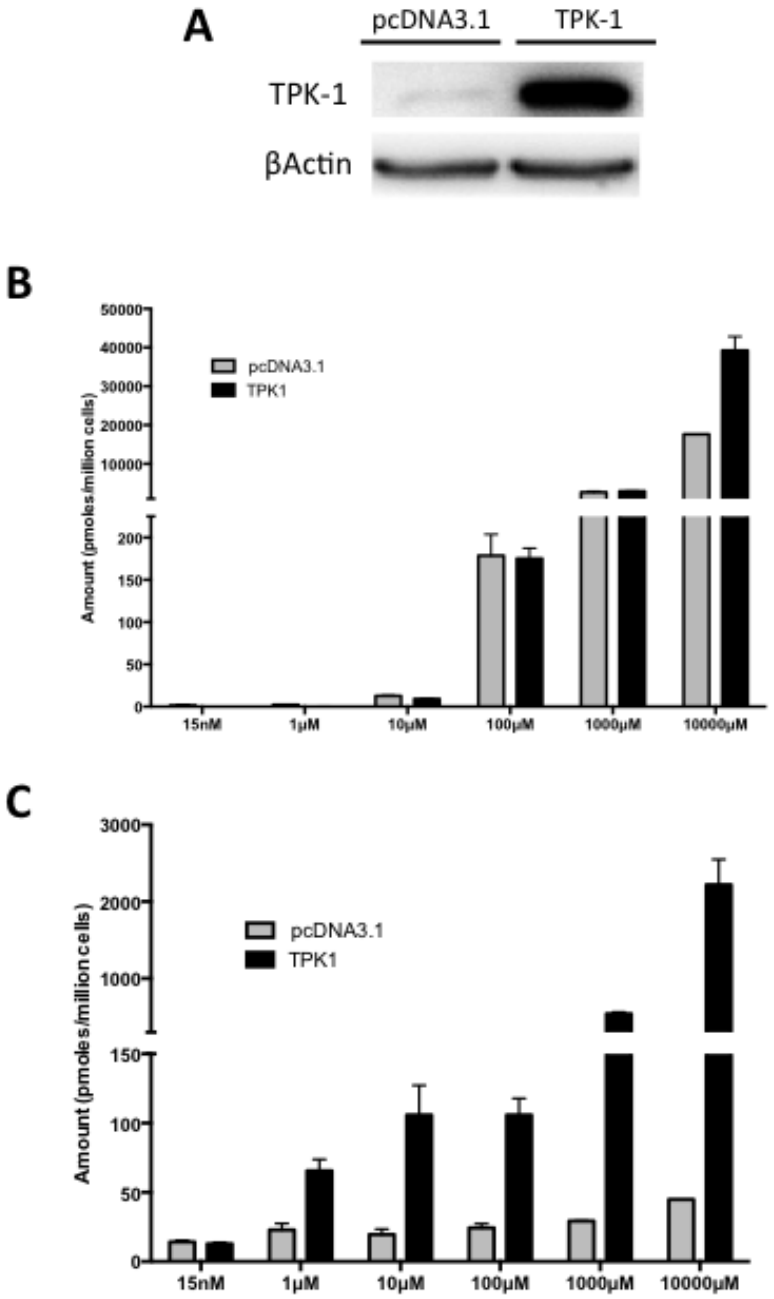

Figure 4.8. Representative Western blot of Panc-1 cells transfected with TPK1 variant 1 or vector control. βActin was used as a loading control. Both vector control and TPK1 over-expressing cells were then cultured in 15nM, 1μM, 10μM, 100μM, 1,000μM, or 10,000μM thiamine for 24 h prior to quantification of intracellular thiamine (**B**) and thiamine pyrophosphate (**C**) using an HPLC assay (n=3).

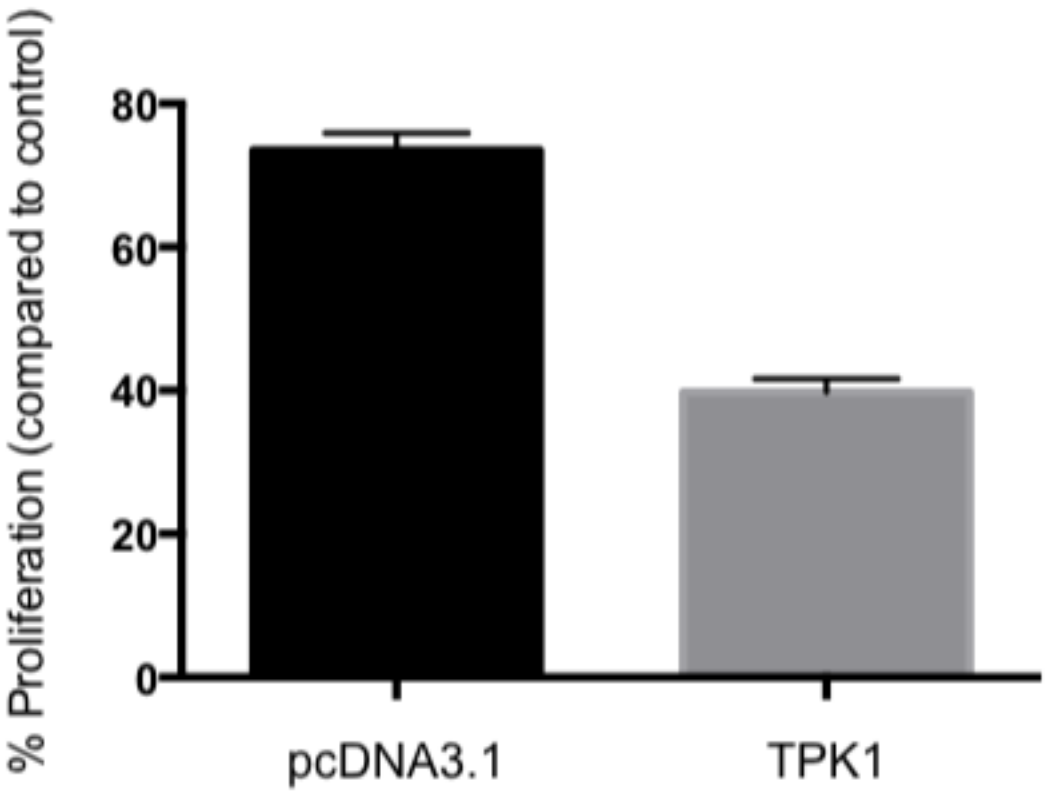

Figure 4.9. Cell proliferation of Panc-1 cells transfected with vector control or TPK1 variant 1. Cells seeded into 96-well plates were treated with 10mM thiamine for 5 days. The extent of cell proliferation was measured using crystal violet. Results are presented as the percent proliferation comparing treated cells to untreated cells (15nM thiamine). Data represents mean ± SD on n=3 independent experiments.

4.2.4. Discussion

In order to further assess the expression and function of TPK1 in hypoxic microenvironments, we utilized additional breast cancer cell model systems. We have found that the estrogen receptor positive breast cancer cell line, BT-474, exhibits an adaptive increase in TPK1 expression similar to that observed in MDA-MB-231 cells. It can therefore be suggested that an increase in thiamine uptake and TPK1 expression support the maintenance of thiamine homeostasis within hypoxic microenvironments (Sweet, Paul *et al.* 2010). However, the function of this increase has yet to be evaluated. The maintenance of thiamine homeostasis under low oxygen conditions may be necessary to support changes in the function of thiamine dependent enzymes, such as transketolase. Bentz *et al.* observed an increase in the expression of transketolase in hypoxic colon cancer cells (Bentz, Cee *et al.* 2013). Further work is necessary to evaluate how alterations in the maintenance of thiamine homeostasis, under low oxygen conditions, support malignant metabolism and proliferation.

Targeting the homeostatic regulation of thiamine represents a potential chemotherapeutic strategy. In Panc-1 cells, an exogenous increase in TPK1 protein expression results in an increase in intracellular TPP levels. Moreover, high dose thiamine supplementation to these cells decreases cell proliferation to a greater degree when compared to vector control cells. Therefore, this treatment strategy may be more effective in cells that demonstrate an increased uptake and conversion of thiamine. However, more work is needed to determine the active species responsible for the reduction in cancer cell proliferation.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1. SUMMARY

Malignant cells exhibit an increased requirement for nutrients and cofactors to support and sustain rapid proliferation. This altered metabolic phenotype is observed in all cancer cells regardless of the tissue of origin. Metabolic alterations are the result of oncogenically driven reprogramming that supporting an increased requirement for energy and biomass. These changes are also critical in supporting the generation of new daughter cells (DeBerardinis, Mancuso *et al.* 2007, DeBerardinis, Lum *et al.* 2008, Denko 2008, Cairns, Harris *et al.* 2011). A direct consequence of rapid proliferation is the need for an adequate supply of nutrients that are necessary to support cellular metabolism. As most nutrients do not passively diffuse across the cell membrane, tumor cells increase nutrient uptake through up-regulation of membrane transporters. For example, vitamin B_1 provides an essential cofactor that supports metabolic reactions within rapidly dividing cells. Therefore, alterations in the intracellular maintenance and activation of this essential vitamin warrant further examination.

Variations in extracellular thiamine availability support malignant progression through increasing proliferation and modulating gene expression. For several years clinicians empirically administered thiamine to cancer patients in an effort to overcome deleterious effects of physiological deficiency. Comin-Anduix *et al.* first evaluated the effects of thiamine supplementation on malignant progression and found a statistically significant increase in tumor

growth in mice that were supplemented with thiamine at a level between 12.5 to 37.5 times the RDA. At 25 times the RDA the tumor volume in these mice grew to 250 times that of control mice administered thiamine at the RDA. However, when compared to the level of tumor proliferation observed in control animals, high dose thiamine supplementation decreased tumor proliferation (Boros, Brandes *et al.* 1998). Therefore, it is necessary to understand the effects of thiamine (Vitamin B1) supplementation on malignant progression. Thiamine not only supports cancer proliferation at low to moderate doses but also decreases proliferation at high doses. Therefore, the overall goal of this research project was to evaluate the effects of high dose thiamine administration on cancer cell proliferation and to determine how changes in tumor microenvironment alter the efficacy of this treatment strategy.

Using both neuroblastoma and pancreatic cancer cell lines we have shown a reduction in cell proliferation following high dose thiamine supplementation. This supplementation resulted in a decrease in pyruvate dehydrogenase phosphorylation, which altered the flow of glucose carbons through the metabolic network. The restoration of normal metabolism was evident through the decrease in glucose consumption and lactate production. The decrease in PDH phosphorylation leads to the subsequent increase in mitochondrial activity. Although many malignant cells have functioning mitochondria the preferred method of generating cellular energy is through rapid glycolysis. The decrease in PDH phosphorylation results in a cascade of events that culminate in cellular apoptosis (Hanberry, Berger *et al.* 2014).

When compared to current xenobiotic chemotherapeutic treatment strategies, such as DCA, high dose thiamine supplementation represents a potentially safe alternative. High dose DCA administration results in toxic neuropathy while thiamine administration well tolerated (Kaufmann, Engelstad *et al.* 2006, Smithline, Donnino *et al.* 2012). Although it can be

hypothesized that high dose thiamine supplementation would not affect normal cells, future work will seek to assess the effects of high dose thiamine on non-cancerous cells. However, as a result of its need for facilitated transport it is also necessary to characterize how malignant alterations in the maintenance of thiamine homeostasis can alter the efficacy of this strategy.

Our group has reported an increase in the gene expression of SLC19A2, SLC25A19, and TPK1 in breast cancer tissue when compared to normal breast tissue (Zastre, Hanberry *et al.* 2013). We have also observed a decrease in the expression of SLC19A3, consistent with what has been reported in breast, colon, and gastric cancers. We also observed an increase in the intracellular levels of thiamine and thiamine phosphorylates when comparing normal breast cells to breast cancer cells (Zastre, Hanberry *et al.* 2013). Alterations in thiamine homeostasis is hypothesized to support the malignant maintenance of energy and the production of biomass (Singleton and Martin 2001). Although these studies begin to define changes in the expression and function of thiamine transporters within malignant cells, alterations in thiamine homeostasis that occur as a result of changes in tumor microenvironment have not been fully characterized.

Using breast cancer cells, Sweet *et al.* identified an adaptive increase in the expression and function of SLC19A3 following hypoxic exposure (Sweet, Paul *et al.* 2010). However, in order for thiamine to support cellular metabolism it must first be converted to its cofactor form, TPP. Therefore, in order to further evaluate changes in in the regulation of thiamine homeostasis during malignant progression we continued to examine alterations within hypoxic microenvironments. We have now shown that hypoxic exposure induces a two-fold increase in the expression of TPK1 in breast cancer cells. Compared to vector control cells cultured at 1μM and 10μM, cells that exogenously over-express TPK1 variant 1 have a greater intracellular concentration of TPP. When compared to cells cultured in normoxia, an increase in TPP was

observed in hypoxic cells cultured in media containing 1μM and 10μM thiamine. Therefore, an increase in TPP is only observed in cells over-expressing TPK1. Furthermore, we have determined that the hypoxia induced increase in TPK1 protein expression is a result of the adaptive regulation of TPK1 protein translation. Further studies will seek to determine the molecular mechanisms by which TPK1 is adaptively regulated, following hypoxic exposure. We then sought to determine how thiamine supplementation affects the expression and function of thiamine homeostasis genes.

Thiamine supplementation results in an increase in intracellular thiamine in all cells regardless of culturing environment. In hypoxia, cells cultured in media containing 10μM thiamine show a further increase in thiamine uptake compared to cells cultured in normoxia alone. In order to evaluate the potential mechanism behind an increase in intracellular thiamine we then examined the membrane expression of thiamine transporters. An equivalent level of THTR1 and THTR2 membrane expression was observed following both the exogenous addition of thiamine and hypoxic exposure. Together, these observations suggest that the expression and function of a yet to be characterized thiamine transporter may be responsible for the increase in intracellular thiamine under these conditions.

In summary, high dose thiamine reduces malignant proliferation by altering malignant metabolism and inducing apoptosis. When compared to current chemotherapeutics, such as DCA, high dose thiamine represents a potentially safe treatment strategy. Furthermore, this approach is supported by malignant alterations in the regulation of thiamine homeostasis. We have shown that malignant cells up-regulate the intracellular uptake and activation of thiamine. Alterations observed in hypoxic microenvironments suggest that this targeted approach may overcome diffusional limitations of chemotherapeutics during solid tumor formation. Although

thiamine supplementation increases intracellular thiamine uptake, the limitation presented by the requirement for facilitated transport results in the need for alternative approaches to bypass this potential impedance. Further studies will seek to both determine how these alterations support cellular metabolism and optimize the chemotherapeutic utilization of high dose thiamine supplementation.

5.2. EXPERIMENTAL LIMITATIONS

The following section details both experimental limitations and assumptions that were made as they pertain to the use of cell culture systems and common methodologies.

5.2.1. Utilization of in vitro cell culture models

The use of in vitro cell culture systems presents limitations in the interpretation of scientific data. We are specifically interested in how alterations in oxygen supply and thiamine levels affect malignant progression. In our studies we utilized breast, neuroblastoma, and pancreatic cancer cells models to assess alterations in gene expression and the potential use of high dose vitamin as a chemotherapeutic strategy. The use of in vitro cell model systems to evaluate these changes represents a 2-dimensional approach where solid tumors proliferate in 3-dimensions.

5.2.2. Hypoxic Conditions

In our experimental setup all cells are exposed to the same amount of nutrients and oxygen. However, within a solid tumor a gradient is established which results in diffusional limitations. Utilization of an *in vitro* cell model does not allow an accurate recapitulation the

oxygen and nutrient exposure within a solid tumor. In our experiments we have selected to use 1% oxygen as it falls in the middle of the range of oxygen levels observed in a solid tumor (0.1-2%) (Vaupel, Kallinowski *et al.* 1989). Additionally, the use of *in vitro* models results in the potential for cells to be exposed to cycles of re-oxygenation during treatments. Cycling between hypoxia and re-oxygenation results in a number of intracellular changes including the alteration of gene transcription (Rupec and Baeuerle 1995, Hung, Charnock-Jones *et al.* 2004, Kim, Ahn *et al.* 2007, Postovit, Abbott *et al.* 2008). Therefore, future work will aim to include the use of in vivo models that more accurately represent diffusional limitations of oxygen observed within a solid tumor.

5.2.3. Nutrient Bioavailability

Using in vitro models provides 100% bioavailability when testing the effects of a treatment on cell proliferation. However, solid tumor formation occurs in 3-dimensions whereas in vitro cell model systems represent a 2-dimensional environment. In addition to limitations in the penetration of oxygen, cells furthest away from vasculature also have limited nutrient availability. Hypoxia induced alterations in thiamine homeostasis illustrate an adaptive increase in the uptake and activation of this essential vitamin. However, the requirement for facilitated transport limits the intracellular availability of thiamine. For this reason we recognize the necessity to study and evaluate compounds which increase the bioavailability of thiamine while limiting toxicity to normal cells. Administration of thiamine analogs, such as lipid-soluble thiamine disulfide S-acyl thiamine derivatives, increases the bioavailability of thiamine when compared to thiamine hydrochloride alone (Volvert, Seyen *et al.* 2008). Therefore utilization of

in vivo models is necessary to further assess the effects of both thiamine and thiamine analog administration on tumor proliferation.

5.3. FUTURE DIRECTIONS

5.3.1. Determination of Active Species

The involvement of membrane bound transporters and subsequent activation results in the dynamic intracellular homeostatic regulation of thiamine. In our studies we have observed a decrease in cancer cell proliferation following high dose thiamine administration. Additionally, we have shown that cancer cells undergo apoptosis through a DCA like mechanism in which the level of phosphorylated PDH is decreased and mitochondria are reactivated. However we have not determined which species of thiamine is responsible for this alteration.

The active form of thiamine, TPP, is a known regulator of PDH phosphorylation by PDKs. Kolobova *et al.* found that the presence of TPP decreases the extent to which PDH can be phosphorylated (Kolobova, Tuganova *et al.* 2001). However, we do not know how thiamine and its other phosphate derivatives specifically effect the inactivation of PDH by PDK enzymes. Additionally, we lack an understanding of how intracellular homeostasis is altered following high dose thiamine administration. Smithline *et al.* observed that thiamine supplementation alone does not result in an increase in intracellular TPP (Smithline, Donnino *et al.* 2012). We have confirmed this finding using MDA-MB-231 breast cancer cells cultured in 15nM, 1 μM, and 10μM thiamine. However, the reduction in cellular proliferation that we observed occurs following administration of more that 1mM thiamine. Therefore it is necessary to further evaluate how thiamine homeostasis changes following high dose administration.

Within cancer cells both thiamine and TPP elicit protective effects against free radical damage. Thiamine acts as an antioxidant, inhibits lipid peroxidation, and reduces free radical oxidation of oleic acids (Lukienko, Mel'nichenko *et al.* 2000). TPP also displays a preventive effect on cisplatin induced oxidative damage in liver tissue (Turan, Siltelioglu Turan *et al.* 2013). Trachootham *et al.* argued that modulating the redox regulatory mechanisms in cancer cells presents a potentially effective strategy in the treatment of cancer (Trachootham, Alexandre *et al.* 2009). Therefore, it can be hypothesized that thiamine and its phosphorylated forms may have multiple intracellular effects resulting in an apoptotic response.

For these reasons further work is necessary to assess the cofactor and non-cofactor role of high dose thiamine and how these individual species reduce cell proliferation. Quantitation of intracellular thiamine and thiamine phosphorylates will help to elucidate alterations in intracellular concentrations following high dose administration. Additionally, the use of activity assays will allow for the functional assessment of thiamine dependent enzymatic activity following high dose supplementation. Finally, assessing the effects of thiamine administration on modulating the level of intracellular ROS would provide insight into a potential non-cofactor role of high dose thiamine in reducing cellular proliferation.

5.3.2. Role in Malignant Progression

Altered cellular metabolism results in the requirement for an adequate supply of nutrient cofactors. In 2001, Boros *et al.* utilized an Ehrlich Ascites tumor mouse model to demonstrate the effects of thiamine supplementation on tumor proliferation. Currently we lack a sufficient understanding of the role of thiamine in malignant progression. Our group has shown an increase in SLC19A2, SLC25A19, and TPK1 gene expression in breast cancer tissue compared

to normal breast tissue (Zastre, Hanberry *et al.* 2013). Additionally, we observed a hypoxia induced increase in SLC19A3 and TPK1 expression, which results in an increase in thiamine uptake and phosphorylation in breast cancer cells (Sweet, Paul *et al.* 2010). However, the functional roles of these adaptive changes are unclear.

Previous research has also shown an increase in ribose production through the non-oxidative portion of the PPP and an increase in glutamine metabolism during malignancy (Eagle, Oyama *et al.* 1956, Kovacevic and McGivan 1983, Boros, Torday *et al.* 2000). These observations suggest that malignant alterations in the uptake and utilization of thiamine may be a critical pro-survival response. Although we have shown an adaptive increase in thiamine homeostasis genes in breast cancer when compared to normal breast cells, no one has examined intratumoral changes in thiamine homeostasis through the analysis of gene expression and quantitation of thiamine uptake and phosphorylation. For these reasons, alterations in thiamine homeostasis during tumor progression warrant further study. Further work is necessary to understand how these alterations support enhanced uptake and altered enzymatic activity during malignant progression.

REFERENCES

"Sidney Farber (1903-1973)." Classics in Oncology: Wiley Online.

Ahmad, I., R. Patel, Y. Liu, L. B. Singh, M. M. Taketo, X. R. Wu, H. Y. Leung and O. J. Sansom (2011). "Ras mutation cooperates with beta-catenin activation to drive bladder tumourigenesis." Cell Death Dis **2**: e124.

Akakura, N., M. Kobayashi, I. Horiuchi, A. Suzuki, J. Wang, J. Chen, H. Niizeki, K. Kawamura, M. Hosokawa and M. Asaka (2001). "Constitutive expression of hypoxia-inducible factor-1alpha renders pancreatic cancer cells resistant to apoptosis induced by hypoxia and nutrient deprivation." Cancer Res **61**(17): 6548-6554.

Alberola-Ila, J. and G. Hernandez-Hoyos (2003). "The Ras/MAPK cascade and the control of positive selection." Immunol Rev **191**: 79-96.

Altschuler, S. J. and L. F. Wu (2010). "Cellular heterogeneity: do differences make a difference?" Cell **141**(4): 559-563.

Ambros, V. (2004). "The functions of animal microRNAs." Nature **431**(7006): 350-355.

Anand, P., A. B. Kunnumakkara, C. Sundaram, K. B. Harikumar, S. T. Tharakan, O. S. Lai, B. Sung and B. B. Aggarwal (2008). "Cancer is a preventable disease that requires major lifestyle changes." Pharm Res **25**(9): 2097-2116.

Anastasiou, D., G. Poulogiannis, J. M. Asara, M. B. Boxer, J. K. Jiang, M. Shen, G. Bellinger, A. T. Sasaki, J. W. Locasale, D. S. Auld, C. J. Thomas, M. G. Vander Heiden and L. C. Cantley (2011). "Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses." Science **334**(6060): 1278-1283.

Aragon-Ching, J. B. and J. A. Zujewski (2007). "CNS metastasis: an old problem in a new guise." Clin Cancer Res **13**(6): 1644-1647.

Arumugam, M., J. Raes and E. Pelletier (2011). "Enterotypes of the human gut microbiome." Nature **473**(7346): 174-180.

Ashokkumar, B., N. D. Vaziri and H. M. Said (2006). "Thiamin uptake by the human-derived renal epithelial (HEK-293) cells: cellular and molecular mechanisms." Am J Physiol Renal Physiol **291**(4): F796-805.

Ashokkumar, B., N. D. Vaziri and H. M. Said (2006). "Thiamin uptake by the human-derived renal epithelial (HEK-293) cells: cellular and molecular mechanisms." American Journal of Physiology-Renal Physiology **291**(4): F796-F805.

Ayyanathan, K., S. Kesaraju, K. Dawson-Scully and H. Weissbach (2012). "Combination of sulindac and dichloroacetate kills cancer cells via oxidative damage." PLoS One **7**(7): e39949.

Bahn, A., Y. Hagos, S. Reuter, D. Balen, H. Brzica, W. Krick, B. C. Burckhardt, I. Sabolic and G. Burckhardt (2008). "Identification of a new urate and high affinity nicotinate transporter, hOAT10 (SLC22A13)." J Biol Chem **283**(24): 16332-16341.

Bailey, S. W. and J. E. Ayling (2009). "The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake." Proc Natl Acad Sci U S A **106**(36): 15424-15429.

Baker, H., O. Frank, T. Chen, S. Feingold, B. Deangelis and E. R. Baker (1981). "Elevated vitamin levels in colon adenocarcinoma as compared with metastatic liver adenocarcinoma from colon primary and normal adjacent tissue." Cancer **47**(12): 2883-2886.

Baker, H., O. Frank, T. Chen, S. Feingold, B. DeAngelis and E. R. Baker (1981). "Elevated vitamin levels in colon adenocarcinoma as compared with metastatic liver adenocarcinoma from colon primary and normal adjacent tissue." Cancer **47**(12): 2883-2886.

Balamurugan, K. and H. M. Said (2002). "Functional role of specific amino acid residues in human thiamine transporter SLC19A2: mutational analysis." Am J Physiol Gastrointest Liver Physiol **283**(1): G37-43.

Barthel, A., S. T. Okino, J. Liao, K. Nakatani, J. Li, J. P. Whitlock, Jr. and R. A. Roth (1999). "Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1." J Biol Chem **274**(29): 20281-20286.

Basturk, O., R. Singh, E. Kaygusuz, S. Balci, N. Dursun, N. Culhaci and N. V. Adsay (2011). "GLUT-1 expression in pancreatic neoplasia: implications in pathogenesis, diagnosis, and prognosis." Pancreas **40**(2): 187-192.

Basu, T. and J. Dickerson (1976). "The thiamin status of early cancer patients with particular reference to those with breast and bronchial carcinomas." Oncology **33**(5-6): 250-252.

Basu, T., J. Dickerson, R. Raven and D. Williams (1974). "The thiamine status of patients with cancer as determined by the red cell transketolase activity." International journal for vitamin and nutrition research. Internationale Zeitschrift für Vitamin-und Ernährungsforschung. Journal international de vitaminologie et de nutrition **44**(1): 53.

Basu, T. K. and J. W. Dickerson (1976). "The thiamin status of early cancer patients with particular reference to those with breast and bronchial carcinomas." Oncology **33**(5-6): 250-252.

Bauer, D. E., G. Hatzivassiliou, F. Zhao, C. Andreadis and C. B. Thompson (2005). "ATP citrate lyase is an important component of cell growth and transformation." Oncogene **24**(41): 6314-6322.

Baumunk, D., U. Reichelt, J. Hildebrandt, H. Krause, J. Ebbing, H. Cash, K. Miller, M. Schostak and S. Weikert (2013). "Expression parameters of the metabolic pathway genes pyruvate dehydrogenase kinase-1 (PDK-1) and DJ-1/PARK7 in renal cell carcinoma (RCC)." World J Urol **31**(5): 1191-1196.

Bentz, S., A. Cee, E. Endlicher, K. A. Wojtal, A. Naami, T. Pesch, S. Lang, P. Schubert, M. Fried, A. Weber, J. F. Coy, S. Goelder, R. Knuchel, M. Hausmann and G. Rogler (2013). "Hypoxia induces the expression of transketolase-like 1 in human colorectal cancer." Digestion **88**(3): 182-192.

Berg, A. and A. de Kok (1997). "2-Oxo acid dehydrogenase multienzyme complexes. The central role of the lipoyl domain." Biol Chem **378**(7): 617-634.

Berg, J. M., J. L. Tymocko and L. Strayer (2002). Biochemistry. New York, WH Freeman.

Berman, A. T., A. D. Thukral, W. T. Hwang, L. J. Solin and N. Vapiwala (2013). "Incidence and patterns of distant metastases for patients with early-stage breast cancer after breast conservation treatment." Clin Breast Cancer **13**(2): 88-94.

Bertout, J. A., S. A. Patel and M. C. Simon (2008). "The impact of O2 availability on human cancer." Nat Rev Cancer **8**(12): 967-975.

Bertram, J. S. (2000). "The molecular biology of cancer." Mol Aspects Med **21**(6): 167-223.

Bettendorff, L. (1994). "The compartmentation of phosphorylated thiamine derivatives in cultured neuroblastoma cells." Biochim Biophys Acta **1222**(1): 7-14.

Bettendorff, L. (1995). "Thiamine homeostasis in neuroblastoma cells." Neurochem Int **26**(3): 295-302.

Bettendorff, L., L. Weekers, P. Wins and E. Schoffeniels (1990). "Injection of sulbutiamine induces an increase in thiamine triphosphate in rat tissues." Biochem Pharmacol **40**(11): 2557-2560.

Bindra, R. S. and P. M. Glazer (2005). "Genetic instability and the tumor microenvironment: towards the concept of microenvironment-induced mutagenesis." Mutat Res **569**(1-2): 75-85.

Bitsch, R., M. Wolf, J. Moller, L. Heuzeroth and D. Gruneklee (1991). "Bioavailability assessment of the lipophilic benfotiamine as compared to a water-soluble thiamin derivative." Ann Nutr Metab **35**(5): 292-296.

Blagosklonny, M. V. (2004). "Antiangiogenic therapy and tumor progression." Cancer Cell **5**(1): 13-17.

Boidot, R., F. Vegran, A. Meulle, A. Le Breton, C. Dessy, P. Sonveaux, S. Lizard-Nacol and O. Feron (2012). "Regulation of monocarboxylate transporter MCT1 expression by p53 mediates inward and outward lactate fluxes in tumors." Cancer Res **72**(4): 939-948.

Bonnet, S., S. L. Archer, J. Allalunis-Turner, A. Haromy, C. Beaulieu, R. Thompson, C. T. Lee, G. D. Lopaschuk, L. Puttagunta, S. Bonnet, G. Harry, K. Hashimoto, C. J. Porter, M. A. Andrade, B. Thebaud and E. D. Michelakis (2007). "A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth." Cancer Cell **11**(1): 37-51.

Boros, L. (1999). "Population thiamine status and varying cancer rates between western, Asian and African countries." Anticancer research **20**(3B): 2245-2248.

Boros, L., J. Brandes, W. Lee, M. Cascante, J. Puigjaner, E. Revesz, T. Bray, W. Schirmer and W. Melvin (1998). "Thiamine supplementation to cancer patients: a double edged sword." Anticancer research **18**(1): 595-602.

Boros, L., P. Lee, J. Brandes, M. Cascante, P. Muscarella, W. Schirmer, W. Melvin and E. Ellison (1998). "Nonoxidative pentose phosphate pathways and their direct role in ribose synthesis in tumors: is cancer a disease of cellular glucose metabolism?" Medical hypotheses **50**(1): 55-59.

Boros, L., J. Puigjaner, M. Cascante, W. Lee, J. Brandes, S. Bassilian, F. Yusuf, R. Williams, P. Muscarella and W. Melvin (1997). "Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation." Cancer research **57**(19): 4242.

Boros, L. G., J. Puigjaner, M. Cascante, W. N. Lee, J. L. Brandes, S. Bassilian, F. I. Yusuf, R. D. Williams, P. Muscarella, W. S. Melvin and W. J. Schirmer (1997). "Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation." Cancer Res **57**(19): 4242-4248.

Boros, L. G., J. S. Torday, S. Lim, S. Bassilian, M. Cascante and W. N. Lee (2000). "Transforming growth factor beta2 promotes glucose carbon incorporation into nucleic acid ribose through the nonoxidative pentose cycle in lung epithelial carcinoma cells." Cancer Res **60**(5): 1183-1185.

Boulware, M. J., V. S. Subramanian, H. M. Said and J. S. Marchant (2003). "Polarized expression of members of the solute carrier SLC19A gene family of water-soluble multivitamin transporters: implications for physiological function." Biochem J **376**(Pt 1): 43-48.

Bowker-Kinley, M. M., W. I. Davis, P. Wu, R. A. Harris and K. M. Popov (1998). "Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex." Biochem J **329 (Pt 1)**: 191-196.

Brody, T. (1999). Nutritional Biochemistry. San Diego, Academic Press.

Broer, S., H. P. Schneider, A. Broer, B. Rahman, B. Hamprecht and J. W. Deitmer (1998). "Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH." Biochem J **333 (Pt 1)**: 167-174.

Bruce, W. R., M. Cirocco, A. Giacca, Y. I. Kim, N. Marcon and S. Minkin (2005). "A pilot randomised controlled trial to reduce colorectal cancer risk markers associated with B-vitamin deficiency, insulin resistance and colonic inflammation." Br J Cancer **93**(6): 639-646.

Bruce, W. R., R. Furrer, N. Shangari, P. J. O'Brien, A. Medline and Y. Wang (2003). "Marginal dietary thiamin deficiency induces the formation of colonic aberrant crypt foci (ACF) in rats." Cancer Lett **202**(2): 125-129.

Bubendorf, L., A. Schopfer, U. Wagner, G. Sauter, H. Moch, N. Willi, T. C. Gasser and M. J. Mihatsch (2000). "Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients." Hum Pathol **31**(5): 578-583.

Cairns, R. A., I. S. Harris and T. W. Mak (2011). "Regulation of cancer cell metabolism." Nat Rev Cancer **11**(2): 85-95.

Califano, J., P. van der Riet, W. Westra, H. Nawroz, G. Clayman, S. Piantadosi, R. Corio, D. Lee, B. Greenberg, W. Koch and D. Sidransky (1996). "Genetic progression model for head and neck cancer: implications for field cancerization." Cancer Res **56**(11): 2488-2492.

Calin, G. A., C. Sevignani, C. D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini and C. M. Croce (2004). "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers." Proc Natl Acad Sci U S A **101**(9): 2999-3004.

Campbell, P. J., E. D. Pleasance, P. J. Stephens, E. Dicks, R. Rance, I. Goodhead, G. A. Follows, A. R. Green, P. A. Futreal and M. R. Stratton (2008). "Subclonal phylogenetic structures in cancer revealed by ultra-deep sequencing." Proc Natl Acad Sci U S A **105**(35): 13081-13086.

Cannizzaro, S. M., R. F. Padera, R. Langer, R. A. Rogers, F. E. Black, M. C. Davies, S. J. Tendler and K. M. Shakesheff (1998). "A novel biotinylated degradable polymer for cell-interactive applications." Biotechnol Bioeng **58**(5): 529-535.

Cantor, J. R. and D. M. Sabatini (2012). "Cancer cell metabolism: one hallmark, many faces." Cancer Discov **2**(10): 881-898.

Carew, J. S. and P. Huang (2002). "Mitochondrial defects in cancer." Mol Cancer **1**: 9.

Carew, J. S., Y. Zhou, M. Albitar, J. D. Carew, M. J. Keating and P. Huang (2003). "Mitochondrial DNA mutations in primary leukemia cells after chemotherapy: clinical significance and therapeutic implications." Leukemia **17**(8): 1437-1447.

Cascante, M., J. Centelles, R. Veech, W. Lee and L. Boros (2000). "Role of thiamin (vitamin B-1) and transketolase in tumor cell proliferation." Nutrition and cancer **36**(2): 150-154.

Cervantes-Laurean, D., N. G. McElvaney and J. Moss (1999). Niacin. Baltimore, Williams & Wilkins.

Chaffer, C. L. and R. A. Weinberg (2011). "A perspective on cancer cell metastasis." Science **331**(6024): 1559-1564.

Chamberlain, B., J. Buttery and P. R. Pannall (1996). "A stable reagent mixture for the whole blood transketolase assay." Annals of clinical biochemistry **33**: 352-354.

Chatterjee, A., S. Dasgupta and D. Sidransky (2011). "Mitochondrial subversion in cancer." Cancer Prev Res (Phila) **4**(5): 638-654.

Chen, C., N. Pore, A. Behrooz, F. Ismail-Beigi and A. Maity (2001). "Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia." J Biol Chem **276**(12): 9519-9525.

Chen, H., J. X. Yue, S. H. Yang, H. Ding, R. W. Zhao and S. Zhang (2009). "Overexpression of transketolase-like gene 1 is associated with cell proliferation in uterine cervix cancer." J Exp Clin Cancer Res **28**: 43.

Chen, J. Z. and F. F. Kadlubar (2004). "Mitochondrial mutagenesis and oxidative stress in human prostate cancer." J Environ Sci Health C Environ Carcinog Ecotoxicol Rev **22**(1): 1-12.

Chen, L., Y. Shu, X. Liang, E. C. Chen, S. W. Yee, A. A. Zur, S. Li, L. Xu, K. R. Keshari, M. J. Lin, H. C. Chien, Y. Zhang, K. M. Morrissey, J. Liu, J. Ostrem, N. S. Younger, J. Kurhanewicz, K. M. Shokat, K. Ashrafi and K. M. Giacomini (2014). "OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin." Proc Natl Acad Sci U S A **111**(27): 9983-9988.

Chen, Y., J. McGee, X. Chen, T. N. Doman, X. Gong, Y. Zhang, N. Hamm, X. Ma, R. E. Higgs, S. V. Bhagwat, S. Buchanan, S. B. Peng, K. A. Staschke, V. Yadav, Y. Yue and H. Kouros-Mehr (2014). "Identification of druggable cancer driver genes amplified across TCGA datasets." PLoS One **9**(5): e98293.

Choi, S. W. and J. B. Mason (2000). "Folate and carcinogenesis: an integrated scheme." J Nutr **130**(2): 129-132.

Chornyy, S., J. Parkhomenko and N. Chorna (2007). "Thiamine deficiency caused by thiamine antagonists triggers upregulation of apoptosis inducing factor gene expression and leads to caspase 3-mediated apoptosis in neuronally differentiated rat PC-12 cells." Acta Biochim Pol **54**(2): 315-322.

Christofk, H. R., M. G. Vander Heiden, M. H. Harris, A. Ramanathan, R. E. Gerszten, R. Wei, M. D. Fleming, S. L. Schreiber and L. C. Cantley (2008). "The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth." Nature **452**(7184): 230-233.

Churchill-Davidson, I., C. Sanger and R. H. Thomlinson (1955). "High-pressure oxygen and radiotherapy." Lancet **268**(6874): 1091-1095.

Ciszak, E. M., L. G. Korotchkina, P. M. Dominiak, S. Sidhu and M. S. Patel (2003). "Structural basis for flip-flop action of thiamin pyrophosphate-dependent enzymes revealed by human pyruvate dehydrogenase." J Biol Chem **278**(23): 21240-21246.

Coghlin, C. and G. I. Murray (2010). "Current and emerging concepts in tumour metastasis." J Pathol **222**(1): 1-15.

Comin-Anduix, B., J. Boren, S. Martinez, C. Moro, J. J. Centelles, R. Trebukhina, N. Petushok, W. N. Lee, L. G. Boros and M. Cascante (2001). "The effect of thiamine supplementation on tumour proliferation. A metabolic control analysis study." Eur J Biochem **268**(15): 4177-4182.

Comín- Anduix, B., J. Boren, S. Martinez, C. Moro, J. J. Centelles, R. Trebukhina, N. Petushok, W. N. P. Lee, L. G. Boros and M. Cascante (2001). "The effect of thiamine supplementation on tumour proliferation." European Journal of Biochemistry **268**(15): 4177-4182.

Crea, F., E. M. Hurt, L. A. Mathews, S. M. Cabarcas, L. Sun, V. E. Marquez, R. Danesi and W. L. Farrar (2011). "Pharmacologic disruption of Polycomb Repressive Complex 2 inhibits tumorigenicity and tumor progression in prostate cancer." Mol Cancer **10**: 40.

Croce, C. M. (2008). "Oncogenes and cancer." N Engl J Med **358**(5): 502-511.

Daily, A., S. Liu, Y. Bae, S. Bhatnagar and J. A. Moscow (2011). "Linear chain PEGylated recombinant Bacillus thiaminolyticus thiaminase I enzyme has growth inhibitory activity against lymphoid leukemia cell lines." Mol Cancer Ther **10**(9): 1563-1570.

Daily, A., S. Liu, S. Bhatnagar, R. G. Karabakhtsian and J. A. Moscow (2012). "Low-thiamine diet increases mammary tumor latency in FVB/N-Tg(MMTVneu) mice." Int J Vitam Nutr Res **82**(4): 298-302.

Dang, C. V., J. W. Kim, P. Gao and J. Yustein (2008). "The interplay between MYC and HIF in cancer." Nat Rev Cancer **8**(1): 51-56.

Dang, C. V., A. Le and P. Gao (2009). "MYC-induced cancer cell energy metabolism and therapeutic opportunities." Clin Cancer Res **15**(21): 6479-6483.

Dawson, D. M., T. L. Goodfriend and N. O. Kaplan (1964). "Lactic Dehydrogenases: Functions of the Two Types Rates of Synthesis of the Two Major Forms Can Be Correlated with Metabolic Differentiation." Science **143**(3609): 929-933.

DeBerardinis, R. J., J. J. Lum, G. Hatzivassiliou and C. B. Thompson (2008). "The biology of cancer: metabolic reprogramming fuels cell growth and proliferation." Cell Metab **7**(1): 11-20.

DeBerardinis, R. J., J. J. Lum, G. Hatzivassiliou and C. B. Thompson (2008). "The biology of cancer: metabolic reprogramming fuels cell growth and proliferation." Cell metabolism **7**(1): 11-20.

DeBerardinis, R. J., A. Mancuso, E. Daikhin, I. Nissim, M. Yudkoff, S. Wehrli and C. B. Thompson (2007). "Beyond aerobic glycolysis: transformed cells can engage in glutamine

metabolism that exceeds the requirement for protein and nucleotide synthesis." Proc Natl Acad Sci U S A **104**(49): 19345-19350.

DeBerardinis, R. J., A. Mancuso, E. Daikhin, I. Nissim, M. Yudkoff, S. Wehrli and C. B. Thompson (2007). "Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis." Proceedings of the National Academy of Sciences **104**(49): 19345-19350.

Deberardinis, R. J., N. Sayed, D. Ditsworth and C. B. Thompson (2008). "Brick by brick: metabolism and tumor cell growth." Curr Opin Genet Dev **18**(1): 54-61.

Decker, L. E. and R. U. Byerrum (1954). "The relationship between dietary riboflavin concentration and the tissue concentration of riboflavin-containing coenzymes and enzymes." J Nutr **53**(2): 303-315.

Denko, N. C. (2008). "Hypoxia, HIF1 and glucose metabolism in the solid tumour." Nat Rev Cancer **8**(9): 705-713.

Denton, R. M. and J. G. McCormack (1980). "On the role of the calcium transport cycle in heart and other mammalian mitochondria." FEBS Lett **119**(1): 1-8.

Desmoulin, S. K., Z. Hou, A. Gangjee and L. H. Matherly (2012). "The human proton-coupled folate transporter: Biology and therapeutic applications to cancer." Cancer Biol Ther **13**(14): 1355-1373.

Desouki, M. M., S. Liao, H. Huang, J. Conroy, N. J. Nowak, L. Shepherd, D. P. Gaile and J. Geradts (2011). "Identification of metastasis-associated breast cancer genes using a high-resolution whole genome profiling approach." Journal of cancer research and clinical oncology **137**(5): 795-809.

Diaz, G. A., M. Banikazemi, K. Oishi, R. J. Desnick and B. D. Gelb (1999). "Mutations in a new gene encoding a thiamine transporter cause thiamine-responsive megaloblastic anaemia syndrome." Nat Genet **22**(3): 309-312.

Diaz-Moralli, S., M. Tarrado-Castellarnau, C. Alenda, A. Castells and M. Cascante (2011). "Transketolase-like 1 expression is modulated during colorectal cancer progression and metastasis formation." PLoS One **6**(9): e25323.

Ding, B. C., J. R. Whetstine, T. L. Witt, J. D. Schuetz and L. H. Matherly (2001). "Repression of human reduced folate carrier gene expression by wild type p53." J Biol Chem **276**(12): 8713-8719.

Dolce, V., G. Fiermonte, M. J. Runswick, F. Palmieri and J. E. Walker (2001). "The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals." Proc Natl Acad Sci U S A **98**(5): 2284-2288.

Dominguez-Sola, D., C. Y. Ying, C. Grandori, L. Ruggiero, B. Chen, M. Li, D. A. Galloway, W. Gu, J. Gautier and R. Dalla-Favera (2007). "Non-transcriptional control of DNA replication by c-Myc." Nature **448**(7152): 445-451.

Downward, J. (2003). "Targeting RAS signalling pathways in cancer therapy." Nat Rev Cancer **3**(1): 11-22.

Dutta, B., W. Huang, M. Molero, R. Kekuda, F. H. Leibach, L. D. Devoe, V. Ganapathy and P. D. Prasad (1999). "Cloning of the human thiamine transporter, a member of the folate transporter family." J Biol Chem **274**(45): 31925-31929.

Eagle, H., V. I. Oyama, M. Levy, C. L. Horton and R. Fleischman (1956). "The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid." J Biol Chem **218**(2): 607-616.

Edinger, A. L. and C. B. Thompson (2002). "Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake." Mol Biol Cell **13**(7): 2276-2288.

Elmadfa, I., D. Majchrzak, P. Rust and D. Genser (2001). "The thiamine status of adult humans depends on carbohydrate intake." International journal for vitamin and nutrition research **71**(4): 217-221.

Elnakat, H. and M. Ratnam (2004). "Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy." Adv Drug Deliv Rev **56**(8): 1067-1084.

Elstrom, R. L., D. E. Bauer, M. Buzzai, R. Karnauskas, M. H. Harris, D. R. Plas, H. Zhuang, R. M. Cinalli, A. Alavi, C. M. Rudin and C. B. Thompson (2004). "Akt stimulates aerobic glycolysis in cancer cells." Cancer Res **64**(11): 3892-3899.

Elwood, P. C., K. Nachmanoff, Y. Saikawa, S. T. Page, P. Pacheco, S. Roberts and K. N. Chung (1997). "The divergent 5' termini of the alpha human folate receptor (hFR) mRNAs originate from two tissue-specific promoters and alternative splicing: characterization of the alpha hFR gene structure." Biochemistry **36**(6): 1467-1478.

Estrella, V., T. Chen, M. Lloyd, J. Wojtkowiak, H. H. Cornnell, A. Ibrahim-Hashim, K. Bailey, Y. Balagurunathan, J. M. Rothberg, B. F. Sloane, J. Johnson, R. A. Gatenby and R. J. Gillies (2013). "Acidity generated by the tumor microenvironment drives local invasion." Cancer Res **73**(5): 1524-1535.

Eudy, J. D., O. Spiegelstein, R. C. Barber, B. J. Wlodarczyk, J. Talbot and R. H. Finnell (2000). "Identification and characterization of the human and mouse SLC19A3 gene: a novel member of the reduced folate family of micronutrient transporter genes." Mol Genet Metab **71**(4): 581-590.

Farber, S. and L. K. Diamond (1948). "Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid." N Engl J Med **238**(23): 787-793.

Figueiredo, J. C., M. V. Grau, R. W. Haile, R. S. Sandler, R. W. Summers, R. S. Bresalier, C. A. Burke, G. E. McKeown-Eyssen and J. A. Baron (2009). "Folic acid and risk of prostate cancer: results from a randomized clinical trial." J Natl Cancer Inst **101**(6): 432-435.

Firth, J. D., B. L. Ebert and P. J. Ratcliffe (1995). "Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements." J Biol Chem **270**(36): 21021-21027.

Fiume, M. Z. and P. Cosmetic Ingredient Review Expert (2001). "Final report on the safety assessment of biotin." Int J Toxicol **20 Suppl 4**: 1-12.

Fleming, J. C., M. P. Steinkamp, R. Kawatsuji, E. Tartaglini, J. L. Pinkus, G. S. Pinkus, M. D. Fleming and E. J. Neufeld (2001). "Characterization of a murine high-affinity thiamine transporter, Slc19a2." Mol Genet Metab **74**(1-2): 273-280.

Fleming, J. C., E. Tartaglini, M. P. Steinkamp, D. F. Schorderet, N. Cohen and E. J. Neufeld (1999). "The gene mutated in thiamine-responsive anaemia with diabetes and deafness (TRMA) encodes a functional thiamine transporter." Nat Genet **22**(3): 305-308.

Flier, J. S., M. M. Mueckler, P. Usher and H. F. Lodish (1987). "Elevated levels of glucose transport and transporter messenger RNA are induced by ras or src oncogenes." Science **235**(4795): 1492-1495.

Foldi, M., E. Stickeler, L. Bau, O. Kretz, D. Watermann, G. Gitsch, G. Kayser, A. Zur Hausen and J. F. Coy (2007). "Transketolase protein TKTL1 overexpression: A potential biomarker and therapeutic target in breast cancer." Oncol Rep **17**(4): 841-845.

Földi, M., E. Stickeler, L. Bau, O. Kretz, D. Watermann, G. Gitsch, G. Kayser, A. zur Hausen and J. F. Coy (2007). "Transketolase protein TKTL1 overexpression: A potential biomarker and therapeutic target in breast cancer." Oncology reports **17**(4): 841-845.

Forbes, N. S., A. L. Meadows, D. S. Clark and H. W. Blanch (2006). "Estradiol stimulates the biosynthetic pathways of breast cancer cells: detection by metabolic flux analysis." Metab Eng **8**(6): 639-652.

Forbes, N. S., A. L. Meadows, D. S. Clark and H. W. Blanch (2006). "Estradiol stimulates the biosynthetic pathways of breast cancer cells: detection by metabolic flux analysis." Metabolic engineering **8**(6): 639-652.

Frank, T., R. Bitsch, J. Maiwald and G. Stein (2000). "High thiamine diphosphate concentrations in erythrocytes can be achieved in dialysis patients by oral administration of benfontiamine." Eur J Clin Pharmacol **56**(3): 251-257.

Fraser, J. L., A. Vanderver, S. Yang, T. Chang, L. Cramp, G. Vezina, U. Lichter-Konecki, K. P. Cusmano-Ozog, P. Smpokou and K. A. Chapman (2014). "Thiamine pyrophosphokinase deficiency causes a Leigh Disease like phenotype in a sibling pair: Identification through whole exome sequencing and management strategies." Molecular Genetics and Metabolism Reports **1**: 66-70.

Fujii, H., M. Yoshida, Z. X. Gong, T. Matsumoto, Y. Hamano, M. Fukunaga, R. H. Hruban, E. Gabrielson and T. Shirai (2000). "Frequent genetic heterogeneity in the clonal evolution of gynecological carcinosarcoma and its influence on phenotypic diversity." Cancer Res **60**(1): 114-120.

Fuss, J. O. and P. K. Cooper (2006). "DNA repair: dynamic defenders against cancer and aging." PLoS Biol **4**(6): e203.

Ganapathy, V., S. Smith and P. Prasad (2004). "SLC19: the folate/thiamine transporter family." Pflügers Archiv European Journal of Physiology **447**(5): 641-646.

Ganapathy, V., S. B. Smith and P. D. Prasad (2004). "SLC19: the folate/thiamine transporter family." Pflugers Arch **447**(5): 641-646.

Gangolf, M., J. Czerniecki, M. Radermecker, O. Detry, M. Nisolle, C. Jouan, D. Martin, F. Chantraine, B. Lakaye and P. Wins (2010). "Thiamine status in humans and content of phosphorylated thiamine derivatives in biopsies and cultured cells." PLoS One **5**(10): e13616.

Gangolf, M., J. Czerniecki, M. Radermecker, O. Detry, M. Nisolle, C. Jouan, D. Martin, F. Chantraine, B. Lakaye, P. Wins, T. Grisar and L. Bettendorff (2010). "Thiamine status in humans and content of phosphorylated thiamine derivatives in biopsies and cultured cells." PLoS One **5**(10): e13616.

Gao, S., L. H. Ding, J. W. Wang, C. B. Li and Z. Y. Wang (2013). "Diet folate, DNA methylation and polymorphisms in methylenetetrahydrofolate reductase in association with the susceptibility to gastric cancer." Asian Pac J Cancer Prev **14**(1): 299-302.

Garber, K. (2006). "Energy deregulation: licensing tumors to grow." Science **312**(5777): 1158-1159.

Garland, P. B. (1964). "Some kinetic properties of pig-heart oxoglutarate dehydrogenase that provide a basis for metabolic control of the enzyme activity and also a stoicheiometric assay for coenzyme A in tissue extracts." Biochem J **92**(2): 10C-12C.

Gatenby, R. A. and R. J. Gillies (2007). "Glycolysis in cancer: a potential target for therapy." Int J Biochem Cell Biol **39**(7-8): 1358-1366.

Geyer, J., M. Netzel, I. Bitsch, T. Frank, R. Bitsch, K. Kramer and P. P. Hoppe (2000). "Bioavailability of water- and lipid-soluble thiamin compounds in broiler chickens." Int J Vitam Nutr Res **70**(6): 311-316.

Ghosal, A., N. Lambrecht, S. B. Subramanya, R. Kapadia and H. M. Said (2013). "Conditional knockout of the Slc5a6 gene in mouse intestine impairs biotin absorption." Am J Physiol Gastrointest Liver Physiol **304**(1): G64-71.

Gille, A., E. T. Bodor, K. Ahmed and S. Offermanns (2008). "Nicotinic acid: pharmacological effects and mechanisms of action." Annu Rev Pharmacol Toxicol **48**: 79-106.

Gleadle, J. M. and P. J. Ratcliffe (1997). "Induction of hypoxia-inducible factor-1, erythropoietin, vascular endothelial growth factor, and glucose transporter-1 by hypoxia: evidence against a regulatory role for Src kinase." Blood **89**(2): 503-509.

Goldman, I. D. and L. H. Matherly (1985). "The cellular pharmacology of methotrexate." Pharmacol Ther **28**(1): 77-102.

Goldman, I. D. and R. Zhao (2002). "Molecular, biochemical, and cellular pharmacology of pemetrexed." Semin Oncol **29**(6 Suppl 18): 3-17.

Gotanda, Y., Y. Akagi, A. Kawahara, T. Kinugasa, T. Yoshida, Y. Ryu, I. Shiratsuchi, M. Kage and K. Shirouzu (2013). "Expression of monocarboxylate transporter (MCT)-4 in colorectal cancer and its role: MCT4 contributes to the growth of colorectal cancer with vascular endothelial growth factor." Anticancer Res **33**(7): 2941-2947.

Gottlieb, E. and I. P. Tomlinson (2005). "Mitochondrial tumour suppressors: a genetic and biochemical update." Nat Rev Cancer **5**(11): 857-866.

Grabellus, F., J. Nagarajah, A. Bockisch, K. W. Schmid and S. Y. Sheu (2012). "Glucose transporter 1 expression, tumor proliferation, and iodine/glucose uptake in thyroid cancer with emphasis on poorly differentiated thyroid carcinoma." Clin Nucl Med **37**(2): 121-127.

Grander, D. (1998). "How do mutated oncogenes and tumor suppressor genes cause cancer?" Med Oncol **15**(1): 20-26.

Gray, L. H. (1961). "Radiobiologic basis of oxygen as a modifying factor in radiation therapy." Am J Roentgenol Radium Ther Nucl Med **85**: 803-815.

Greb, A. and R. Bitsch (1998). "Comparative bioavailability of various thiamine derivatives after oral administration." Int J Clin Pharmacol Ther **36**(4): 216-221.

Grivicich, I., A. Regner, A. B. da Rocha, L. B. Grass, P. A. Alves, G. B. Kayser, G. Schwartsmann and J. A. Henriques (2005). "Irinotecan/5-fluorouracil combination induces alterations in mitochondrial membrane potential and caspases on colon cancer cell lines." Oncol Res **15**(7-8): 385-392.

Guo, J. Y., H. Y. Chen, R. Mathew, J. Fan, A. M. Strohecker, G. Karsli-Uzunbas, J. J. Kamphorst, G. Chen, J. M. Lemons, V. Karantza, H. A. Coller, R. S. Dipaola, C. Gelinas, J. D. Rabinowitz and E. White (2011). "Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis." Genes Dev **25**(5): 460-470.

Guppy, M., E. Greiner and K. Brand (1993). "The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes." Eur J Biochem **212**(1): 95-99.

Hagenbuch, B. and C. Gui (2008). "Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family." Xenobiotica **38**(7-8): 778-801.

Hainaut, P. and A. Plymoth (2013). "Targeting the hallmarks of cancer: towards a rational approach to next-generation cancer therapy." Curr Opin Oncol **25**(1): 50-51.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hanberry, B. S., R. Berger and J. A. Zastre (2014). "High-dose vitamin B1 reduces proliferation in cancer cell lines analogous to dichloroacetate." Cancer Chemother Pharmacol.

Hatzivassiliou, G., F. Zhao, D. E. Bauer, C. Andreadis, A. N. Shaw, D. Dhanak, S. R. Hingorani, D. A. Tuveson and C. B. Thompson (2005). "ATP citrate lyase inhibition can suppress tumor cell growth." Cancer Cell **8**(4): 311-321.

He, M., Q. Y. Wang, Q. Q. Yin, J. Tang, Y. Lu, C. X. Zhou, C. W. Duan, D. L. Hong, T. Tanaka, G. Q. Chen and Q. Zhao (2013). "HIF-1alpha downregulates miR-17/20a directly targeting p21 and STAT3: a role in myeloid leukemic cell differentiation." Cell Death Differ **20**(3): 408-418.

Hess, K. R., G. R. Varadhachary, S. H. Taylor, W. Wei, M. N. Raber, R. Lenzi and J. L. Abbruzzese (2006). "Metastatic patterns in adenocarcinoma." Cancer **106**(7): 1624-1633.

Higdon, J. (2014). "Riboflavin." Micronutrient Information Center.

Hitosugi, T., L. Zhou, S. Elf, J. Fan, H. B. Kang, J. H. Seo, C. Shan, Q. Dai, L. Zhang, J. Xie, T. L. Gu, P. Jin, M. Aleckovic, G. LeRoy, Y. Kang, J. A. Sudderth, R. J. DeBerardinis, C. H. Luan, G. Z. Chen, S. Muller, D. M. Shin, T. K. Owonikoko, S. Lonial, M. L. Arellano, H. J. Khoury, F. R. Khuri, B. H. Lee, K. Ye, T. J. Boggon, S. Kang, C. He and J. Chen (2012). "Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth." Cancer Cell **22**(5): 585-600.

Holland-Frei (2003). Holland-Frei Cancer Medicine, BC Decker.

Hoyumpa, A. M., Jr. (1980). "Mechanisms of thiamin deficiency in chronic alcoholism." Am J Clin Nutr **33**(12): 2750-2761.

Hsu, P. P. and D. M. Sabatini (2008). "Cancer cell metabolism: Warburg and beyond." Cell **134**(5): 703-707.

Hu, L. H., J. H. Yang, D. T. Zhang, S. Zhang, L. Wang, P. C. Cai, J. F. Zheng and J. S. Huang (2007). "The TKTL1 gene influences total transketolase activity and cell proliferation in human colon cancer LoVo cells." Anticancer Drugs **18**(4): 427-433.

Huang, B., R. Gudi, P. Wu, R. A. Harris, J. Hamilton and K. M. Popov (1998). "Isoenzymes of pyruvate dehydrogenase phosphatase. DNA-derived amino acid sequences, expression, and regulation." J Biol Chem **273**(28): 17680-17688.

Huang, B., P. Wu, K. M. Popov and R. A. Harris (2003). "Starvation and diabetes reduce the amount of pyruvate dehydrogenase phosphatase in rat heart and kidney." Diabetes **52**(6): 1371-1376.

Hung, T. H., D. S. Charnock-Jones, J. N. Skepper and G. J. Burton (2004). "Secretion of tumor necrosis factor-alpha from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: a potential mediator of the inflammatory response in preeclampsia." Am J Pathol **164**(3): 1049-1061.

Hur, H., Y. Xuan, Y. B. Kim, G. Lee, W. Shim, J. Yun, I. H. Ham and S. U. Han (2013). "Expression of pyruvate dehydrogenase kinase-1 in gastric cancer as a potential therapeutic target." Int J Oncol **42**(1): 44-54.

Ikehata, M., K. Ueda and S. Iwakawa (2012). "Different involvement of DNA methylation and histone deacetylation in the expression of solute-carrier transporters in 4 colon cancer cell lines." Biol Pharm Bull **35**(3): 301-307.

Ikehata, M., K. Ueda and S. Iwakawa (2012). "Different involvement of DNA methylation and histone deacetylation in the expression of solute-carrier transporters in 4 colon cancer cell lines." Biological and Pharmaceutical Bulletin **35**(3): 301-307.

Ishikawa, K., K. Takenaga, M. Akimoto, N. Koshikawa, A. Yamaguchi, H. Imanishi, K. Nakada, Y. Honma and J. Hayashi (2008). "ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis." Science **320**(5876): 661-664.

Iyer, N. V., L. E. Kotch, F. Agani, S. W. Leung, E. Laughner, R. H. Wenger, M. Gassmann, J. D. Gearhart, A. M. Lawler, A. Y. Yu and G. L. Semenza (1998). "Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha." Genes Dev **12**(2): 149-162.

Iyer, R. R., A. Pluciennik, V. Burdett and P. L. Modrich (2006). "DNA mismatch repair: functions and mechanisms." Chem Rev **106**(2): 302-323.

Jacobson, E. L., W. M. Shieh and A. C. Huang (1999). "Mapping the role of NAD metabolism in prevention and treatment of carcinogenesis." Mol Cell Biochem **193**(1-2): 69-74.

Jaconello, P. (1992). "Niacin versus niacinamide." CMAJ **147**(7): 990.

Jansen, G. (19991). "Receptor- and carrier-mediated transport systems for folates and antifolates. Exploitation for folate chemotherapy and immunotherapy."

Journe, F., G. Laurent, C. Chaboteaux, D. Nonclercq, V. Durbecq, D. Larsimont and J.-J. Body (2008). "Farnesol, a mevalonate pathway intermediate, stimulates MCF-7 breast cancer cell growth through farnesoid-X-receptor-mediated estrogen receptor activation." Breast cancer research and treatment **107**(1): 49-61.

Jozwiak, P., A. Krzeslak, L. Pomorski and A. Lipinska (2012). "Expression of hypoxia-related glucose transporters GLUT1 and GLUT3 in benign, malignant and non-neoplastic thyroid lesions." Mol Med Rep **6**(3): 601-606.

Jr., G. F. C. (2008). The vitamins: Fundamental Aspects in Nutrition and Health, Elsevier Academic Press.

Jun, Y. J., S. M. Jang, H. L. Han, K. H. Lee, K. S. Jang and S. S. Paik (2011). "Clinicopathologic significance of GLUT1 expression and its correlation with Apaf-1 in colorectal adenocarcinomas." World J Gastroenterol **17**(14): 1866-1873.

Kabat, G. C., A. B. Miller, M. Jain and T. E. Rohan (2008). "Dietary intake of selected B vitamins in relation to risk of major cancers in women." Br J Cancer **99**(5): 816-821.

Kang, J. and D. C. Samuels (2008). "The evidence that the DNC (SLC25A19) is not the mitochondrial deoxyribonucleotide carrier." Mitochondrion **8**(2): 103-108.

Karpov, L. M., A. Rozanov and T. O. Filippova (1986). "[Characteristic features of 35S-benzoylthiamine monophosphate in albino mice]." Vopr Med Khim **32**(4): 136-139.

Karpova, T., S. Danchuk, E. Kolobova and K. M. Popov (2003). "Characterization of the isozymes of pyruvate dehydrogenase phosphatase: implications for the regulation of pyruvate dehydrogenase activity." Biochim Biophys Acta **1652**(2): 126-135.

Kauffman, F. C. (1972). "The quantitative histochemistry of enzymes of the pentose phosphate pathway in the central nervous system of the rat." J Neurochem **19**(1): 1-9.

Kaufmann, P., K. Engelstad, Y. Wei, S. Jhung, M. C. Sano, D. C. Shungu, W. S. Millar, X. Hong, C. L. Gooch, X. Mao, J. M. Pascual, M. Hirano, P. W. Stacpoole, S. DiMauro and D. C. De Vivo (2006). "Dichloroacetate causes toxic neuropathy in MELAS: a randomized, controlled clinical trial." Neurology **66**(3): 324-330.

Kaul, L., M. Y. Heshmat, J. Kovi, M. A. Jackson, A. G. Jackson, G. W. Jones, M. Edson, J. P. Enterline, R. G. Worrell and S. L. Perry (1987). "The role of diet in prostate cancer." Nutr Cancer **9**(2-3): 123-128.

Kelley, K. M., B. G. Rowan and M. Ratnam (2003). "Modulation of the folate receptor alpha gene by the estrogen receptor: mechanism and implications in tumor targeting." Cancer Res **63**(11): 2820-2828.

Kelley, R. I., D. Robinson, E. G. Puffenberger, K. A. Strauss and D. H. Morton (2002). "Amish lethal microcephaly: a new metabolic disorder with severe congenital microcephaly and 2-ketoglutaric aciduria." Am J Med Genet **112**(4): 318-326.

Kim, J. W. and C. V. Dang (2006). "Cancer's molecular sweet tooth and the Warburg effect." Cancer Res **66**(18): 8927-8930.

Kim, J. W., P. Gao, Y. C. Liu, G. L. Semenza and C. V. Dang (2007). "Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1." Mol Cell Biol **27**(21): 7381-7393.

Kim, J. W., I. Tchernyshyov, G. L. Semenza and C. V. Dang (2006). "HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia." Cell Metab **3**(3): 177-185.

Kim, Y. I. (1999). "Folate and carcinogenesis: evidence, mechanisms, and implications." J Nutr Biochem **10**(2): 66-88.

Kim, Y. J., J. Y. Ahn, P. Liang, C. Ip, Y. Zhang and Y. M. Park (2007). "Human prx1 gene is a target of Nrf2 and is up-regulated by hypoxia/reoxygenation: implication to tumor biology." Cancer Res **67**(2): 546-554.

Kimura, M. and Y. Itokawa (1983). "Determination of thiamin and thiamin phosphate esters in blood by liquid chromatography with post-column derivatization." Clin Chem **29**(12): 2073-2075.

Kirkland, J. B. (2012). "Niacin requirements for genomic stability." Mutat Res **733**(1-2): 14-20.

Knoechel, T. R., A. D. Tucker, C. M. Robinson, C. Phillips, W. Taylor, P. J. Bungay, S. A. Kasten, T. E. Roche and D. G. Brown (2006). "Regulatory roles of the N-terminal domain based on crystal structures of human pyruvate dehydrogenase kinase 2 containing physiological and synthetic ligands." Biochemistry **45**(2): 402-415.

Koike, K., M. Hamada, N. Tanaka, K. I. Otsuka, K. Ogasahara and M. Koike (1974). "Properties and subunit composition of the pig heart 2-oxoglutarate dehydrogenase." J Biol Chem **249**(12): 3836-3842.

Koike, M. and K. Koike (1976). "Structure, assembly and function of mammalian alpha-keto acid dehydrogenase complexes." Adv Biophys: 187-227.

Kolobova, E., A. Tuganova, I. Boulatnikov and K. M. Popov (2001). "Regulation of pyruvate dehydrogenase activity through phosphorylation at multiple sites." Biochem J **358**(Pt 1): 69-77.

Kong, D., E. Heath, W. Chen, M. L. Cher, I. Powell, L. Heilbrun, Y. Li, S. Ali, S. Sethi, O. Hassan, C. Hwang, N. Gupta, D. Chitale, W. A. Sakr, M. Menon and F. H. Sarkar (2012). "Loss of let-7 up-regulates EZH2 in prostate cancer consistent with the acquisition of cancer stem cell signatures that are attenuated by BR-DIM." PLoS One **7**(3): e33729.

Konishi, N., Y. Hiasa, H. Matsuda, M. Tao, T. Tsuzuki, I. Hayashi, Y. Kitahori, T. Shiraishi, R. Yatani, J. Shimazaki and et al. (1995). "Intratumor cellular heterogeneity and alterations in ras oncogene and p53 tumor suppressor gene in human prostate carcinoma." Am J Pathol **147**(4): 1112-1122.

Koppenol, W. H., P. L. Bounds and C. V. Dang (2011). "Otto Warburg's contributions to current concepts of cancer metabolism." Nature Reviews Cancer **11**(5): 325-337.

Koppenol, W. H., P. L. Bounds and C. V. Dang (2011). "Otto Warburg's contributions to current concepts of cancer metabolism." Nat Rev Cancer **11**(5): 325-337.

Korotchkina, L. G. and M. S. Patel (1995). "Mutagenesis studies of the phosphorylation sites of recombinant human pyruvate dehydrogenase. Site-specific regulation." J Biol Chem **270**(24): 14297-14304.

Korotchkina, L. G. and M. S. Patel (2001). "Probing the mechanism of inactivation of human pyruvate dehydrogenase by phosphorylation of three sites." J Biol Chem **276**(8): 5731-5738.

Korotchkina, L. G. and M. S. Patel (2001). "Site specificity of four pyruvate dehydrogenase kinase isoenzymes toward the three phosphorylation sites of human pyruvate dehydrogenase." J Biol Chem **276**(40): 37223-37229.

Koukourakis, M. I., A. Giatromanolaki, A. L. Harris and E. Sivridis (2006). "Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma." Cancer Res **66**(2): 632-637.

Koukourakis, M. I., A. Giatromanolaki, C. Simopoulos, A. Polychronidis and E. Sivridis (2005). "Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer." Clin Exp Metastasis **22**(1): 25-30.

Koukourakis, M. I., A. Giatromanolaki, E. Sivridis, G. Bougioukas, V. Didilis, K. C. Gatter, A. L. Harris, Tumour and G. Angiogenesis Research (2003). "Lactate dehydrogenase-5 (LDH-5) overexpression in non-small-cell lung cancer tissues is linked to tumour hypoxia, angiogenic factor production and poor prognosis." Br J Cancer **89**(5): 877-885.

Koukourakis, M. I., A. Giatromanolaki, E. Sivridis, K. C. Gatter and A. L. Harris (2005). "Pyruvate dehydrogenase and pyruvate dehydrogenase kinase expression in non small cell lung cancer and tumor-associated stroma." Neoplasia (New York, NY) **7**(1): 1.

Koukourakis, M. I., A. Giatromanolaki, E. Sivridis, K. C. Gatter, A. L. Harris, Tumor and G. Angiogenesis Research (2005). "Pyruvate dehydrogenase and pyruvate dehydrogenase kinase expression in non small cell lung cancer and tumor-associated stroma." Neoplasia **7**(1): 1-6.

Koukourakis, M. I., A. Giatromanolaki, S. Winter, R. Leek, E. Sivridis and A. L. Harris (2009). "Lactate dehydrogenase 5 expression in squamous cell head and neck cancer relates to prognosis following radical or postoperative radiotherapy." Oncology **77**(5): 285-292.

Kovacevic, Z. and J. D. McGivan (1983). "Mitochondrial metabolism of glutamine and glutamate and its physiological significance." Physiol Rev **63**(2): 547-605.

Kreso, A., C. A. O'Brien, P. van Galen, O. I. Gan, F. Notta, A. M. Brown, K. Ng, J. Ma, E. Wienholds, C. Dunant, A. Pollett, S. Gallinger, J. McPherson, C. G. Mullighan, D. Shibata and J. E. Dick (2013). "Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer." Science **339**(6119): 543-548.

Krockenberger, M., J. B. Engel, M. Schmidt, N. Kohrenhagen, S. F. Hausler, Y. Dombrowski, M. Kapp, J. Dietl and A. Honig (2010). "Expression of transketolase-like 1 protein (TKTL1) in human endometrial cancer." Anticancer Res **30**(5): 1653-1659.

Krockenberger, M., A. Honig, L. Rieger, J. F. Coy, M. Sutterlin, M. Kapp, E. Horn, J. Dietl and U. Kammerer (2007). "Transketolase-like 1 expression correlates with subtypes of ovarian cancer and the presence of distant metastases." Int J Gynecol Cancer **17**(1): 101-106.

Kung, H. N., J. R. Marks and J. T. Chi (2011). "Glutamine synthetase is a genetic determinant of cell type-specific glutamine independence in breast epithelia." PLoS Genet **7**(8): e1002229.

Laforenza, U., C. Patrini, C. Alvisi, A. Faelli, A. Licandro and G. Rindi (1997). "Thiamine uptake in human intestinal biopsy specimens, including observations from a patient with acute thiamine deficiency." Am J Clin Nutr **66**(2): 320-326.

Langbein, S., M. Zerilli, A. Zur Hausen, W. Staiger, K. Rensch-Boschert, N. Lukan, J. Popa, M. Ternullo, A. Steidler and C. Weiss (2006). "Expression of transketolase TKTL1 predicts colon and urothelial cancer patient survival: Warburg effect reinterpreted." British journal of cancer **94**(4): 578-585.

Langbein, S., M. Zerilli, A. Zur Hausen, W. Staiger, K. Rensch-Boschert, N. Lukan, J. Popa, M. P. Ternullo, A. Steidler, C. Weiss, R. Grobholz, F. Willeke, P. Alken, G. Stassi, P. Schubert and J. F. Coy (2006). "Expression of transketolase TKTL1 predicts colon and urothelial cancer patient survival: Warburg effect reinterpreted." Br J Cancer **94**(4): 578-585.

Lawlis, V. B. and T. E. Roche (1981). "Inhibition of bovine kidney alpha-ketoglutarate dehydrogenase complex by reduced nicotinamide adenine dinucleotide in the presence or absence of calcium ion and effect of adenosine 5'-diphosphate on reduced nicotinamide adenine dinucleotide inhibition." Biochemistry **20**(9): 2519-2524.

Lee, B. Y., K. Yanamandra and J. A. Bocchini, Jr. (2005). "Thiamin deficiency: a possible major cause of some tumors? (review)." Oncol Rep **14**(6): 1589-1592.

Lemos, C., A. Faria, M. Meireles, F. Martel, R. Monteiro and C. Calhau (2012). "Thiamine is a substrate of organic cation transporters in Caco-2 cells." European Journal of Pharmacology **682**(1): 37-42.

Lemos, C., A. Faria, M. Meireles, F. Martel, R. Monteiro and C. Calhau (2012). "Thiamine is a substrate of organic cation transporters in Caco-2 cells." Eur J Pharmacol **682**(1-3): 37-42.

Leuchtenberger, R., C. Leuchtenberger, D. Laszlo and R. Lewisohn (1945). "The Influence of "Folic Acid" on Spontaneous Breast Cancers in Mice." Science **101**(2611): 46.

Li, C., Z. Xiao, Z. Chen, X. Zhang, J. Li, X. Wu, X. Li, H. Yi, M. Li, G. Zhu and S. Liang (2006). "Proteome analysis of human lung squamous carcinoma." Proteomics **6**(2): 547-558.

Li, F., Y. Wang, K. I. Zeller, J. J. Potter, D. R. Wonsey, K. A. O'Donnell, J. W. Kim, J. T. Yustein, L. A. Lee and C. V. Dang (2005). "Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis." Mol Cell Biol **25**(14): 6225-6234.

Li, G. M. (2008). "Mechanisms and functions of DNA mismatch repair." Cell Res **18**(1): 85-98.

Lin, H., F. Hsieh, H. Song and J. Lin (2005). "Elevated phosphorylation and activation of PDK-1/AKT pathway in human breast cancer." British journal of cancer **93**(12): 1372-1381.

Lindhurst, M. J., G. Fiermonte, S. Song, E. Struys, F. De Leonardis, P. L. Schwartzberg, A. Chen, A. Castegna, N. Verhoeven and C. K. Mathews (2006). "Knockout of Slc25a19 causes mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia." Proceedings of the National Academy of Sciences **103**(43): 15927-15932.

Lindhurst, M. J., G. Fiermonte, S. Song, E. Struys, F. De Leonardis, P. L. Schwartzberg, A. Chen, A. Castegna, N. Verhoeven, C. K. Mathews, F. Palmieri and L. G. Biesecker (2006). "Knockout of Slc25a19 causes mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia." Proc Natl Acad Sci U S A **103**(43): 15927-15932.

Liu, H., N. Savaraj, W. Priebe and T. J. Lampidis (2002). "Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: a strategy for solid tumor therapy (Model C)." Biochem Pharmacol **64**(12): 1745-1751.

Liu, J. Y., D. E. Timm and T. D. Hurley (2006). "Pyrithiamine as a substrate for thiamine pyrophosphokinase." J Biol Chem **281**(10): 6601-6607.

Liu, S., H. Huang, X. Lu, M. Golinski, S. Comesse, D. Watt, R. Grossman and J. Moscow (2003). "Down-regulation of thiamine transporter THTR2 gene expression in breast cancer and its association with resistance to apoptosis." Molecular Cancer Research **1**(9): 665.

Liu, S., H. Huang, X. Lu, M. Golinski, S. Comesse, D. Watt, R. B. Grossman and J. A. Moscow (2003). "Down-regulation of thiamine transporter THTR2 gene expression in breast cancer and its association with resistance to apoptosis." Mol Cancer Res **1**(9): 665-673.

Liu, S., N. R. Monks, J. W. Hanes, T. P. Begley, H. Yu and J. A. Moscow (2010). "Sensitivity of breast cancer cell lines to recombinant thiaminase I." Cancer Chemother Pharmacol **66**(1): 171-179.

Liu, X., E. K. Lam, X. Wang, J. Zhang, Y. Y. Cheng, Y. W. Lam, E. K. Ng, J. Yu, F. K. Chan, H. Jin and J. J. Sung (2009). "Promoter hypermethylation mediates downregulation of thiamine receptor SLC19A3 in gastric cancer." Tumour Biol **30**(5-6): 242-248.

Liu, X., E. K.-Y. Lam, X. Wang, J.-B. Zhang, Y.-Y. Cheng, Y. W. Lam, E. K.-O. Ng, J. Yu, F. K.-L. Chan, H.-C. Jin and J. J.-Y. Sung (2009). "Promoter Hypermethylation Mediates Downregulation of Thiamine Receptor SLC19A3 in Gastric Cancer." Tumor Biol **30**(5-6): 242-248.

Locasale, J. W., A. R. Grassian, T. Melman, C. A. Lyssiotis, K. R. Mattaini, A. J. Bass, G. Heffron, C. M. Metallo, T. Muranen, H. Sharfi, A. T. Sasaki, D. Anastasiou, E. Mullarky, N. I. Vokes, M. Sasaki, R. Beroukhim, G. Stephanopoulos, A. H. Ligon, M. Meyerson, A. L. Richardson, L. Chin, G. Wagner, J. M. Asara, J. S. Brugge, L. C. Cantley and M. G. Vander Heiden (2011). "Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis." Nat Genet **43**(9): 869-874.

Lu, C. W., S. C. Lin, K. F. Chen, Y. Y. Lai and S. J. Tsai (2008). "Induction of pyruvate dehydrogenase kinase-3 by hypoxia-inducible factor-1 promotes metabolic switch and drug resistance." J Biol Chem **283**(42): 28106-28114.

Lu, C. W., S. C. Lin, C. W. Chien, S. C. Lin, C. T. Lee, B. W. Lin, J. C. Lee and S. J. Tsai (2011). "Overexpression of pyruvate dehydrogenase kinase 3 increases drug resistance and early recurrence in colon cancer." Am J Pathol **179**(3): 1405-1414.

Lu, J. and E. L. Frank (2008). "Rapid HPLC measurement of thiamine and its phosphate esters in whole blood." Clinical chemistry **54**(5): 901-906.

Lucock, M. (2000). "Folic acid: nutritional biochemistry, molecular biology, and role in disease processes." Mol Genet Metab **71**(1-2): 121-138.

Lukienko, P., N. Mel'nichenko, I. Zverinskii and S. Zabrodskaya (2000). "Antioxidant properties of thiamine." Bulletin of experimental biology and medicine **130**(3): 874-876.

Lukienko, P. I., N. G. Mel'nichenko, I. V. Zverinskii and S. V. Zabrodskaya (2000). "Antioxidant properties of thiamine." Bull Exp Biol Med **130**(9): 874-876.

Luo, S., V. S. Kansara, X. Zhu, N. K. Mandava, D. Pal and A. K. Mitra (2006). "Functional characterization of sodium-dependent multivitamin transporter in MDCK-MDR1 cells and its utilization as a target for drug delivery." Mol Pharm **3**(3): 329-339.

Luo, X. M., S. H. Zhou and J. Fan (2010). "Glucose transporter-1 as a new therapeutic target in laryngeal carcinoma." J Int Med Res **38**(6): 1885-1892.

Macheda, M. L., S. Rogers and J. D. Best (2005). "Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer." J Cell Physiol **202**(3): 654-662.

Macintosh, C. A., M. Stower, N. Reid and N. J. Maitland (1998). "Precise microdissection of human prostate cancers reveals genotypic heterogeneity." Cancer Res **58**(1): 23-28.

Madhok, B. M., S. Yeluri, S. L. Perry, T. A. Hughes and D. G. Jayne (2010). "Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells." Br J Cancer **102**(12): 1746-1752.

Maher, J. C., A. Krishan and T. J. Lampidis (2004). "Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions." Cancer Chemother Pharmacol **53**(2): 116-122.

Maley, C. C., P. C. Galipeau, J. C. Finley, V. J. Wongsurawat, X. Li, C. A. Sanchez, T. G. Paulson, P. L. Blount, R. A. Risques, P. S. Rabinovitch and B. J. Reid (2006). "Genetic clonal diversity predicts progression to esophageal adenocarcinoma." Nat Genet **38**(4): 468-473.

Marchant, J. S., V. S. Subramanian, I. Parker and H. M. Said (2002). "Intracellular trafficking and membrane targeting mechanisms of the human reduced folate carrier in Mammalian epithelial cells." J Biol Chem **277**(36): 33325-33333.

Marek, M., K. Kaiser and H. J. Gruber (1997). "Biotin-pyrene conjugates with poly(ethylene glycol) spacers are convenient fluorescent probes for avidin and streptavidin." Bioconjug Chem **8**(4): 560-566.

Marin-Hernandez, A., S. Rodriguez-Enriquez, P. A. Vital-Gonzalez, F. L. Flores-Rodriguez, M. Macias-Silva, M. Sosa-Garrocho and R. Moreno-Sanchez (2006). "Determining and understanding the control of glycolysis in fast-growth tumor cells. Flux control by an over-expressed but strongly product-inhibited hexokinase." FEBS J **273**(9): 1975-1988.

Marin-Valencia, I., C. Yang, T. Mashimo, S. Cho, H. Baek, X. L. Yang, K. N. Rajagopalan, M. Maddie, V. Vemireddy, Z. Zhao, L. Cai, L. Good, B. P. Tu, K. J. Hatanpaa, B. E. Mickey, J. M. Mates, J. M. Pascual, E. A. Maher, C. R. Malloy, R. J. Deberardinis and R. M. Bachoo (2012). "Analysis of tumor metabolism reveals mitochondrial glucose oxidation in genetically diverse human glioblastomas in the mouse brain in vivo." Cell Metab **15**(6): 827-837.

Martin, P. R., C. K. Singleton and S. Hiller-Sturmhofel (2003). "The role of thiamine deficiency in alcoholic brain disease." Alcohol Research and Health **27**(2): 134-142.

Martin, P. R., C. K. Singleton and S. Hiller-Sturmhofel (2003). "The role of thiamine deficiency in alcoholic brain disease." Alcohol Res Health **27**(2): 134-142.

Marusyk, A. and K. Polyak (2010). "Tumor heterogeneity: causes and consequences." Biochim Biophys Acta **1805**(1): 105-117.

Maslova, A. O., L. E. Meshalkina and G. A. Kochetov (2012). "Computer modeling of transketolase-like protein, TKTL1, a marker of certain tumor tissues." Biochemistry (Mosc) **77**(3): 296-299.

Matherly, L. H. and D. I. Goldman (2003). "Membrane transport of folates." Vitam Horm **66**: 403-456.

Matherly, L. H., Z. Hou and Y. Deng (2007). "Human reduced folate carrier: translation of basic biology to cancer etiology and therapy." Cancer Metastasis Rev **26**(1): 111-128.

Mathew, R. and E. White (2011). "Autophagy, stress, and cancer metabolism: what doesn't kill you makes you stronger." Cold Spring Harb Symp Quant Biol **76**: 389-396.

Mathupala, S., Y. Ko and P. Pedersen (2006). "Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria." Oncogene **25**(34): 4777-4786.

Mathupala, S. P., A. Rempel and P. L. Pedersen (2001). "Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions." J Biol Chem **276**(46): 43407-43412.

Mayr, J. A., P. Freisinger, K. Schlachter, B. Rolinski, F. A. Zimmermann, T. Scheffner, T. B. Haack, J. Koch, U. Ahting and H. Prokisch (2011). "Thiamine pyrophosphokinase deficiency in

encephalopathic children with defects in the pyruvate oxidation pathway." The American Journal of Human Genetics **89**(6): 806-812.

Mayr, J. A., P. Freisinger, K. Schlachter, B. Rolinski, F. A. Zimmermann, T. Scheffner, T. B. Haack, J. Koch, U. Ahting, H. Prokisch and W. Sperl (2011). "Thiamine pyrophosphokinase deficiency in encephalopathic children with defects in the pyruvate oxidation pathway." Am J Hum Genet **89**(6): 806-812.

Mazure, N. M. and J. Pouyssegur (2010). "Hypoxia-induced autophagy: cell death or cell survival?" Curr Opin Cell Biol **22**(2): 177-180.

Mazurek, S. (2011). "Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells." Int J Biochem Cell Biol **43**(7): 969-980.

McCormack, J. G. and R. M. Denton (1979). "The effects of calcium ions and adenine nucleotides on the activity of pig heart 2-oxoglutarate dehydrogenase complex." Biochem J **180**(3): 533-544.

McFate, T., A. Mohyeldin, H. Lu, J. Thakar, J. Henriques, N. D. Halim, H. Wu, M. J. Schell, T. M. Tsang, O. Teahan, S. Zhou, J. A. Califano, N. H. Jeoung, R. A. Harris and A. Verma (2008). "Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells." J Biol Chem **283**(33): 22700-22708.

Meador, K., D. Loring, M. Nichols, E. Zamrini, M. Rivner, H. Posas, E. Thompson and E. Moore (1993). "Preliminary findings of high-dose thiamine in dementia of Alzheimer's type." J Geriatr Psychiatry Neurol **6**(4): 222-229.

Medina, R. A. and G. I. Owen (2002). "Glucose transporters: expression, regulation and cancer." Biol Res **35**(1): 9-26.

Menon, I. and J. Quastel (1966). "Transport and metabolism of thiamine in Ehrlich ascites-carcinoma cells." Biochem. J **99**: 766-775.

Meshalkina, L. E., V. L. Drutsa, O. N. Koroleva, O. N. Solovjeva and G. A. Kochetov (2013). "Is transketolase-like protein, TKTL1, transketolase?" Biochim Biophys Acta **1832**(3): 387-390.

Metallo, C. M., P. A. Gameiro, E. L. Bell, K. R. Mattaini, J. Yang, K. Hiller, C. M. Jewell, Z. R. Johnson, D. J. Irvine, L. Guarente, J. K. Kelleher, M. G. Vander Heiden, O. Iliopoulos and G. Stephanopoulos (2012). "Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia." Nature **481**(7381): 380-384.

Michael, G. and D. Schomburg (2013). Biochemical pathways: an atlas of biochemistry and molecular biology, John Wiley & Sons.

Michelakis, E. D., L. Webster and J. R. Mackey (2008). "Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer." Br J Cancer **99**(7): 989-994.

Minko, T., P. V. Paranjpe, B. Qiu, A. Lalloo, R. Won, S. Stein and P. J. Sinko (2002). "Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethylene glycol) conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells." Cancer Chemother Pharmacol **50**(2): 143-150.

Mishra, P. R. and N. K. Jain (2002). "Biotinylated methotrexate loaded erythrocytes for enhanced liver uptake. 'A study on the rat'." Int J Pharm **231**(2): 145-153.

Mitschke, L., C. Parthier, K. Schroder-Tittmann, J. Coy, S. Ludtke and K. Tittmann (2010). "The crystal structure of human transketolase and new insights into its mode of action." J Biol Chem **285**(41): 31559-31570.

Monahan, B. P. and C. J. Allegra (2001). Antifolates.

Moreno-Sanchez, R., S. Rodriguez-Enriquez, A. Marin-Hernandez and E. Saavedra (2007). "Energy metabolism in tumor cells." FEBS J **274**(6): 1393-1418.

Morris, H. P. and W. V. B. Robertson (1943). "Growth rate and number of spontaneous mammary carcinomas and riboflavin concentration of liver, muscle, and tumor of C3H mice as influenced by dietary riboflavin." Journal of the National Cancer Institutes **3**: 479-489.

Moscow, J. A., M. Gong, R. He, M. K. Sgagias, K. H. Dixon, S. L. Anzick, P. S. Meltzer and K. H. Cowan (1995). "Isolation of a gene encoding a human reduced folate carrier (RFC1) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells." Cancer Res **55**(17): 3790-3794.

Mullen, A. R., W. W. Wheaton, E. S. Jin, P. H. Chen, L. B. Sullivan, T. Cheng, Y. Yang, W. M. Linehan, N. S. Chandel and R. J. DeBerardinis (2012). "Reductive carboxylation supports growth in tumour cells with defective mitochondria." Nature **481**(7381): 385-388.

Na, K., T. Bum Lee, K. H. Park, E. K. Shin, Y. B. Lee and H. K. Choi (2003). "Self-assembled nanoparticles of hydrophobically-modified polysaccharide bearing vitamin H as a targeted anti-cancer drug delivery system." Eur J Pharm Sci **18**(2): 165-173.

Nabokina, S. M., K. Inoue, V. S. Subramanian, J. E. Valle, H. Yuasa and H. M. Said (2014). "Molecular identification and functional characterization of the human colonic thiamine pyrophosphate transporter." J Biol Chem **289**(7): 4405-4416.

Nabokina, S. M., S. R. Senthilkumar and H. M. Said (2011). "Tspan-1 interacts with the thiamine transporter-1 in human intestinal epithelial cells and modulates its stability." American Journal of Physiology-Gastrointestinal and Liver Physiology **301**(5): G808-G813.

Nabokina, S. M., V. S. Subramanian, J. E. Valle and H. M. Said (2013). "Adaptive regulation of human intestinal thiamine uptake by extracellular substrate level: a role for THTR-2 transcriptional regulation." Am J Physiol Gastrointest Liver Physiol **305**(8): G593-599.

Nabokina, S. M., J. E. Valle and H. M. Said (2013). "Characterization of the human mitochondrial thiamine pyrophosphate transporter SLC25A19 minimal promoter: a role for NF-Y in regulating basal transcription." Gene **528**(2): 248-255.

Negrini, S., V. G. Gorgoulis and T. D. Halazonetis (2010). "Genomic instability--an evolving hallmark of cancer." Nat Rev Mol Cell Biol **11**(3): 220-228.

Neufeld, E. J., J. C. Fleming, E. Tartaglini and M. P. Steinkamp (2001). "Thiamine-responsive megaloblastic anemia syndrome: a disorder of high-affinity thiamine transport." Blood Cells Mol Dis **27**(1): 135-138.

Nguyen, D. X. and J. Massague (2007). "Genetic determinants of cancer metastasis." Nat Rev Genet **8**(5): 341-352.

Niewisch, M. R., Z. Kuci, H. Wolburg, M. Sautter, L. Krampen, B. Deubzer, R. Handgretinger and G. Bruchelt (2012). "Influence of dichloroacetate (DCA) on lactate production and oxygen consumption in neuroblastoma cells: is DCA a suitable drug for neuroblastoma therapy?" Cell Physiol Biochem **29**(3-4): 373-380.

Nishizuka, Y. and O. Hayaishi (1963). "Studies on the Biosynthesis of Nicotinamide Adenine Dinucleotide. I. Enzymic Synthesis of Niacin Ribonucleotides from 3-Hydroxyanthranilic Acid in Mammalian Tissues." J Biol Chem **238**: 3369-3377.

Nosaka, K., M. Onozuka, N. Kakazu, S. Hibi, H. Nishimura, H. Nishino and T. Abe (2001). "Isolation and characterization of a human thiamine pyrophosphokinase cDNA." Biochim Biophys Acta **1517**(2): 293-297.

Novello, F. and P. McLean (1968). "The pentose phosphate pathway of glucose metabolism. Measurement of the non-oxidative reactions of the cycle." Biochem J **107**(6): 775-791.

Oommen, A. M., J. B. Griffin, G. Sarath and J. Zempleni (2005). "Roles for nutrients in epigenetic events." J Nutr Biochem **16**(2): 74-77.

Ostrovskii Iu, M., R. V. Trebukhina and G. N. Mikhal'tsevich (1979). "[Tumor and body interrelationships in 14C-thiamine utilization]." Biull Eksp Biol Med **88**(12): 708-710.

Owen, O. E., S. C. Kalhan and R. W. Hanson (2002). "The key role of anaplerosis and cataplerosis for citric acid cycle function." J Biol Chem **277**(34): 30409-30412.

Panberthy, W. T. and J. B. Kirkland (2012). Niacin, International Life Sciences Institute.

Papandreou, I., R. A. Cairns, L. Fontana, A. L. Lim and N. C. Denko (2006). "HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption." Cell Metab **3**(3): 187-197.

Papandreou, I., T. Goliasova and N. C. Denko (2011). "Anticancer drugs that target metabolism: Is dichloroacetate the new paradigm?" Int J Cancer **128**(5): 1001-1008.

Parker, N., M. J. Turk, E. Westrick, J. D. Lewis, P. S. Low and C. P. Leamon (2005). "Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay." Anal Biochem **338**(2): 284-293.

Patel, M., R. K. Vadlapatla, S. Shah and A. K. Mitra (2012). "Molecular expression and functional activity of sodium dependent multivitamin transporter in human prostate cancer cells." Int J Pharm **436**(1-2): 324-331.

Patel, M. S. and T. E. Roche (1990). "Molecular biology and biochemistry of pyruvate dehydrogenase complexes." FASEB J **4**(14): 3224-3233.

Patra, K. C., Q. Wang, P. T. Bhaskar, L. Miller, Z. Wang, W. Wheaton, N. Chandel, M. Laakso, W. J. Muller, E. L. Allen, A. K. Jha, G. A. Smolen, M. F. Clasquin, R. B. Robey and N. Hay (2013). "Hexokinase 2 is required for tumor initiation and maintenance and its systemic deletion is therapeutic in mouse models of cancer." Cancer Cell **24**(2): 213-228.

Pelicano, H., D. S. Martin, R. H. Xu and P. Huang (2006). "Glycolysis inhibition for anticancer treatment." Oncogene **25**(34): 4633-4646.

Pelicano, H., R. H. Xu, M. Du, L. Feng, R. Sasaki, J. S. Carew, Y. Hu, L. Ramdas, L. Hu, M. J. Keating, W. Zhang, W. Plunkett and P. Huang (2006). "Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism." J Cell Biol **175**(6): 913-923.

Perham, R. N. (1991). "Domains, motifs, and linkers in 2-oxo acid dehydrogenase multienzyme complexes: a paradigm in the design of a multifunctional protein." Biochemistry **30**(35): 8501-8512.

Petros, J. A., A. K. Baumann, E. Ruiz-Pesini, M. B. Amin, C. Q. Sun, J. Hall, S. Lim, M. M. Issa, W. D. Flanders, S. H. Hosseini, F. F. Marshall and D. C. Wallace (2005). "mtDNA mutations increase tumorigenicity in prostate cancer." Proc Natl Acad Sci U S A **102**(3): 719-724.

Pfeiffer, T., S. Schuster and S. Bonhoeffer (2001). "Cooperation and competition in the evolution of ATP-producing pathways." Science **292**(5516): 504-507.

Pierce, B. (2005). Genetics: A conceptual approach, W. H. Freeman and Company.

Pillai, M. R., P. Chacko, L. A. Kesari, P. G. Jayaprakash, H. N. Jayaram and A. C. Antony (2003). "Expression of folate receptors and heterogeneous nuclear ribonucleoprotein E1 in women with human papillomavirus mediated transformation of cervical tissue to cancer." J Clin Pathol **56**(8): 569-574.

Pinard, M. F., J. Jolivet, M. Ratnam, I. Kathmann, C. Molthoff, R. Westerhof, J. H. Schornagel and G. Jansen (1996). "Functional aspects of membrane folate receptors in human breast cancer cells with transport-related resistance to methotrexate." Cancer Chemother Pharmacol **38**(3): 281-288.

Pitkin, R., L. Allen, L. Bailey and M. Bernfield (2000). Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Washington, D.C.

Possemato, R., K. M. Marks, Y. D. Shaul, M. E. Pacold, D. Kim, K. Birsoy, S. Sethumadhavan, H. K. Woo, H. G. Jang, A. K. Jha, W. W. Chen, F. G. Barrett, N. Stransky, Z. Y. Tsun, G. S. Cowley, J. Barretina, N. Y. Kalaany, P. P. Hsu, K. Ottina, A. M. Chan, B. Yuan, L. A. Garraway, D. E. Root, M. Mino-Kenudson, E. F. Brachtel, E. M. Driggers and D. M. Sabatini (2011). "Functional genomics reveal that the serine synthesis pathway is essential in breast cancer." Nature **476**(7360): 346-350.

Postovit, L. M., D. E. Abbott, S. L. Payne, W. W. Wheaton, N. V. Margaryan, R. Sullivan, M. K. Jansen, K. Csiszar, M. J. Hendrix and D. A. Kirschmann (2008). "Hypoxia/reoxygenation: a dynamic regulator of lysyl oxidase-facilitated breast cancer migration." J Cell Biochem **103**(5): 1369-1378.

Powers, H. J., M. H. Hill, M. Welfare, A. Spiers, W. Bal, J. Russell, Y. Duckworth, E. Gibney, E. A. Williams and J. C. Mathers (2007). "Responses of biomarkers of folate and riboflavin status to folate and riboflavin supplementation in healthy and colorectal polyp patients (the FAB2 Study)." Cancer Epidemiol Biomarkers Prev **16**(10): 2128-2135.

Prasad, P. D., S. Ramamoorthy, F. H. Leibach and V. Ganapathy (1995). "Molecular cloning of the human placental folate transporter." Biochem Biophys Res Commun **206**(2): 681-687.

Qin, X., Y. Cui, L. Shen, N. Sun, Y. Zhang, J. Li, X. Xu, B. Wang, X. Xu, Y. Huo and X. Wang (2013). "Folic acid supplementation and cancer risk: a meta-analysis of randomized controlled trials." Int J Cancer **133**(5): 1033-1041.

Queiros, O., A. Preto, A. Pacheco, C. Pinheiro, J. Azevedo-Silva, R. Moreira, M. Pedro, Y. H. Ko, P. L. Pedersen, F. Baltazar and M. Casal (2012). "Butyrate activates the monocarboxylate transporter MCT4 expression in breast cancer cells and enhances the antitumor activity of 3-bromopyruvate." J Bioenerg Biomembr **44**(1): 141-153.

Rais, B., B. Comin, J. Puigjaner, J. L. Brandes, E. Creppy, D. Saboureau, R. Ennamany, W. N. Lee, L. G. Boros and M. Cascante (1999). "Oxythiamine and dehydroepiandrosterone induce a G1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle." FEBS Lett **456**(1): 113-118.

Rajgopal, A., A. Edmondnson, I. D. Goldman and R. Zhao (2001). "SLC19A3 encodes a second thiamine transporter ThTr2." Biochim Biophys Acta **1537**(3): 175-178.

Rajgopal, A., E. E. Sierra, R. Zhao and I. D. Goldman (2001). "Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities." Am J Physiol Cell Physiol **281**(5): C1579-1586.

Ralph, S. J., S. Rodriguez-Enriquez, J. Neuzil, E. Saavedra and R. Moreno-Sanchez (2010). "The causes of cancer revisited: "mitochondrial malignancy" and ROS-induced oncogenic

transformation - why mitochondria are targets for cancer therapy." Mol Aspects Med **31**(2): 145-170.

Ramanathan, A., C. Wang and S. L. Schreiber (2005). "Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements." Proc Natl Acad Sci U S A **102**(17): 5992-5997.

Ramjaun, A. R. and J. Downward (2007). "Ras and phosphoinositide 3-kinase: partners in development and tumorigenesis." Cell Cycle **6**(23): 2902-2905.

Ramos-Montoya, A., W. N. Lee, S. Bassilian, S. Lim, R. V. Trebukhina, M. V. Kazhyna, C. J. Ciudad, V. Noe, J. J. Centelles and M. Cascante (2006). "Pentose phosphate cycle oxidative and nonoxidative balance: A new vulnerable target for overcoming drug resistance in cancer." Int J Cancer **119**(12): 2733-2741.

Randle, P. J. (1986). "Fuel selection in animals." Biochem Soc Trans **14**(5): 799-806.

Rardin, M. J., S. E. Wiley, R. K. Naviaux, A. N. Murphy and J. E. Dixon (2009). "Monitoring phosphorylation of the pyruvate dehydrogenase complex." Anal Biochem **389**(2): 157-164.

Rathmell, J. C., C. J. Fox, D. R. Plas, P. S. Hammerman, R. M. Cinalli and C. B. Thompson (2003). "Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival." Mol Cell Biol **23**(20): 7315-7328.

Raz, T., V. Labay, D. Baron, R. Szargel, Y. Anbinder, T. Barrett, W. Rabl, M. B. Viana, H. Mandel, A. Baruchel, J. M. Cayuela and N. Cohen (2000). "The spectrum of mutations, including four novel ones, in the thiamine-responsive megaloblastic anemia gene SLC19A2 of eight families." Hum Mutat **16**(1): 37-42.

Reidling, J. C. and H. M. Said (2005). "Adaptive regulation of intestinal thiamin uptake: molecular mechanism using wild-type and transgenic mice carrying hTHTR-1 and-2 promoters." American Journal of Physiology-Gastrointestinal and Liver Physiology **288**(6): G1127-G1134.

Reinicke, K., P. Sotomayor, P. Cisterna, C. Delgado, F. Nualart and A. Godoy (2012). "Cellular distribution of Glut-1 and Glut-5 in benign and malignant human prostate tissue." J Cell Biochem **113**(2): 553-562.

Richardson, A. D., C. Yang, A. Osterman and J. W. Smith (2008). "Central carbon metabolism in the progression of mammary carcinoma." Breast cancer research and treatment **110**(2): 297-307.

Richardson, M. M., L. K. Jennings and X. A. Zhang (2011). "Tetraspanins and tumor progression." Clinical & experimental metastasis **28**(3): 261-270.

Rindi, G. and U. Laforenza (2000). "Thiamine intestinal transport and related issues: recent aspects." Proc Soc Exp Biol Med **224**(4): 246-255.

Rindi, G. and U. Laforenza (2000). "Thiamine intestinal transport and related issues: recent aspects." Proceedings of the Society for Experimental Biology and Medicine **224**(4): 246-255.

Robey, R. B. and N. Hay (2006). "Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt." Oncogene **25**(34): 4683-4696.

Roche, T. E. and Y. Hiromasa (2007). "Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer." Cell Mol Life Sci **64**(7-8): 830-849.

Rodriguez-Enriquez, S., A. Marin-Hernandez, J. C. Gallardo-Perez and R. Moreno-Sanchez (2009). "Kinetics of transport and phosphorylation of glucose in cancer cells." J Cell Physiol **221**(3): 552-559.

Ross, J. S., S. M. Ali, K. Wang, G. Palmer, R. Yelensky, D. Lipson, V. A. Miller, D. Zajchowski, L. K. Shawver and P. J. Stephens (2013). "Comprehensive genomic profiling of epithelial ovarian cancer by next generation sequencing-based diagnostic assay reveals new routes to targeted therapies." Gynecol Oncol **130**(3): 554-559.

Rupec, R. A. and P. A. Baeuerle (1995). "The genomic response of tumor cells to hypoxia and reoxygenation. Differential activation of transcription factors AP-1 and NF-kappa B." Eur J Biochem **234**(2): 632-640.

Russell-Jones, G., K. McTavish, J. McEwan, J. Rice and D. Nowotnik (2004). "Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumours." J Inorg Biochem **98**(10): 1625-1633.

Said, H. M. (1999). "Cellular uptake of biotin: mechanisms and regulation." J Nutr **129**(2S Suppl): 490S-493S.

Said, H. M., K. Balamurugan, V. S. Subramanian and J. S. Marchant (2004). "Expression and functional contribution of hTHTR-2 in thiamin absorption in human intestine." Am J Physiol Gastrointest Liver Physiol **286**(3): G491-498.

Said, H. M., K. Balamurugan, V. S. Subramanian and J. S. Marchant (2004). "Expression and functional contribution of hTHTR-2 in thiamin absorption in human intestine." American Journal of Physiology-Gastrointestinal and Liver Physiology **286**(3): G491-G498.

Said, H. M., N. Chatterjee, R. U. Haq, V. S. Subramanian, A. Ortiz, L. H. Matherly, F. M. Sirotnak, C. Halsted and S. A. Rubin (2000). "Adaptive regulation of intestinal folate uptake: effect of dietary folate deficiency." Am J Physiol Cell Physiol **279**(6): C1889-1895.

Said, H. M., A. Ortiz, C. K. Kumar, N. Chatterjee, P. K. Dudeja and S. Rubin (1999). "Transport of thiamine in human intestine: mechanism and regulation in intestinal epithelial cell model Caco-2." Am J Physiol **277**(4 Pt 1): C645-651.

Said, H. M., A. Ortiz, C. K. Kumar, N. Chatterjee, P. K. Dudeja and S. Rubin (1999). "Transport of thiamine in human intestine: mechanism and regulation in intestinal epithelial cell model Caco-2." American Journal of Physiology-Cell Physiology **277**(4): C645.

Sakashita, M., N. Aoyama, R. Minami, S. Maekawa, K. Kuroda, D. Shirasaka, T. Ichihara, Y. Kuroda, S. Maeda and M. Kasuga (2001). "Glut1 expression in T1 and T2 stage colorectal carcinomas: its relationship to clinicopathological features." Eur J Cancer **37**(2): 204-209.

Sale, G. J. and P. J. Randle (1981). "Analysis of site occupancies in [32P]phosphorylated pyruvate dehydrogenase complexes by aspartyl-prolyl cleavage of tryptic phosphopeptides." Eur J Biochem **120**(3): 535-540.

Samowitz, W. S. and M. L. Slattery (1999). "Regional reproducibility of microsatellite instability in sporadic colorectal cancer." Genes Chromosomes Cancer **26**(2): 106-114.

Sanioto, S. M., H. Reinauer and S. Hollmann (1977). "Thiamine pyrophosphokinase activity in liver, heart and brain crude extracts of control and thiamine deficient rats." Int J Vitam Nutr Res **47**(4): 315-324.

Santidrian, A. F., A. Matsuno-Yagi, M. Ritland, B. B. Seo, S. E. LeBoeuf, L. J. Gay, T. Yagi and B. Felding-Habermann (2013). "Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression." J Clin Invest **123**(3): 1068-1081.

Sauter, G., H. Moch, T. C. Gasser, M. J. Mihatsch and F. M. Waldman (1995). "Heterogeneity of chromosome 17 and erbB-2 gene copy number in primary and metastatic bladder cancer." Cytometry **21**(1): 40-46.

Scatena, R. (2012). "Mitochondria and cancer: a growing role in apoptosis, cancer cell metabolism and dedifferentiation." Adv Exp Med Biol **942**: 287-308.

Schänzer, A., B. Döring, M. Ondrouschek, S. Goos, B. K. Garvalov, J. Geyer, T. Acker, B. Neubauer and A. Hahn (2014). "Stress- Induced Upregulation of SLC19A3 is Impaired in Biotin- Thiamine- Responsive Basal Ganglia Disease." Brain Pathology **24**(3): 270-279.

Scheerger, S. B. and J. Zempleni (2003). "Expression of oncogenes depends on biotin in human small cell lung cancer cells NCI-H69." Int J Vitam Nutr Res **73**(6): 461-467.

Schmid, U., H. Stopper, A. Heidland and N. Schupp (2008). "Benfotiamine exhibits direct antioxidative capacity and prevents induction of DNA damage in vitro." Diabetes Metab Res Rev **24**(5): 371-377.

Schmid, U., H. Stopper, A. Heidland and N. Schupp (2008). "Benfotiamine exhibits direct antioxidative capacity and prevents induction of DNA damage in vitro." Diabetes/metabolism research and reviews **24**(5): 371-377.

Schmidt, M., H. Voelker, M. Kapp, M. Krockenberger, J. Dietl and U. Kammerer (2010). "Glycolytic phenotype in breast cancer: activation of Akt, up-regulation of GLUT1, TKTL1 and down-regulation of M2PK." Journal of Cancer Research and Clinical Oncology **136**(2): 219-225.

Schmidt, M., H. U. Voelker, M. Kapp, M. Krockenberger, J. Dietl and U. Kammerer (2010). "Glycolytic phenotype in breast cancer: activation of Akt, up-regulation of GLUT1, TKTL1 and down-regulation of M2PK." J Cancer Res Clin Oncol **136**(2): 219-225.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative CT method." Nature protocols **3**(6): 1101-1108.

Schneider, S., S. Ludtke, K. Schroder-Tittmann, C. Wechsler, D. Meyer and K. Tittmann (2012). "A delta38 deletion variant of human transketolase as a model of transketolase-like protein 1 exhibits no enzymatic activity." PLoS One **7**(10): e48321.

Schoental, R. (1977). "The role of nicotinamide and of certain other modifying factors in diethylnitrosamine carcinogenesis: fusaria mycotoxins and "spontaneous" tumors in animals and man." Cancer **40**(4 Suppl): 1833-1840.

Schultz, H., D. Kahler, D. Branscheid, E. Vollmer, P. Zabel and T. Goldmann (2008). "TKTL1 is overexpressed in a large portion of non-small cell lung cancer specimens." Diagn Pathol **3**: 35.

Schultz, H., D. Kähler, D. Branscheid, E. Vollmer, P. Zabel and T. Goldmann (2008). "TKTL1 is overexpressed in a large portion of non-small cell lung cancer specimens." Diagn Pathol **3**(35): 10.

Schwaab, J., K. Horisberger, P. Strobel, B. Bohn, D. Gencer, G. Kahler, P. Kienle, S. Post, F. Wenz, W. K. Hofmann, R. D. Hofheinz and P. Erben (2011). "Expression of Transketolase like gene 1 (TKTL1) predicts disease-free survival in patients with locally advanced rectal cancer receiving neoadjuvant chemoradiotherapy." BMC Cancer **11**: 363.

Scott, J. M. (1999). "Folate and vitamin B12." Proc Nutr Soc **58**(2): 441-448.

Seligmann, H., R. Levi, A. M. Konijn and M. Prokocimer (2001). "Thiamine deficiency in patients with B-chronic lymphocytic leukaemia: a pilot study." Postgrad Med J **77**(911): 582-585.

Semenza, G. L. (2012). "Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy." Trends Pharmacol Sci **33**(4): 207-214.

Semenza, G. L., P. H. Roth, H. M. Fang and G. L. Wang (1994). "Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1." J Biol Chem **269**(38): 23757-23763.

Serkova, N. and L. G. Boros (2005). "Detection of resistance to imatinib by metabolic profiling: clinical and drug development implications." Am J Pharmacogenomics **5**(5): 293-302.

Shime, H., M. Yabu, T. Akazawa, K. Kodama, M. Matsumoto, T. Seya and N. Inoue (2008). "Tumor-secreted lactic acid promotes IL-23/IL-17 proinflammatory pathway." J Immunol **180**(11): 7175-7183.

Shipitsin, M., L. L. Campbell, P. Argani, S. Weremowicz, N. Bloushtain-Qimron, J. Yao, T. Nikolskaya, T. Serebryiskaya, R. Beroukhim, M. Hu, M. K. Halushka, S. Sukumar, L. M. Parker, K. S. Anderson, L. N. Harris, J. E. Garber, A. L. Richardson, S. J. Schnitt, Y. Nikolsky, R. S. Gelman and K. Polyak (2007). "Molecular definition of breast tumor heterogeneity." Cancer Cell **11**(3): 259-273.

Shujuan, Y., Z. Jianxing and C. Xin-Yue (2013). "Methylenetetrahydrofolate reductase genetic polymorphisms and esophageal squamous cell carcinoma susceptibility: A meta-analysis of case-control studies." Pak J Med Sci **29**(2): 693-698.

Simon, H.-U., A. Haj-Yehia and F. Levi-Schaffer (2000). "Role of reactive oxygen species (ROS) in apoptosis induction." Apoptosis **5**(5): 415-418.

Singh, I. N., P. G. Sullivan, Y. Deng, L. H. Mbye and E. D. Hall (2006). "Time course of post-traumatic mitochondrial oxidative damage and dysfunction in a mouse model of focal traumatic brain injury: implications for neuroprotective therapy." J Cereb Blood Flow Metab **26**(11): 1407-1418.

Singleton, C. K. and P. R. Martin (2001). "Molecular mechanisms of thiamine utilization." Curr Mol Med **1**(2): 197-207.

Smith, C. M., J. Bryla and J. R. Williamson (1974). "Regulation of mitochondrial alpha-ketoglutarate metabolism by product inhibition at alpha-ketoglutarate dehydrogenase." J Biol Chem **249**(5): 1497-1505.

Smith, T. A. (1999). "Facilitative glucose transporter expression in human cancer tissue." Br J Biomed Sci **56**(4): 285-292.

Smithline, H. A., M. Donnino and D. J. Greenblatt (2012). "Pharmacokinetics of high-dose oral thiamine hydrochloride in healthy subjects." BMC Clin Pharmacol **12**: 4.

Soga, T., M. Kamohara, J. Takasaki, S. Matsumoto, T. Saito, T. Ohishi, H. Hiyama, A. Matsuo, H. Matsushime and K. Furuichi (2003). "Molecular identification of nicotinic acid receptor." Biochem Biophys Res Commun **303**(1): 364-369.

Spronck, J. C., J. L. Nickerson and J. B. Kirkland (2007). "Niacin deficiency alters p53 expression and impairs etoposide-induced cell cycle arrest and apoptosis in rat bone marrow cells." Nutr Cancer **57**(1): 88-99.

Staiger, W. I., J. F. Coy, R. Grobholz, R. D. Hofheinz, N. Lukan, S. Post, M. H. Schwarzbach and F. Willeke (2006). "Expression of the mutated transketolase TKTL1, a molecular marker in gastric cancer." Oncol Rep **16**(4): 657-661.

Stasinopoulos, I., N. Mori and Z. M. Bhujwalla (2008). "The malignant phenotype of breast cancer cells is reduced by COX-2 silencing." Neoplasia **10**(11): 1163-1169.

Stockwin, L. H., S. X. Yu, S. Borgel, C. Hancock, T. L. Wolfe, L. R. Phillips, M. G. Hollingshead and D. L. Newton (2010). "Sodium dichloroacetate selectively targets cells with defects in the mitochondrial ETC." Int J Cancer **127**(11): 2510-2519.

Subramanian, V. S., J. S. Marchant, I. Parker and H. M. Said (2003). "Cell Biology of the Human Thiamine Transporter-1 (hTHTR1) Intracellular Trafficking and Membrane targeting mechanisms." Journal of Biological Chemistry **278**(6): 3976-3984.

Subramanian, V. S., J. S. Marchant, I. Parker and H. M. Said (2003). "Cell biology of the human thiamine transporter-1 (hTHTR1). Intracellular trafficking and membrane targeting mechanisms." J Biol Chem **278**(6): 3976-3984.

Subramanian, V. S., J. S. Marchant and H. M. Said (2006). "Targeting and trafficking of the human thiamine transporter-2 in epithelial cells." Journal of Biological Chemistry **281**(8): 5233-5245.

Sun, R. C., M. Fadia, J. E. Dahlstrom, C. R. Parish, P. G. Board and A. C. Blackburn (2010). "Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth in vitro and in vivo." Breast Cancer Res Treat **120**(1): 253-260.

Sun, W., Y. Liu, C. A. Glazer, C. Shao, S. Bhan, S. Demokan, M. Zhao, M. A. Rudek, P. K. Ha and J. A. Califano (2010). "TKTL1 is activated by promoter hypomethylation and contributes to head and neck squamous cell carcinoma carcinogenesis through increased aerobic glycolysis and HIF1alpha stabilization." Clin Cancer Res **16**(3): 857-866.

Suva, M. L., N. Riggi, M. Janiszewska, I. Radovanovic, P. Provero, J. C. Stehle, K. Baumer, M. A. Le Bitoux, D. Marino, L. Cironi, V. E. Marquez, V. Clement and I. Stamenkovic (2009). "EZH2 is essential for glioblastoma cancer stem cell maintenance." Cancer Res **69**(24): 9211-9218.

Sweet, R., A. Paul and J. Zastre (2010). "Hypoxia induced upregulation and function of the thiamine transporter, SLC19A3 in a breast cancer cell line." Cancer Biology & Therapy **10**(11): 1101-1111.

Sweet, R., A. Paul and J. Zastre (2010). "Hypoxia induced upregulation and function of the thiamine transporter, SLC19A3 in a breast cancer cell line." Cancer Biol Ther **10**(11): 1101-1111.

Tabarki, B., S. Al-Shafi, S. Al-Shahwan, Z. Azmat, A. Al-Hashem, N. Al-Adwani, N. Biary, M. Al-Zawahmah, S. Khan and G. Zuccoli (2013). "Biotin-responsive basal ganglia disease revisited: clinical, radiologic, and genetic findings." Neurology **80**(3): 261-267.

Tallaksen, C., T. Bøhmer, J. Karlsen and H. Bell (1997). "Determination of thiamin and its phosphate esters in human blood, plasma, and urine." Methods in enzymology **279**: 67.

Taylor, R. W. and D. M. Turnbull (2005). "Mitochondrial DNA mutations in human disease." Nat Rev Genet **6**(5): 389-402.

Teague, W. M., F. H. Pettit, T. L. Wu, S. R. Silberman and L. J. Reed (1982). "Purification and properties of pyruvate dehydrogenase phosphatase from bovine heart and kidney." Biochemistry **21**(22): 5585-5592.

Thomson, A. D. (2000). "Mechanisms of vitamin deficiency in chronic alcohol misusers and the development of the Wernicke-Korsakoff syndrome." Alcohol Alcohol Suppl **35**(1): 2-7.

Thomson, A. D., H. Baker and C. M. Leevy (1970). "Patterns of 35S-thiamine hydrochloride absorption in the malnourished alcoholic patient." J Lab Clin Med **76**(1): 34-45.

Thomson, A. D. and C. M. Leevy (1972). "Observations on the mechanism of thiamine hydrochloride absorption in man." Clin Sci **43**(2): 153-163.

Tiev, K. P., J. Cabane and J. C. Imbert (1999). "[Treatment of chronic postinfectious fatigue: randomized double-blind study of two doses of sulbutiamine (400-600 mg/day) versus placebo]." Rev Med Interne **20**(10): 912-918.

Tolar, J. and J. P. Neglia (2003). "Transplacental and other routes of cancer transmission between individuals." J Pediatr Hematol Oncol **25**(6): 430-434.

Trachootham, D., J. Alexandre and P. Huang (2009). "Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?" Nat Rev Drug Discov **8**(7): 579-591.

Trachootham, D., J. Alexandre and P. Huang (2009). "Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?" Nature reviews Drug discovery **8**(7): 579-591.

Trebukhina, R. V., Y. M. Ostrovsky, V. S. Shapot, G. N. Mikhaltsevich and V. N. Tumanov (1984). "Turnover of [14C]thiamin and activities of thiamin pyrophosphate-dependent enzymes in tissues of mice with Ehrlich ascites carcinoma." Nutr Cancer **6**(4): 260-273.

Tsao, S. M., M. C. Yin and W. H. Liu (2007). "Oxidant stress and B vitamins status in patients with non-small cell lung cancer." Nutr Cancer **59**(1): 8-13.

Turan, M. I., I. Siltelioglu Turan, R. Mammadov, K. Altinkaynak and A. Kisaoglu (2013). "The effect of thiamine and thiamine pyrophosphate on oxidative liver damage induced in rats with cisplatin." Biomed Res Int **2013**: 783809.

Ullah, M. S., A. J. Davies and A. P. Halestrap (2006). "The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism." J Biol Chem **281**(14): 9030-9037.

Vadlapudi, A. D., R. K. Vadlapatla and A. K. Mitra (2012). "Sodium dependent multivitamin transporter (SMVT): a potential target for drug delivery." Curr Drug Targets **13**(7): 994-1003.

Vadlapudi, A. D., R. K. Vadlapatla, D. Pal and A. K. Mitra (2013). "Biotin uptake by T47D breast cancer cells: functional and molecular evidence of sodium-dependent multivitamin transporter (SMVT)." Int J Pharm **441**(1-2): 535-543.

van den Brenk, H. A., V. Moore, C. Sharpington and C. Orton (1972). "Production of metastases by a primary tumour irradiated under aerobic and anaerobic conditions in vivo." Br J Cancer **26**(5): 402-412.

Van Reeth, O. (1999). "Pharmacologic and therapeutic features of sulbutiamine." Drugs Today (Barc) **35**(3): 187-192.

van Sluis, R., Z. M. Bhujwalla, N. Raghunand, P. Ballesteros, J. Alvarez, S. Cerdan, J. P. Galons and R. J. Gillies (1999). "In vivo imaging of extracellular pH using 1H MRSI." Magn Reson Med **41**(4): 743-750.

van Zaanen, H. C. and J. van der Lelie (1992). "Thiamine deficiency in hematologic malignant tumors." Cancer **69**(7): 1710-1713.

Vander Heiden, M. G., L. C. Cantley and C. B. Thompson (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science **324**(5930): 1029-1033.

Vaupel, P., F. Kallinowski and P. Okunieff (1989). "Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review." Cancer Res **49**(23): 6449-6465.

Volker, H. U., M. Scheich, B. Schmausser, U. Kammerer and M. Eck (2007). "Overexpression of transketolase TKTL1 is associated with shorter survival in laryngeal squamous cell carcinomas." Eur Arch Otorhinolaryngol **264**(12): 1431-1436.

Volvert, M. L., S. Seyen, M. Piette, B. Evrard, M. Gangolf, J. C. Plumier and L. Bettendorff (2008). "Benfotiamine, a synthetic S-acyl thiamine derivative, has different mechanisms of action and a different pharmacological profile than lipid-soluble thiamine disulfide derivatives." BMC Pharmacol **8**: 10.

Vos, T. A., J. E. Ros, R. Havinga, H. Moshage, F. Kuipers, P. L. Jansen and M. Muller (1999). "Regulation of hepatic transport systems involved in bile secretion during liver regeneration in rats." Hepatology **29**(6): 1833-1839.

Wakil, S. J. and D. M. Gibson (1960). "Studies on the mechanism of fatty acid synthesis VIII. The participation of protein-bound biotin in the biosynthesis of fatty acids." Biochimica et Biophysica Acta **41**(1): 122-129.

Wallace, D. C. (2012). "Mitochondria and cancer." Nat Rev Cancer **12**(10): 685-698.

Wanders, D. and R. L. Judd (2011). "Future of GPR109A agonists in the treatment of dyslipidaemia." Diabetes Obes Metab **13**(8): 685-691.

Wang, J., X. Zhang, D. Ma, W. N. Lee, J. Xiao, Y. Zhao, V. L. Go, Q. Wang, Y. Yen, R. Recker and G. G. Xiao (2013). "Inhibition of transketolase by oxythiamine altered dynamics of protein signals in pancreatic cancer cells." Exp Hematol Oncol **2**: 18.

Wanka, C., J. P. Steinbach and J. Rieger (2012). "Tp53-induced glycolysis and apoptosis regulator (TIGAR) protects glioma cells from starvation-induced cell death by up-regulating respiration and improving cellular redox homeostasis." J Biol Chem **287**(40): 33436-33446.

Warburg, O. (1923). "The Metabolism of Tumors." Biochem **142**: 317-333.

Warburg, O. H. and F. Dickens (1931). "metabolism of tumours."

Webb, M. E., A. Marquet, R. R. Mendel, F. Rebeille and A. G. Smith (2007). "Elucidating biosynthetic pathways for vitamins and cofactors." Nat Prod Rep **24**(5): 988-1008.

Weber, C. E. and P. C. Kuo (2012). "The tumor microenvironment." Surg Oncol **21**(3): 172-177.

Weinhouse, S. (1976). "The Warburg hypothesis fifty years later." Z Krebsforsch Klin Onkol Cancer Res Clin Oncol **87**(2): 115-126.

Weinstein, S. J., T. J. Hartman, R. Stolzenberg-Solomon, P. Pietinen, M. J. Barrett, P. R. Taylor, J. Virtamo and D. Albanes (2003). "Null association between prostate cancer and serum folate, vitamin B(6), vitamin B(12), and homocysteine." Cancer Epidemiol Biomarkers Prev **12**(11 Pt 1): 1271-1272.

Weissbach, H. and R. T. Taylor (1970). "Roles of vitamin B 12 and folic acid in methionine synthesis." Vitam Horm **28**: 415-440.

Wen, Y. Y., S. J. Yang, J. X. Zhang and X. Y. Chen (2013). "Methylenetetrahydrofolate reductase genetic polymorphisms and esophageal squamous cell carcinoma susceptibility: a meta-analysis of case-control studies." Asian Pac J Cancer Prev **14**(1): 21-25.

Wieman, H. L., J. A. Wofford and J. C. Rathmell (2007). "Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking." Mol Biol Cell **18**(4): 1437-1446.

Wigfield, S., S. Winter, A. Giatromanolaki, J. Taylor, M. Koukourakis and A. Harris (2008). "PDK-1 regulates lactate production in hypoxia and is associated with poor prognosis in head and neck squamous cancer." British journal of cancer **98**(12): 1975-1984.

Williams, F. M. and W. F. Flintoff (1995). "Isolation of a human cDNA that complements a mutant hamster cell defective in methotrexate uptake." J Biol Chem **270**(7): 2987-2992.

Wise, A., S. M. Foord, N. J. Fraser, A. A. Barnes, N. Elshourbagy, M. Eilert, D. M. Ignar, P. R. Murdock, K. Steplewski, A. Green, A. J. Brown, S. J. Dowell, P. G. Szekeres, D. G. Hassall, F. H. Marshall, S. Wilson and N. B. Pike (2003). "Molecular identification of high and low affinity receptors for nicotinic acid." J Biol Chem **278**(11): 9869-9874.

Wise, D. R., R. J. DeBerardinis, A. Mancuso, N. Sayed, X.-Y. Zhang, H. K. Pfeiffer, I. Nissim, E. Daikhin, M. Yudkoff and S. B. McMahon (2008). "Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction." Proceedings of the National Academy of Sciences **105**(48): 18782-18787.

Wise, D. R., R. J. DeBerardinis, A. Mancuso, N. Sayed, X. Y. Zhang, H. K. Pfeiffer, I. Nissim, E. Daikhin, M. Yudkoff, S. B. McMahon and C. B. Thompson (2008). "Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction." Proc Natl Acad Sci U S A **105**(48): 18782-18787.

Wise, D. R., P. S. Ward, J. E. Shay, J. R. Cross, J. J. Gruber, U. M. Sachdeva, J. M. Platt, R. G. DeMatteo, M. C. Simon and C. B. Thompson (2011). "Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability." Proc Natl Acad Sci U S A **108**(49): 19611-19616.

Wong, S. C., S. A. Proefke, A. Bhushan and L. H. Matherly (1995). "Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective Chinese hamster ovary cells." J Biol Chem **270**(29): 17468-17475.

Wood, T. (1985). The pentose phosphate pathway. Orlando, FL, Academic Press, Inc.

Xie, J., B. S. Wang, D. H. Yu, Q. Lu, J. Ma, H. Qi, C. Fang and H. Z. Chen (2011). "Dichloroacetate shifts the metabolism from glycolysis to glucose oxidation and exhibits synergistic growth inhibition with cisplatin in HeLa cells." Int J Oncol **38**(2): 409-417.

Xu, L., R. Tong, D. M. Cochran and R. K. Jain (2005). "Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model." Cancer Res **65**(13): 5711-5719.

Xu, X., A. Zur Hausen, J. F. Coy and M. Lochelt (2009). "Transketolase-like protein 1 (TKTL1) is required for rapid cell growth and full viability of human tumor cells." Int J Cancer **124**(6): 1330-1337.

Xu, X., A. zur Hausen, J. F. Coy and M. Löchelt (2009). "Transketolase like protein 1 (TKTL1) is required for rapid cell growth and full viability of human tumor cells." International Journal of Cancer **124**(6): 1330-1337.

Yabu, M., H. Shime, H. Hara, T. Saito, M. Matsumoto, T. Seya, T. Akazawa and N. Inoue (2011). "IL-23-dependent and -independent enhancement pathways of IL-17A production by lactic acid." Int Immunol **23**(1): 29-41.

Yamamoto, S., K. Inoue, K. Y. Ohta, R. Fukatsu, J. Y. Maeda, Y. Yoshida and H. Yuasa (2009). "Identification and functional characterization of rat riboflavin transporter 2." J Biochem **145**(4): 437-443.

Yang, H. T., P. C. Chao and M. C. Yin (2013). "Riboflavin at high doses enhances lung cancer cell proliferation, invasion, and migration." J Food Sci **78**(2): H343-349.

Yang, S., X. Wang, G. Contino, M. Liesa, E. Sahin, H. Ying, A. Bause, Y. Li, J. M. Stommel, G. Dell'antonio, J. Mautner, G. Tonon, M. Haigis, O. S. Shirihai, C. Doglioni, N. Bardeesy and A. C. Kimmelman (2011). "Pancreatic cancers require autophagy for tumor growth." Genes Dev **25**(7): 717-729.

Yang, Y. A., W. F. Han, P. J. Morin, F. J. Chrest and E. S. Pizer (2002). "Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase." Exp Cell Res **279**(1): 80-90.

Yang, Z. J., C. E. Chee, S. Huang and F. A. Sinicrope (2011). "The role of autophagy in cancer: therapeutic implications." Mol Cancer Ther **10**(9): 1533-1541.

Yeaman, S. J., E. T. Hutcheson, T. E. Roche, F. H. Pettit, J. R. Brown, L. J. Reed, D. C. Watson and G. H. Dixon (1978). "Sites of phosphorylation on pyruvate dehydrogenase from bovine kidney and heart." Biochemistry **17**(12): 2364-2370.

Yin, G., H. Ming, X. Zheng, Y. Xuan, J. Liang and X. Jin (2012). "Methylenetetrahydrofolate reductase C677T gene polymorphism and colorectal cancer risk: A case-control study." Oncol Lett **4**(2): 365-369.

Yonezawa, A., S. Masuda, T. Katsura and K. Inui (2008). "Identification and functional characterization of a novel human and rat riboflavin transporter, RFT1." Am J Physiol Cell Physiol **295**(3): C632-641.

Yoshioka, K. (1984). "Some properties of the thiamine uptake system in isolated rat hepatocytes." Biochimica et Biophysica Acta (BBA)-Biomembranes **778**(1): 201-209.

Yoshioka, K. (1984). "Some properties of the thiamine uptake system in isolated rat hepatocytes." Biochim Biophys Acta **778**(1): 201-209.

Youle, R. J. and D. P. Narendra (2011). "Mechanisms of mitophagy." Nat Rev Mol Cell Biol **12**(1): 9-14.

Youngberg, J. T. and R. A. Myers (1990). "Complications from hyperbaric oxygen therapy?" Ann Emerg Med **19**(11): 1356-1357.

Yuan, W., S. Wu, J. Guo, Z. Chen, J. Ge, P. Yang, B. Hu and Z. Chen (2010). "Silencing of TKTL1 by siRNA inhibits proliferation of human gastric cancer cells in vitro and in vivo." Cancer Biol Ther **9**(9): 710-716.

Yuvaraj, S., V. G. Premkumar, K. Vijayasarathy, S. G. Gangadaran and P. Sachdanandam (2008). "Augmented antioxidant status in Tamoxifen treated postmenopausal women with breast cancer on co-administration with Coenzyme Q10, Niacin and Riboflavin." Cancer Chemother Pharmacol **61**(6): 933-941.

Zastre, J. A., B. S. Hanberry, R. L. Sweet, A. C. McGinnis, K. R. Venuti, M. G. Bartlett and R. Govindarajan (2013). "Up-regulation of vitamin B1 homeostasis genes in breast cancer." J Nutr Biochem **24**(9): 1616-1624.

Zastre, J. A., R. L. Sweet, B. S. Hanberry and S. Ye (2013). "Linking vitamin B1 with cancer cell metabolism." Cancer Metab **1**(1): 16.

Zaugg, K., Y. Yao, P. T. Reilly, K. Kannan, R. Kiarash, J. Mason, P. Huang, S. K. Sawyer, B. Fuerth, B. Faubert, T. Kalliomaki, A. Elia, X. Luo, V. Nadeem, D. Bungard, S. Yalavarthi, J. D. Growney, A. Wakeham, Y. Moolani, J. Silvester, A. Y. Ten, W. Bakker, K. Tsuchihara, S. L. Berger, R. P. Hill, R. G. Jones, M. Tsao, M. O. Robinson, C. B. Thompson, G. Pan and T. W. Mak (2011). "Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress." Genes Dev **25**(10): 1041-1051.

Zhang, S., J. H. Yang, C. K. Guo and P. C. Cai (2007). "Gene silencing of TKTL1 by RNAi inhibits cell proliferation in human hepatoma cells." Cancer Lett **253**(1): 108-114.

Zhang, S., J. X. Yue, J. H. Yang, P. C. Cai and W. J. Kong (2008). "Overexpression of transketolase protein TKTL1 is associated with occurrence and progression in nasopharyngeal carcinoma." Cancer biology & therapy **7**(4): 1-6.

Zhang, S., J. X. Yue, J. H. Yang, P. C. Cai and W. J. Kong (2008). "Overexpression of transketolase protein TKTL1 is associated with occurrence and progression in nasopharyngeal carcinoma: a potential therapeutic target in nasopharyngeal carcinoma." Cancer Biol Ther **7**(4): 517-522.

Zhao, F., A. Mancuso, T. V. Bui, X. Tong, J. J. Gruber, C. R. Swider, P. V. Sanchez, J. J. Lum, N. Sayed, J. V. Melo, A. E. Perl, M. Carroll, S. W. Tuttle and C. B. Thompson (2010). "Imatinib resistance associated with BCR-ABL upregulation is dependent on HIF-1alpha-induced metabolic reprograming." Oncogene **29**(20): 2962-2972.

Zhao, R., F. Gao and I. D. Goldman (2001). "Molecular cloning of human thiamin pyrophosphokinase1." Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression **1517**(2): 320-322.

Zhao, R., F. Gao and I. D. Goldman (2002). "Reduced folate carrier transports thiamine monophosphate: an alternative route for thiamine delivery into mammalian cells." American Journal of Physiology-Cell Physiology **282**(6): C1512-C1517.

Zhao, R., F. Gao and I. D. Goldman (2002). "Reduced folate carrier transports thiamine monophosphate: an alternative route for thiamine delivery into mammalian cells." Am J Physiol Cell Physiol **282**(6): C1512-1517.

Zhao, R., F. Gao, Y. Wang, G. A. Diaz, B. D. Gelb and I. D. Goldman (2001). "Impact of the reduced folate carrier on the accumulation of active thiamin metabolites in murine leukemia cells." J Biol Chem **276**(2): 1114-1118.

Zhao, R., R. G. Russell, Y. Wang, L. Liu, F. Gao, B. Kneitz, W. Edelmann and I. D. Goldman (2001). "Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs." J Biol Chem **276**(13): 10224-10228.

Zhao, Y. B., H. Y. Yang, X. W. Zhang and G. Y. Chen (2005). "Mutation in D-loop region of mitochondrial DNA in gastric cancer and its significance." World J Gastroenterol **11**(21): 3304-3306.

Zhong, H., K. Chiles, D. Feldser, E. Laughner, C. Hanrahan, M. M. Georgescu, J. W. Simons and G. L. Semenza (2000). "Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics." Cancer Res **60**(6): 1541-1545.

Zhou, F., A. C. Lee, K. Krafczyk, L. Zhu and M. Murray (2011). "Protein kinase C regulates the internalization and function of the human organic anion transporting polypeptide 1A2." British journal of pharmacology **162**(6): 1380-1388.

Zhou, Z. H., D. B. McCarthy, C. M. O'Connor, L. J. Reed and J. K. Stoops (2001). "The remarkable structural and functional organization of the eukaryotic pyruvate dehydrogenase complexes." Proc Natl Acad Sci U S A **98**(26): 14802-14807.

Zhu, W., W. Qin, P. Bradley, A. Wessel, C. L. Puckett and E. R. Sauter (2005). "Mitochondrial DNA mutations in breast cancer tissue and in matched nipple aspirate fluid." Carcinogenesis **26**(1): 145-152.

Zielinska-Dawidziak, M., K. Grajek, A. Olejnik, K. Czaczyk and W. Grajek (2008). "Transport of high concentration of thiamin, riboflavin and pyridoxine across intestinal epithelial cells Caco-2." Journal of nutritional science and vitaminology **54**(6): 423-429.

Zielinska-Dawidziak, M., K. Grajek, A. Olejnik, K. Czaczyk and W. Grajek (2008). "Transport of high concentration of thiamin, riboflavin and pyridoxine across intestinal epithelial cells Caco-2." J Nutr Sci Vitaminol (Tokyo) **54**(6): 423-429.

APPENDIX

Up-regulation of Vitamin B1 Homeostasis Genes in Breast Cancer

Zastre, J. A., B. S. Hanberry, R. L. Sweet, A. C. McGinnis, K. R. Venuti, M. G. Bartlett and R. Govindarajan (2013). "Up-regulation of vitamin B1 homeostasis genes in breast cancer." The Journal of nutritional biochemistry. Reprinted from Elsevier with permission obtained through the Copy Rights Clearance center (License # 3267750813698).

A.1. Abstract

An increased carbon flux and exploitation of metabolic pathways for the rapid generation of biosynthetic precursors is a common phenotype observed in breast cancer. To support this metabolic phenotype, cancer cells adaptively regulate the expression of glycolytic enzymes and nutrient transporters. However, activity of several enzymes involved in glucose metabolism requires an adequate supply of cofactors. In particular, vitamin B1 (thiamine) is utilized as an essential cofactor for metabolic enzymes that intersect at critical junctions within the glycolytic network. Intracellular availability of thiamine is facilitated by the activity of thiamine transporters and thiamine pyrophosphokinase-1 (TPK-1). Therefore, the objective of this study was to establish if the cellular determinants regulating thiamine homeostasis differ between breast cancer and normal breast epithelia. Employing cDNA arrays of breast cancer and normal breast epithelial tissues, SLC19A2, SLC25A19, and TPK-1 were found to be significantly upregulated. Similarly, upregulation was also observed in breast cancer cell lines compared to human mammary epithelial cells. Thiamine transport assays and quantitation of intracellular thiamine and thiamine pyrophosphate established a significantly greater extent of thiamine transport and free thiamine levels in breast cancer cell lines compared to HMECs. Overall, these findings demonstrate an adaptive response by breast cancer cells to increase cellular availability of thiamine.

A.2. INTRODUCTION

Vitamin B1 (thiamine or thiamin) is an essential micronutrient that is fundamentally required to sustain the bioenergetic and biosynthetic needs of all cells. Thiamine, and in particular thiamine pyrophosphate, is a critical cofactor for three key enzymes involved in

intracellular glucose metabolism, pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH), and transketolase (TKT). Maintenance of thiamine homeostasis is primarily achieved through the intracellular uptake of thiamine by the thiamine transporters THTR1 and THTR2, encoded by the SLC19A2 and SLC19A3 genes, respectively (Ganapathy, Smith *et al.* 2004). Once inside the cell, thiamine is converted to the active coenzyme, thiamine pyrophosphate (TPP) by thiamine pyrophosphokinase-1 (TPK-1). Transport of TPP across the mitochondrial membrane to support PDH and **a**-KGDH activity is facilitated by the thiamine pyrophosphate carrier (TPC), which is encoded by the SLC25A19 gene (previously identified as a deoxynucleotide carrier or DNC) (Lindhurst, Fiermonte *et al.* 2006).

The inability of mammals to synthesize thiamine necessitates a tightly regulated homeostasis system to conserve or increase thiamine availability during nutritional, metabolic, and pathological stress. In cancer, abnormal cellular metabolism is a hallmark of nearly all tumors regardless of tissue or cellular origin. First described by Otto Warburg over 80 years ago, cancer cells exhibit a high rate of fermentative glycolysis in the presence of adequate oxygen (Warburg and Dickens 1931). This aerobic glycolysis, termed the "Warburg effect" is characterized by a high rate of glucose utilization that provides a significant metabolic advantage for cancer cells to generate energy and increase biomass for cell division (Koppenol, Bounds *et al.* 2011). During high glucose utilization, thiamine status is reduced demonstrating an integral connection between thiamine supply and metabolic flux (Elmadfa, Majchrzak *et al.* 2001). Clinically, thiamine deficiencies have been reported in early and advanced breast cancer patients as measured by the "TPP effect" (Basu and Dickerson 1976). When supplemented 12-25 times the recommended daily allowance to mice, thiamine stimulated the growth of an Ehrlich's ascites tumor model (Boros, Lee *et al.* 1998). To compensate for reduced cellular levels of thiamine,

adaptive responses attempt to increase intracellular thiamine through the up-regulation of thiamine transporter expression (Reidling and Said 2005). Thus, the amplified carbon flux observed in cancer cells may impose a requirement for a coordinated enhancement in cellular thiamine supply.

One possible requirement for an increase in thiamine supply by cancer cells may involve *de novo* nucleotide synthesis to support rapid cell division. The thiamine-dependent enzyme transketolase utilizes intermediates from the Embden-Meyerof pathway for the production of ribose 5-phosphate (R5-P), which forms the pentose sugar backbone in the synthesis of nucleotides. Although non-tumorigenic cells primarily generate R5-P through the non-thiamine dependent oxidative portion of the pentose phosphate pathway (PPP), cancer cells exploit the non-oxidative portion of the PPP involving transketolase for R5-P production (Cascante, Centelles *et al.* 2000, Langbein, Zerilli *et al.* 2006). Transketolase activity has been shown to supply ~85% of the C5-ribose sugars for nucleic acid synthesis in cancer cells (Boros, Puigjaner *et al.* 1997). Transketolase isoforms have also been demonstrated to be over-expressed in a range of cancers, including breast, and are critically important for tumor cell proliferation (Földi, Stickeler *et al.* 2007{Langbein, 2006 #313, Schultz, Kähler *et al.* 2008, Zhang, Yue *et al.* 2008, Xu, zur Hausen *et al.* 2009). Additionally, glutaminolysis in cancer cells contributes to anaplerotic reactions that re-supply the TCA cycle with a-ketoglutarate (DeBerardinis, Mancuso *et al.* 2007). In the oxidative direction, a-ketoglutarate is converted to succinyl-CoA via the thiamine-dependent enzyme, α-KGDH with subsequent reactions forming malate. Malate can be used in cataplerotic reactions to produce the amino acid aspartate and NADPH, which are essential for the formation of purine and pyrimidine nucleotides {DeBerardinis, 2007 #3178;Tong, 2009 #3098}. Malignant progression models tracking C^{13}-glucose metabolism

during normal, transformation, and metastatic stages of breast cancer have established a progressive increase in the carbon flux through the PPP and TCA cycle (Richardson, Yang *et al.* 2008). This metabolic phenotype is facilitated by overexpression of the glucose transporter, GLUT1 and the thiamine-dependent transketolase like-1 enzyme TKTL1, as well as an increase in a-ketoglutarate production via glutaminolysis (Forbes, Meadows *et al.* 2006, Schmidt, Voelker *et al.* 2010). Thus, thiamine-dependent enzyme activity in the PPP and TCA cycle are essential for the generation of the extensive biosynthetic precursors that are required during cancer cell proliferation.

In the absence of an increase in dietary thiamine intake, adaptive up-regulation of thiamine homeostasis genes may be a vital response by cancer cells to increase intracellular thiamine levels. Therefore, the objective of the present study was to establish if the expression of thiamine homeostasis genes and overall cellular thiamine status differ between breast cancer and normal breast epithelia cells and tissues. Techniques such as qRTPCR, Western blot, immunofluorescence, transport assays, and quantification of intracellular thiamine and thiamine phosphorylates were employed to investigate gene expression, cellular localization, activity and thiamine status.

A.3. MATERIALS AND METHODS

A.3.1. Material

All cell culture reagents including trypsin/EDTA, penicillin/streptomycin, RPMI 1640, DMEM:F12 (1:1) media and donor horse serum were obtained from Mediatech (Manassas, VA). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Dartmouth, MA). Cell culture treated flasks, plates and dishes were from Greiner Bio-one (Monroe, NC). Radiolabeled ^{3}H-

Thiamine hydrochloride with a specific activity of 20 Ci/mmol was purchased from American Radiolabeled Chemicals (St. Louis, MO). Amiloride hydrochloride hydrate, oxythiamine chloride hydrochloride, pyrithiamine hydrobromide, and thiamine monophosphate was purchased from Sigma-Aldrich (St. Louis, MO). Thiamine pyrophosphate were from TCI (Tokyo, Japan).

A.3.2. Cell culture

The estrogen receptor alpha positive (ERα+) BT474 and MCF7 and estrogen receptor alpha negative (ERα-) BT20 and MDA-MB-231 breast cancer cell lines were obtained from ATCC (Manassas, VA), and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin. Primary human mammary epithelial cells (HMEC) isolated from adult female breast tissue were obtained from Lonza (Walkersville, MD). HMEC were cultured according to the supplier protocol using mammary epithelial cell growth media (MEGM) containing supplied supplements (Lonza). The non-tumorigenic breast epithelial cell lines MCF10A and MCF12A (ATCC, Manassas, VA) were used in this study as a model system of a non-cancer cell type. Both MCF10A and MCF12A were maintained in DMEM:F12 (Mediatech) supplemented with 5% horse serum, 1% penicillin/streptomycin, 20 ng/mL EGF (Peprotech), 0.5 mg/mL Hydrocortisone (Sigma-Aldrich), 100 ng/mL cholera toxin (Sigma-Aldrich) and 10 ug/mL insulin (Sigma-Aldrich). Thiamine content in media(s) was standardized to the level reported in RPMI 1640 media (3 mM) by supplementing with thiamine hydrochloride (Sigma-Aldrich). All cells were cultured at 37^0C in a humidified atmosphere of 5% CO_2 in air.

A.3.2. Breast Cancer cDNA Array

Expression levels of SLC19A2, SLC19A3, SLC25A19 and TPK-1 were determined in human breast cancer and normal breast epithelial tissues by real-time PCR using the TissueScan Breast Cancer cDNA Array panel II (Origene, Rockville, MD). The cDNA array consisted of 48 clinical samples normalized to b-actin. See supplier's homepage http://www.origene.com/qPCR/Tissue-qPCR-Arrays.aspx for details on tissues and pathology. Primers for each gene were designed using the Roche Universal Probe Library website to correspond with a specific 8-9 nucleotide hydrolysis probe labeled at the 5' end with fluorescein (FAM). Probes used were #72 for SLC19A2, #5 for SLC19A3, #34 for SLC25A19, and #42 for TPK-1. The b-actin reference assay kit supplied by Roche Applied Science was used as the internal control gene. Gene expression was normalized to b-actin using the $2^{-\Delta Ct}$ method and the fold expression comparing normal and tumor tissue was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

A.3.4. Real-time quantitative PCR (qRT-PCR)

RNA (2mg) isolated from cell lines (EZNA Total RNA Kit I, Omega Bio-tek, Norcross, GA) was reverse transcribed to cDNA using a mixture of random and oligo-dT primers following qRT-PCR for SLC19A2, SLC19A3, SLC25A19, and TPK-1 using a LightCycler® 480 II (Roche Applied Science, Indianapolis, IN). Primer-probe combinations for each gene are as described above and include probe #3 for SLC19A1. The Tata-box-binding protein (TBP) reference assay kit supplied by Roche Applied Science was used as a housekeeping gene. Fold expression comparing HMEC with breast cancer cell lines was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

A.3.5. Western blotting

The protein expression of THTR1, THTR2, TPC, and TPK-1 was assessed between breast epithelial and cancer cells by Western blot. For whole cell lysates, cells cultured until approximately 80% confluency were washed with ice-cold phosphate-buffered saline (PBS). Cells were lysed using 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in a pH=8.5 50mM Tris, 250mM NaCl, 1mM EDTA buffer supplemented with protease inhibitor cocktail (EMD Biosciences, La Jolla, CA). Lysates were centrifuged at 16,000xg for 20min at 4^0C and the supernatant was collected. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Isolation of plasma membrane associated THTR1 and THTR2 was performed by cell surface biotinylation using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). Briefly, cells were grown to ~80% confluency in T-75 cm^2 flasks and washed twice with ice cold PBS. A solution (8mL) of the cell impermeable Sulfo-NHS-SS-biotin (0.25mg/mL) was added to each flask and incubated at 4^0C for 30min with gentle shaking followed by addition of a quenching solution provided by the manufacturer. Cells were then scraped and centrifuged at 500xg for 3min. The supernatant was discarded and cells were washed twice with PBS. The cell pellet was lysed using the supplied lysis buffer with protease inhibitors and sonicated on ice for five 1-sec bursts at low power (Branson Sonifer 150). Cells were then incubated on ice for 30min with occasional mixing and centrifuged at 10,000xg for 2min at 4^0C. The resulting supernatant was added to a NeutrAvidin Agarose column and incubated for 60min at room temperature with end-over-end mixing. The column was centrifuged for 1 min at 1,000xg, flow through was discarded and the column was rinsed with wash buffer containing protease inhibitors. SDS-PAGE Sample buffer containing 50mM DTT was added to the column and incubated for 60min

at room temperature with end-over-end mixing. The column was centrifuged for 2min at 1,000xg to elute the cell surface protein fraction followed by quantification using the Pierce Reducing Agent Compatible BCA Protein Assay Kit (Thermo Scientific).

Whole cell lysates (50mg) and cell surface protein fractions (50mg) were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). The membrane was blocked with 5% non-fat dry milk in Tris buffered saline-tween 20 (TBS-T) for 1h. The membrane was then immunoblotted overnight with a 1:500 dilution of THTR1 (Alpha Diagnostics, San Antonio, TX), THTR2 (Proteintech Group, Chicago, IL), TPC (Abgent, San Diego, CA), or TPK-1 (Genetex, Irvine, CA) antibodies. Beta-actin (1:1000) was used as housekeeping protein (Thermo Scientific). The blot was then washed with TBS-T (3 x 10min) followed by 1h incubation with 1:15,000 goat anti-rabbit-HRP secondary antibody (Millipore, Billerica, MA). Blots were then washed three times for 10min each with TBS-T and visualized using Supersignal West Pico (Thermo Scientific) and captured with a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry was performed using Fluorchem SP software.

A.3.6. Immuno-fluorescence

All cells were grown to 80-90% confluency on glass cover slips. Cells were fixed with 2% paraformaldehyde in 100 mM potassium phosphate buffer for 10min, then blocked and permeabilized with 1% horse serum and 0.2% Triton X-100 for 30min. Cells were incubated with antibodies for THTR1 (1:1000) (Alpha Diagnostic) or THTR2 (1:1000) (Proteintech Group) in a solution containing 0.5% Tween-20 and 1% horse serum for 1h at room temperature. Phosphate buffered saline containing 0.02% Tween-20 (PBS-T) was used to wash the cells three

times 15min each. Cells were incubated with secondary antibody (1:1000) (goat anti-rabbit IgG) conjugated with Alexa 594 (Invitrogen, Molecular Probes) for 1h and then washed 3 times with PBS-T 15min each. Cover slips were mounted using ProLong Gold anti-fade reagent with DAPI (Invitrogen, Eugene, OR) on glass slides. Immunostained cells were imaged with a Nikon TM Eclipse fluorescence microscope (Nikon Insturments Inc., Melville, NY) and analyzed using Nikon TiE software. To differentiate, THTR1 was visualized in green and THTR2 was visualized in red.

To examine intracellular localization with lysozymes, BT474 cells were stained with LysoTracker ® Red DND-99 (Invitrogen) as per manufacturer's protocol. Briefly, BT474 cells grown to 80-90% confluence on glass cover slips were incubated with 75nM LysoTracker ® probe for 30min at 37°C. Media was then removed and cells were fixed and co-stained for THTR1 as described above.

A.3.7. Transport assay

Uptake studies were performed in 24 well plates on cells grown to approximately 80-90% confluency. Hanks Balanced Salt Solution (HBSS) (0.95mM $CaCl_2$, 0.49mM $MgCl_2$-$6H_2O$, 0.40mM $MgSO_4$-$7H_2O$, 5.36mM KCl, 0.44mM KH_2PO_4, 1.36mM NaCl, 0.34mM Na_2HPO_4-$7H_2O$, 5.0mM dextrose, 4.2mM sodium bicarbonate, 10mM hepes) pH=7.3 at 37^0C was used as the transport buffer. All experiments examining pH-dependent thiamine transport utilized HBSS buffered with 10mM MES at pH=5.0. Inhibition of 5nM ^{3}H-thiamine transport was evaluated using excess unlabeled thiamine (10mM) or known transport inhibitors, amiloride, pyrithiamine, and oxythiamine all at 100µM and pH=7.3 transport buffer. Prior to uptake, cells were washed twice with transport buffer then incubated for 15min in HBSS buffer, with or without inhibitors,

at 37^0C. After which, 5nM ^{3}H-thiamine, with or without inhibitors was added to the cells and uptake allowed to proceed for 5min at 37^0C. Washing the cells twice with ice-cold HBSS buffer terminated the uptake.

Since BT474 cells did not adhere extensively during transport assays performed in 24-well plates, it was necessary to conduct thiamine uptake using the oil stop method as previously described (Sweet, Paul *et al.* 2010). Briefly, BT474 cells were trypsinized prior to uptake studies and the transport assay conducted while the cells were in suspension. Cells were centrifuged at 500xg for 3min, and the cell pellet washed with transport buffer and allowed to equilibrate in transport buffer with or without inhibitors for 15min at 37^0C. Cells were then centrifuged at 500xg for 3min and suspended with 5nM ^{3}H-thiamine, with or without inhibitors. Cells were periodically inverted to maintain the cells in suspension over the course of the 5min uptake time. Transport was terminated by centrifuging (1500xg for 3min) the cell suspension through a layer of silicone oil:liquid paraffin (85:15).

To quantify thiamine uptake, cells were lysed with 1% Triton X-100 in 50mM Tris, 250mM NaCl, and 1mM EDTA pH=7.8 buffer. Cell lysates were collected and centrifuged at 10,000xg for 5min to remove cellular debris and aliquots used for liquid scintillation counting using a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter (Brea, CA). Total protein was measured for normalization using the BCA Protein Assay (Thermo Scientific Rockford, Il).

A.3.8. Determination of thiamine and thiamine phosphate derivatives

Quantitation of intracellular thiamine, TMP, and TPP was performed as previously described with minor modifications (Lu and Frank 2008, Gangolf, Czerniecki *et al.* 2010). Once cells reached approximately 75% confluency, cell culture media was changed with fresh media

and maintained in culture at 37^0C for an additional 24h. Cells were harvested using trypsin/EDTA and washed three times with ice cold PBS. Total cell count was determined using a hemocytometer and the cell pellet suspended in 500µL of ice cold 15% trichloroacetic acid (Sigma-Aldrich). The cell sample was vortexed briefly and set on ice for 3min. Trichloroacetic acid was removed from the supernatant using 3 x 1mL extractions (10,000xg for 6min at 4^0C) with isopropyl ether.

To quantify thiamine and its phosphate esters by HPLC, cell extracts or thiamine, TMP, and TPP standards were derivatized to a fluorescent thiochrome using potassium ferricyanide. Prior to analysis, 80µL of cell sample or standards was mixed with 50µL of a 5mM potassium ferricyanide solution in 15% NaOH and 20µL methanol.

HPLC was performed using a Synergi 4 micron Max RP 80 Å, 4.6 x 150mm column (Phenomenex, Torrance, CA). Mobile phase buffer A consisted of 10% methanol in 25mM sodium phosphate dibasic pH=7 and mobile phase buffer B was 70% methanol in 25mM sodium phosphate dibasic pH=7. Separation of thiamine and derivatives was performed on an Agilent 1100 equipped with a G1321A fluorescence detector at a flow rate of 1 mL/min with a gradient of 15 to 20% B for 3 min then 20 to 55% B for 9 min. Sample injection volume was 30 µL and the compounds were detected at an excitation λ=375 nm and emission λ= 435 nm.

A.3.9. Statistical Analysis

All experiments were performed with a minimum of three independent experiments unless otherwise stated. Statistical significance was evaluated between groups using unpaired student t-test with a significance level of $p<0.05$ using Graphpad Prism 5.

A.4. RESULTS

A.4.1. Comparative Gene Expression Between Normal and Breast Cancer Tissue

Real time qPCR arrays containing cDNA from isolated normal and breast cancer tissues were utilized to compare the gene expression of SLC19A2, SLC19A3, SLC25A19, and TPK-1. Results in Figure A.1 display box and whisker plots with error bars set at the 10^{th} and 90^{th} percentiles comparing the expression in normal tissue to total tumor tissue. Since the loss of ERa (estrogen receptor alpha) expression and over-expression of HER2 (Human epidermal growth factor receptor 2) has been linked with disease progression, results were also separated based on ERa and HER2 expression stated in pathology reports provided by the supplier. In the 43 tumor tissue samples, 27 were ERα(+), 10 ERα(-), 11 HER2(+), and 5 were not stated and excluded from the differential phenotype analysis. We did not differentiate between co-expression of ERα and HER2 due to the small number of samples for these groupings. For SLC19A2, a significant increase in expression was found when comparing total tumor versus normal tissue, corresponding to a fold change of 6.5. Additionally, SLC19A2 expression was significantly greater for ERα(+) and HER2(+) tissues but not significant in ERα(-) phenotypes. In contrast, no significant change (1.3 fold decrease) in SLC19A3 expression was found in tumor versus normal tissue that was consistent across ERα and HER2 status. Expression of SLC25A19 was significantly upregulated in tumor tissue with a fold change of 5.9 and significantly up-regulated regardless of ERa and HER2 status. Similarly, a significant increase in TPK-1 expression was observed, corresponding to a fold change of 2.3, which was also significantly upregulated regardless of ERα and HER2 status.

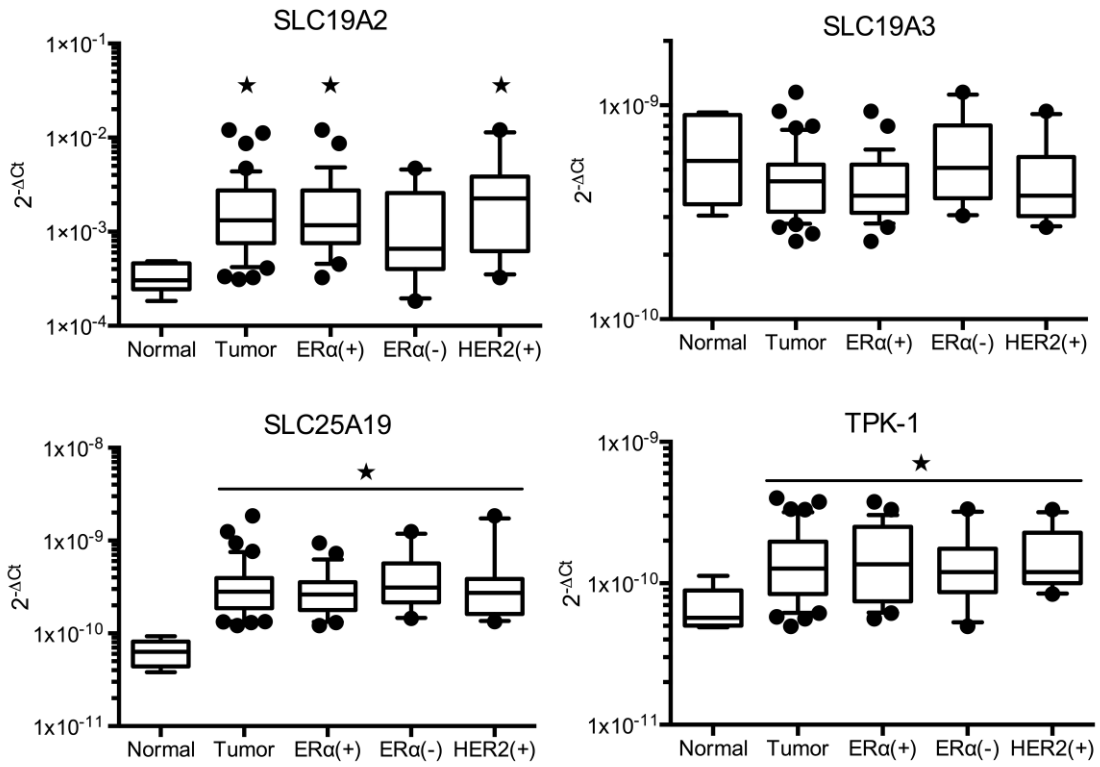

Figure A.1: Expression of thiamine homeostasis genes, SLC19A2, SLC19A3, SLC25A19 and TPK-1 in breast cancer and normal tissues from 48 clinical samples. Gene expression levels were determined by qRTPCR using TissueScan Breast Cancer cDNA Array II. Results are expressed as the Log2 gene expression normalized to actin and displayed using box and whisker plots with error bars set at the 10^{th} and 90^{th} percentiles. Total tumor tissue was further differentiated by ERα and HER2 status. (★) Statistically significant differences ($p<0.05$) compared to normal tissue.

A.4.2. Comparative Gene Expression Between HMEC and Breast Cancer Cell Lines

Figure A.2 displays the gene expression profile of thiamine transporters, TPK-1, and the folate transporter SLC19A1 in several common breast cancer cell lines compared to primary human mammary epithelial cells (HMEC). Two non-tumorigenic breast epithelial cell lines, MCF10A and MCF12A were included in the analysis as both are commonly used as "normal" cells in comparative studies with breast cancer cells. The ERα(+) cell lines BT474 and MCF7 exhibited a greater expression of SLC19A2 compared to HMEC with fold changes of 15.9 and 9.4, respectively. In contrast, SLC19A3 expression was lower for BT474 and MCF7 with fold decreases of 6.3 and 38.9, respectively. The mitochondrial TPP transporter SLC25A19 was overexpressed in BT474 (33.1 fold) and MCF7 (37.8 fold), and TPK-1 was increased 2.6 fold and 1.7 fold for BT474 and MCF7 cells, respectively. The folate transporter SLC19A1 was substantially higher in BT474 (175 fold) and MCF7 (167 fold) cells compared with HMEC. In comparison, the ERα(-) cells BT20 and MDA-MB-231 exhibited increases in the fold expression for SLC19A2 and SLC25A19 but to a lower extent than the ERα(+) cell lines. Expression changes of TPK1 were higher only for MDA-MB-231 (1.9 fold) but unchanged for BT20 (1.2 fold decrease) compared with HMEC. For SLC19A1, MDA-MB-231 was downregulated by 6.2 fold and BT20 was upregulated 140 fold. The expression of the non-tumorigenic cell line MCF12A was similar for all genes to HMEC, while MCF10A exhibited greater expression for SLC25A19 (20.1 fold), TPK-1 (4.7 fold), and SLC19A1 (31.5 fold).

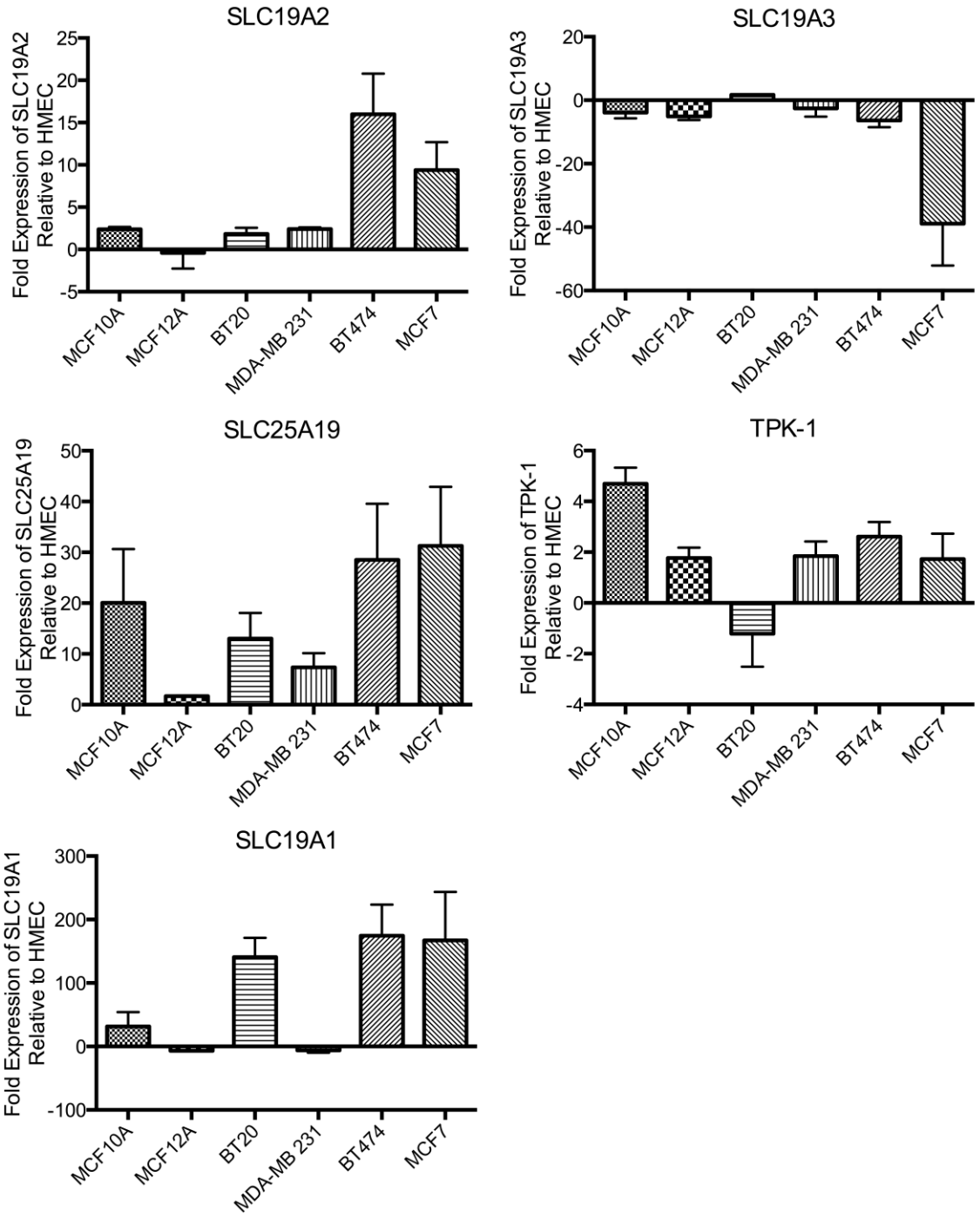

Figure A.2: Expression of thiamine homeostasis genes, SLC19A2, SLC19A3, SLC25A19, TPK-1, and SLC19A1 in immortalized non-tumorigenic breast epithelia and breast cancer cell lines compared to primary human mammary epithelial cells (HMECs). Gene expression levels were determined by qRTPCR using TBP as housekeeping gene. Results are expressed as the fold change in expression relative to HMEC and represent N=4-6 independent RNA samples. Fold change values less than one were transformed to the negative reciprocal to establish the fold expression.

A.4.3. Protein Expression of THTR1/2, SLC25A19, and TPK-1 in HMEC and Breast Cancer Cells

To establish the relative expression of thiamine transporters and TPK-1 at the protein level, whole cell lysates of all cell lines were evaluated using Western blotting. Results in Figure A.3A show representative Western blots and relative density measurements of whole cell lysates for THTR1, THTR2, TPC, and TPK-1. For both THTR1 and THTR2, expression was greater in all breast cancer cell lines tested compared to HMECs except for BT20 exhibiting lower levels of THTR1 and no detectable THTR2. Expression of TPC was also higher in cancer cell lines compared with HMEC. In contrast, all breast cancer cells express TPK-1 with similar band intensity to HMEC except for BT20 that showed the highest expression. Expression of all four proteins in the non-transformed cell lines MCF12A and MCF10A was consistent with HMEC, except for a greater band intensity of THTR2 in MCF12A and TPC for both MCF12A and MCF10A.

Since the functional activity of THTR1 and THTR2 is confined to the plasma membrane, we next tested for the cell surface localization of THTR1 and THTR2. To do so, we utilized cell surface biotinylation techniques to isolate plasma membrane localized THTR1 and THTR2. Figure A.3B shows the cell surface detection of both THTR1 and THTR2 in the various cell lines. HEK293 cells were used as a positive control since previous studies has identified both THTR1 and THTR2 to be functionally localized at the plasma membrane (Subramanian, Marchant *et al.* 2003, Ashokkumar, Vaziri *et al.* 2006). No expression of intracellular TPK-1 was found in the cell surface biotinylation preparations, confirming selectivity of immunoblots for the cell surface fraction (data not shown). Consistent with total SLC19A2 (THTR1) gene expression (Fig. A.2), a high level of cell surface expression for THTR1 was found in ERα(+)

BT474 and MCF7 cells. Interestingly, THTR1 was not detected at the cell surface of HMEC and was either limited in detection or undetectable in both ERα(-) cells MDA MB-231 and BT20, respectively. For THTR2, all cell lines had detectable cell surface expression with varied amounts except no surface expression was detected for BT20 cells.

A.4.4. Cellular Localization of THTR1 and THTR2

To corroborate the cell surface expression of THTR1 and THTR2, we performed immunofluorescence studies in HMEC, MCF12A, and breast cancer cell lines. Consistent with the plasma membrane biotinylation studies (Fig. A.3B), THTR1 was readily detected on the plasma membrane for MCF12A, BT474, and MCF7 cells (Fig. A.4A). The most intense cell surface expression was noticed in MCF7 cells although significant heterogeneity was noticed with a certain fraction of cells displaying cell surface expression and others displaying intracytoplasmic staining. Also consistent with the membrane biotinylation studies (Fig. A.3B), limited to no cell surface staining for THTR1 was discernable for HMECs, BT20, and MDA-MB-231 cells (Fig. A.4A). In contrast to the cell surface localization (Fig. A.3B), THTR2 appeared to be predominantly confined intracellularly with minimal membrane localization discernable by immunofluorescence for all cell lines evaluated (Fig. A.4A).

To detect the identity of the cytoplasmic compartments in which THTR1 was localized, we performed co-localization analyses of THTR1 with markers for various organelles (viz., ER, mitochondria, and lysosomes). Our results indicated intracytoplasmic THTR1 in BT474 was predominantly localized in the lysosomes as judged by significant co-localization of THTR1 with a lysotracker dye (Fig. A.4B) and lack of co-localization with markers of other organelles (data not shown).

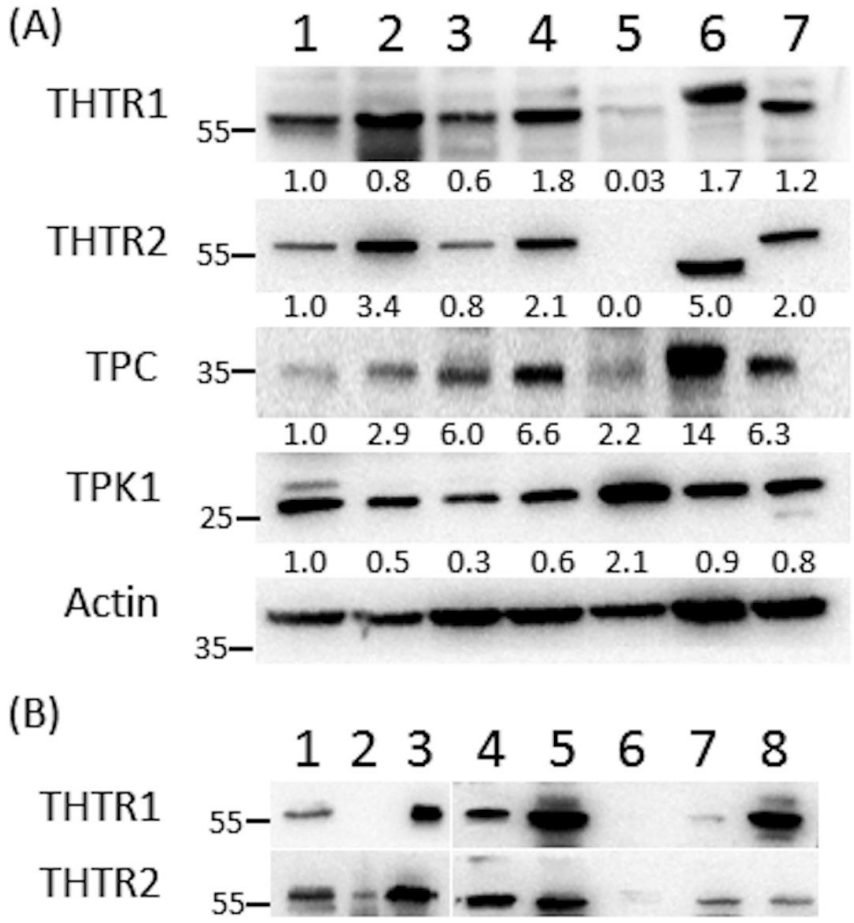

Figure A.3: Representative Western blots of THTR1, THTR2, TPC and TPK-1 in immortalized non-tumorigenic breast epithelia, breast cancer cell lines, and HMECs. (A) Whole cell lysate preparations of (1) HMEC, (2) MCF12A, (3) MCF10A, (4) BT474, (5) BT20, (6) MDA-MB-231, (7) MCF7 were immunofractionated on a 10% SDS-Page gel. Beta-actin was used as housekeeping protein. The fold change in protein expression measured by densitometry is denoted underneath each blot. (B) Cell surface expression of THTR1 and THTR2 isolated using cell surface biotinylation of plasma membrane proteins from (1) HEK293, (2) HMEC, (3) MCF12A, (4) MCF10A, (5) BT474, (6) BT20, (7) MDA-MB-231, (8) MCF7.

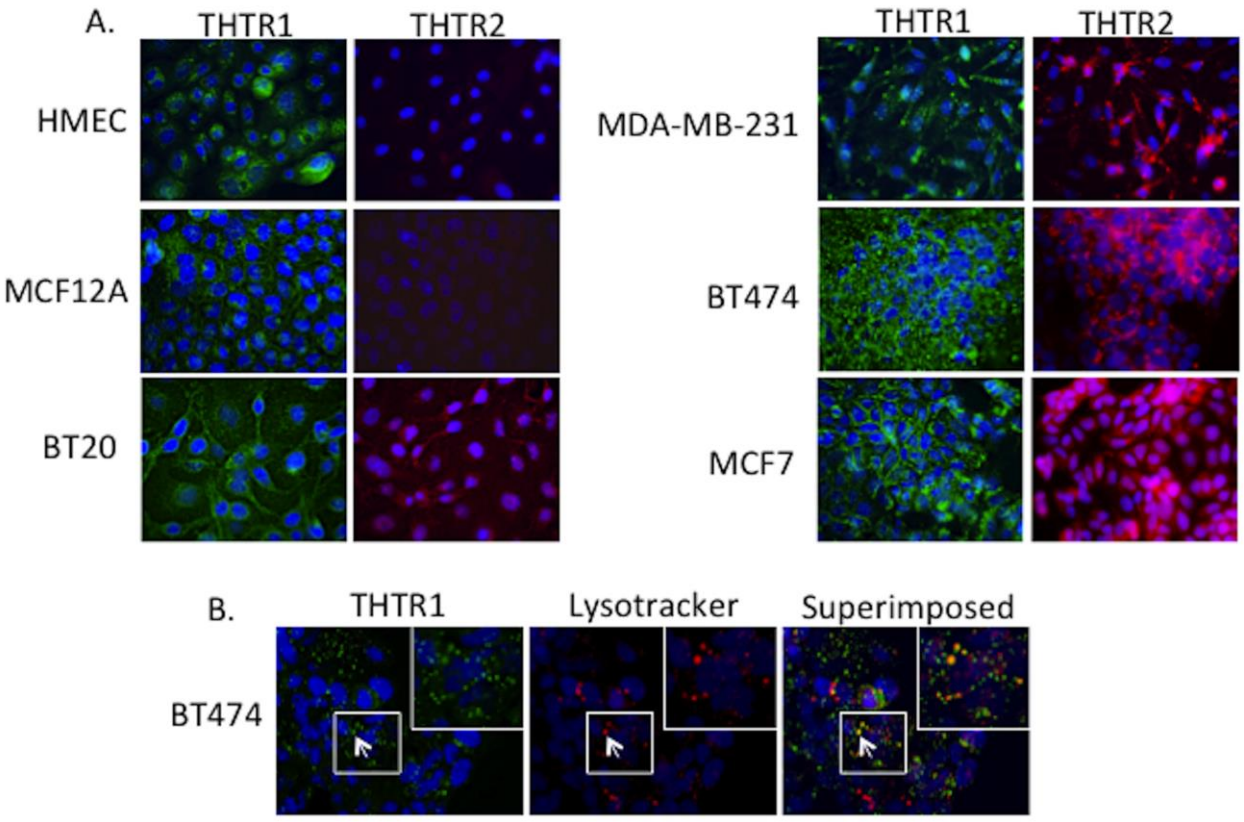

Figure A.4: Cellular localization of THTR1 and THTR2 by Immunofluorescence. (A) Localization of THTR1 and THTR2 in breast cancer cell lines compared with HMECs and MCF12A cells. (B) Co-localization of Lysotracker dye with THTR1 in BT474 cells.

A.4.5. Thiamine Transport in HMEC and Breast Cancer Cell Lines

Transport assays were performed with or without known inhibitors of thiamine transport (amiloride, pyrithiamine and oxythiamine) and excess thiamine (10mM) to establish if a selective uptake process for thiamine exists and if breast cancer cells exhibit a greater extent of thiamine transport. Compared to HMEC, all non-tumorigenic and breast cancer cell lines evaluated demonstrated a significantly greater extent of thiamine uptake (Fig. A.5). The effect of excess unlabeled thiamine (10mM) on transport was evaluated to establish if thiamine transport uses a specific/saturable transport process. For all cell lines, excess unlabeled thiamine significantly decreased the uptake of labeled thiamine (Fig. A.5). To further confirm a specific transport process, known transport inhibitors, amiloride, oxythiamine and pyrithiamine (all at 100 mM) were used (Menon and Quastel 1966, Ashokkumar, Vaziri *et al.* 2006). Moreover, the effect of reduced pH on thiamine transport was determined at pH=5.0, since thiamine transport was previously shown to be pH dependent (Ashokkumar, Vaziri *et al.* 2006). Table 1 displays the uptake level of thiamine in each cell line comparing thiamine alone (5nM) with inhibitors and at pH=5.0. Both inhibitors and acidic pH reduced thiamine transport to varying degrees depending on the cell line (Table A.1). Although trending towards a decrease in transport with reduced pH and inhibitors, no statistically significant differences were found for BT474 cells except for pyrithiamine compared to thiamine alone. In addition, inhibitors and reduced pH did not significantly limit thiamine transport in BT20 and MDA MB-231 cells (Table A.1).

Table A.1. Uptake of 5 nM H3-thiamine uptake in breast cancer and breast epithelial cell lines. Thiamine uptake was performed at 37°C for 5 min in either pH=7.4 transport buffer (5 nM) or pH=5 buffer, and in the presence of amiloride, oxythiamine or pyrithiamine all at 100 μM in pH=7.4 buffer. All experiments are expressed as the mean±S.E.M. of N= 6 independent experiments. Statistical significance (Pb.05) was determined using unpaired Student's t test compared to thiamine uptake (5 nM).

Cell line	Treatment	Uptake (fmol/mg protein)	$P<.05$
HMEC	5 nM	50.6±0.6	
	pH=5	44.1±2.9	Y
	Amiloride	37.9±0.9	Y
	Oxythiamine	33.8±0.6	Y
	Pyrithiamine	23.7±1.1	Y
MCF10A	5 nM	72.5±6.8	
	pH=5	35.6±3.5	Y
	Amiloride	48.0±6.4	Y
	Oxythiamine	47.9±8.3	Y
	Pyrithiamine	36.6±9.1	Y
MCF12A	5 nM	87.4±3.8	
	pH=5	55.6±1.9	Y
	Amiloride	40.8±3.5	Y
	Oxythiamine	43.4±0.8	Y
	Pyrithiamine	12.4±0.7	Y
BT474	5 nM	148±21	
	pH=5	121±8.2	N
	Amiloride	104±21	N
	Oxythiamine	97.1±19	N
	Pyrithiamine	76.3±22	Y
MCF-7	5 nM	61.1±1.2	
	pH=5	43.4±1.8	Y
	Amiloride	40.6±1.1	Y
	Oxythiamine	29.5±2.5	Y
	Pyrithiamine	17.2±2.1	Y
BT-20	5 nM	72.9±9.3	
	pH=5	46.8±5.5	N
	Amiloride	79.6±6.1	N
	Oxythiamine	69.1±16	N
	Pyrithiamine	61.7±17	N
MDA-MB-231	5 nM	72.8±5.3	
	pH=5	69.0±14	N
	Amiloride	62.0±8.9	N
	Oxythiamine	54.4±7.9	N
	Pyrithiamine	43.2±8.6	Y

A.4.6. Quantitation of Thiamine and Thiamine Phosphate esters in Normal Breast Epithelia and Breast Cancer Cell lines

Figure A.6 shows the intracellular free thiamine, TMP and TPP content in cell lines determined by HPLC fluorescence chromatography. Except in MCF7 cells, the free thiamine content was significantly greater in all cell lines tested including the "normal" breast epithelial cell lines MCF10A and MCF12A compared to HMECs. The amount of TPP was significantly greater only in BT474 cells compared to HMECs. Both MDA-MB-231 and BT20 exhibited a significantly lower level of TMP then HMEC cells. When combined, the overall thiamine status was significantly greater for MDA-MB-231 and BT474 cells compared to HMECs, but was significantly lower for MCF7 (Fig. A.6).

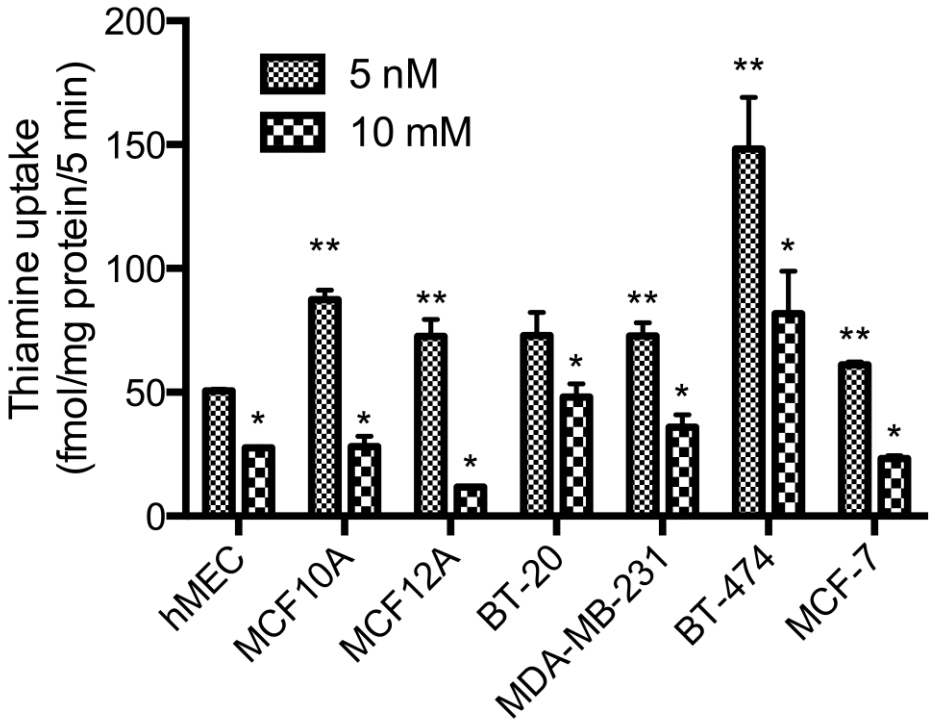

Figure A.5: Uptake of thiamine by breast cancer (BT20, MDA-MB-231, BT474, MCF7) and normal breast epithelial cell lines (HMEC, MCF10A, MCF12A). Thiamine uptake was performed for 5min at 37^0C in pH=7.4 transport buffer with or without excess (10mM) thiamine. Results reported as the mean +/- SEM normalized to total protein with N=6 independent experiments. Statistical significant ($p<0.05$) comparisons between 5nM and with 10mM thiamine (★), and between the uptake of 5nM thiamine in HMEC cells (★★).

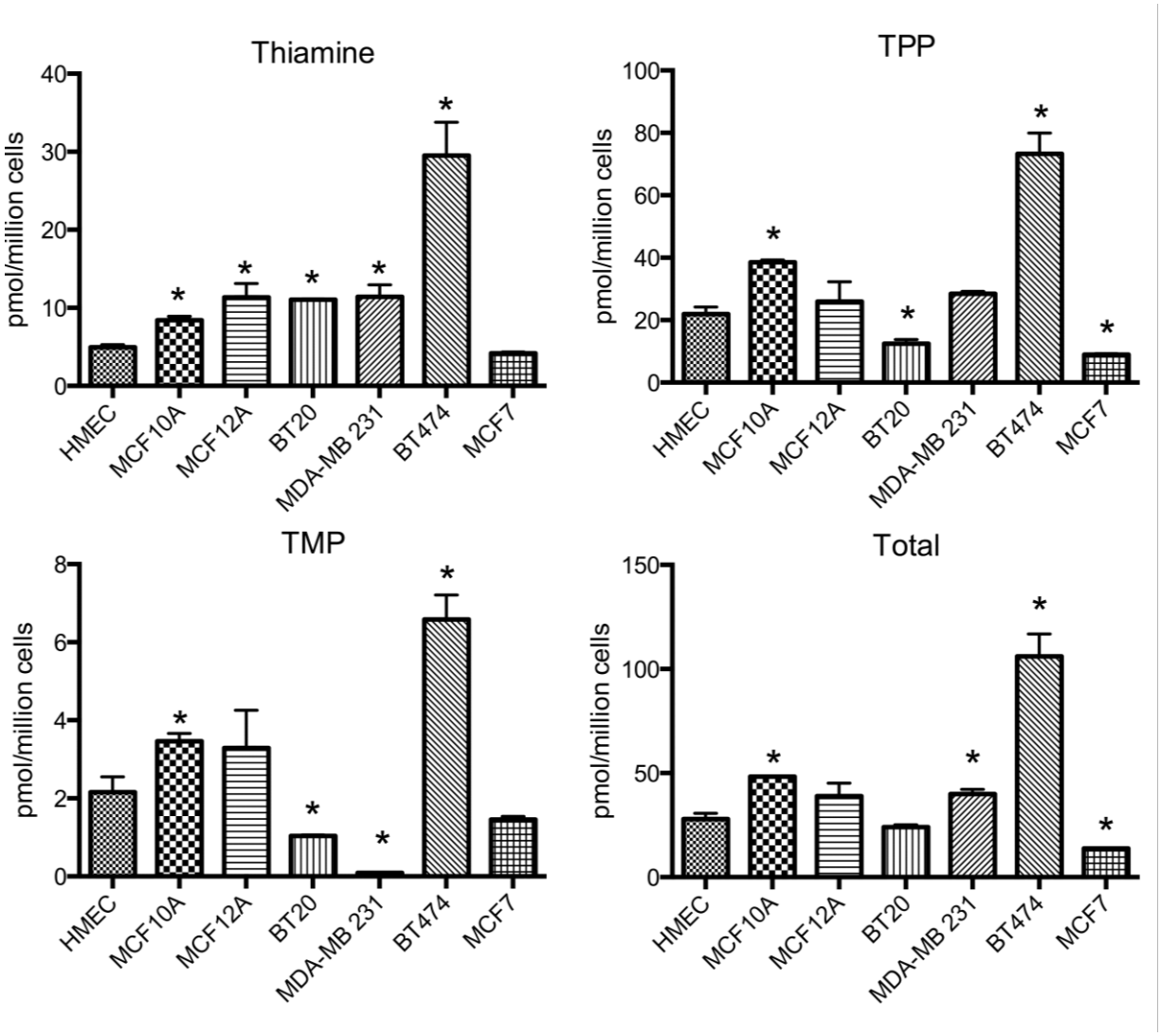

Figure A.6: Quantitation of intracellular thiamine and thiamine phosphate esters, TPP and TMP in breast cancer and normal breast epithelial cell lines. Thiamine and thiamine phosphates in cell lysates were derivatized to a thiochrome derivative for HPLC separation and detection by fluorescence. Quantitation was achieved using standard curves of T, TMP, and TPP. Results expressed as the mean +/- SD normalized cell count (per million) with N=3 independent experiments. (✶) Statistically significant ($p<0.05$) comparisons to HMEC.

A.5. DISCUSSION

The high proliferation rate of cancer cells requires a large quantity of amino acids, glucose, glutamine, NADPH, and other growth factors for the biosynthesis of proteins, lipids, and nucleotides to generate daughter cells. This metabolically demanding process is supported in part by oncogene-mediated regulation of nutrient transport processes to increase cellular supply of carbon sources {Wise (Wise, DeBerardinis *et al.* 2008), 2008 #3293;Kim, 2004 #3294}. This also may necessitate a coordinated enhancement in coenzyme supply to support enzyme activity. Using cDNA qRT-PCR arrays we have established that SLC19A2, SLC25A19, and TPK-1, three genes involved in thiamine transport and activation, are over-expressed in clinical breast cancer tissue samples. To our knowledge this is the first report on the expression differences for the thiamine activating enzyme TPK-1 and the TPP mitochondrial transporter (TPC) SLC25A19 in normal and cancer tissues. Our finding of an approximately 6 and 2 fold increase in SLC25A19 and TPK-1 expression in tumor tissue respectively, highlights a potentially important contribution of these thiamine homeostasis genes in support of cancer cell metabolism.

To further expand on these findings we compared the gene and protein expression of thiamine transporters and TPK-1 in several common breast cancer cell lines to primary HMECs. A similar trend of gene over-expression of SLC19A2, SLC25A19, and TPK-1 and down-regulation of SLC19A3 was observed in the majority of breast cancer cell lines tested compared with clinical samples. When compared to HMEC cells, gene and protein expression levels of the thiamine homeostasis genes were similar to non-tumorigenic MCF12A cell line. Thus the use of the immortalized “normal” breast epithelial MCF12A cells may be a suitable model for thiamine homeostasis comparisons with tumorigenic cell lines. In 3 of the 4 cancer cell lines evaluated, protein expression of THTR1 and TPC was greater than the non-tumor tissue control, consistent

with the relative increase in gene expression. The moderate increase in gene expression of TPK-1 is reflected in the similar level of protein expression. However, BT20 cells exhibited a lower level of gene expression for TPK-1 but a high level of protein expression compared to HMEC. Similarly, protein expression of THTR2 in several cancer cell lines was greater than HMECs in contradiction to the relatively low levels of gene expression. Previous reports have demonstrated a decrease in SLC19A3 gene expression in breast, gastric, and colon cancers but no correlation with THTR2 protein expression has been reported to date (Liu, Huang *et al.* 2003, Liu, Lam *et al.* 2009, Ikehata, Ueda *et al.* 2012). Therefore, the uncoupling of protein expression with gene expression may suggest additional regulatory mechanisms involving translational control of thiamine homeostasis proteins.

A functional requirement for thiamine transport into the cell via THTR1 and THTR2 is localization in the plasma membrane. Both THTR1 and THTR2 have been characterized to be located on the plasma membrane of intestinal, hepatic, and renal epithelial cells (Subramanian, Marchant *et al.* 2003, Subramanian, Marchant *et al.* 2006). Cell surface biotinylation studies demonstrated limited plasma membrane localization for both THTR1 and THTR2 in ERα(-) compared to ERα (+) breast cancer cells. Immunofluorescence studies confirmed our findings with MDA-MB-231 and BT20 cells exhibiting predominantly internal staining of THTR1/2 with BT474 and MCF7 cells having both internal and membrane expression. To date, there is limited knowledge on the signaling pathways or interacting proteins that mediate THTR1 and THTR2 intracellular trafficking to the cell membrane. Recently, tetraspanin-1 (TSPAN1) has been shown to be an auxiliary protein that is co-localized with THTR1 at the plasma membrane that enhances THTR1 stability (Nabokina, Senthilkumar *et al.* 2011). Tetraspanin proteins are important in cell motility and invasion and are associated with acting as a tumor metastasis suppressor

(Richardson, Jennings *et al.* 2011). Interestingly, the expression of TSPAN1 was shown to correlate with ERα status in breast cancer cells, with ERα (-) showing limited expression and ERα (+) cells the highest level of TSPAN1 expression (Desouki, Liao *et al.* 2011). Therefore, reduced expression of TSPAN1 in MDA-MB-231 and BT20 cells may explain the lack of membrane localization of THTR1.

It is unclear why THTR1 and THTR2 expression was predominantly internal in primary HMECs. HMECs were only used within 3-5 passages after receipt from the supplier and routinely exhibited an internal THTR1/2 localization pattern. Since the cellular localization of THTR1 and THTR2 in mammary epithelia is unknown, it is speculative to suggest that localization will be consistent with the findings in other epithelial cells, such as from renal and intestinal cells (Subramanian, Marchant *et al.* 2003, Subramanian, Marchant *et al.* 2006). Our finding that THTR1 was co-localized with lysozymes suggests a continuous turnover of plasma membrane associated THTR1, which may explain the extensive punctated staining in ERα(+) breast cancer cells. It is possible that our cell surface biotinylation and immunofluorescence assays were not able to capture membrane staining due to a rapid cycling of THTR1 at the plasma membrane. Furthermore, it is unclear if the cellular localization pattern of THTR1/2 in HMECs is consistent with mammary tissue or the result of the isolation and subsequent culturing of primary cells. In contrast, the immortalized, but non-tumorigenic MCF12A cells commonly used as "normal" breast epithelial cells exhibited extensive plasma membrane localization of THTR1 and THTR2 by cell surface biotinylation and immunofluorescence. Thus, signaling pathways altered during immortalization procedures for MCF12A cells or during tumorigenesis in mammary epithelial cells may be responsible for an increased membrane localization of THTR1 compared to HMECs. Previous findings by Said et al. have established that

Ca^{2+}/calmodulin kinase inhibition reduces thiamine uptake, possibly through decreased cell surface localization of either THTR1 or THTR2 (Said, Ortiz *et al.* 1999, Ashokkumar, Vaziri *et al.* 2006). Activation and/or inhibition of protein kinase pathways have been shown to modulate membrane trafficking and transport activity of other solute carrier transporters, such as OAT1 and OATP1A2 (Wise, DeBerardinis *et al.* 2008, Zhou, Lee *et al.* 2011). Therefore, determining the signaling pathways that regulate membrane localization and expression of THTR1 and THTR2 will greatly increase our understanding of thiamine homeostasis in cancer.

The expression and localization pattern of THTR1 and THTR2 in breast cancer cells and HMECs may suggest reduced transport capability in cells with no detectable cell surface expression. Overall, thiamine transport was significantly greater in the majority of breast cancer cell lines evaluated compared to HMECs. Moreover, thiamine uptake assays with excess cold thiamine established that a selective transport process for thiamine was functional, even if no detectable THTR1 or THTR2 was observed on the cell surface, as in the case for BT20 and MDA-MB-231. Inhibition of thiamine transport at lower pH and the use of known transport inhibitors further confirmed functional thiamine transport. Although the transport mechanism of thiamine is primarily facilitated through THTR1 and THTR2, high concentrations of thiamine have been demonstrated to cross plasma membranes through a passive transport process (Rindi and Laforenza 2000, Zielinska-Dawidziak, Grajek *et al.* 2008). The reported Km of THTR1 is in the low mM range (~2.6 mM) and THTR2 in the low nM (~27 nM), thus our transport assays using 5 nM are well below saturation for both transporters and a substantial contribution by passive diffusion maybe ruled out (Said, Balamurugan *et al.* 2004, Ashokkumar, Vaziri *et al.* 2006). Recently, transport of thiamine across intestinal epithelial has also been found to be mediated by organic cation transporters (OCT1/3) (Lemos, Faria *et al.* 2012). Therefore, the

observed transport by HMEC, BT20, and MDA-MB-231 cells are more likely to be the result of low or undetectable levels of THTR1 and THTR2 at the plasma membrane or via other transporters.

To establish if the overexpression of the thiamine homeostasis genes in breast cancer cells correlates with an overall increase in the thiamine status, the level of free thiamine, TMP and TPP was quantified. When summed, the overall thiamine status was significantly upregulated in 2 of the 4 cancer cells evaluated. However, breast cancer cells exhibited a greater intracellular pool of free thiamine that did not correlate with an overall increase in TPP within the majority of cell lines. Although the levels of TPP were not reported, the greater accumulation of free thiamine in our breast cancer cells was similar to that observed in isolated colon adenocarcinomas relative to un-invaded control tissue (Baker, Frank *et al.* 1981). No correlation between TPK-1 gene and protein expression could be established to describe the level of TPP within the breast cancer cells. For instance, BT20 has the greatest TPK-1 expression at the protein level but exhibited a low level of intracellular TPP. Since, binding of TPP to apoenzymes is a 1:1 stoichiometry, the intracellular level of TPP maybe an indirect measure of the relative expression level of thiamine-dependent enzymes. Thus the relative expression of PDH, TKT, and a-KGDH may vary depending on the metabolic phenotype of the cell and be an important contributor to the intracellular level of TPP. Additionally, TMP can be produced from the dephosphorylation of TPP by thiamine pyrophosphatase, TMP is also found extracellular at levels approximating that of free thiamine (Tallaksen, Bøhmer *et al.* 1997, Rindi and Laforenza 2000, Zhao, Gao *et al.* 2002). The origin of TMP in the plasma has been suggested to be from the efflux of TMP out of the cells by the reduced folate transporter (RFC-1), encoded by the SLC19A1 gene (Zhao, Gao *et al.* 2001, Zhao, Gao *et al.* 2002). The high level of gene

expression of SLC19A1 in MCF7 cells may have reduced the level of TMP and forced the equilibrium of free thiamine to be ultimately converted to TMP and effluxed out of the cell. However, the reduced expression of SLC19A1 does not explain the relatively low level of TMP and high amount of free thiamine in MDA-MB-231 cells. Therefore, the potential impact of SLC19A1 on the efflux of TMP and the overall thiamine status may be minimal.

Why cancer cells exhibited a greater accumulation of thiamine and not the active coenzyme TPP is unclear. TPP is the only known metabolite of thiamine functioning as an enzyme cofactor but the high level of free thiamine may indicate a potentially important non-cofactor role of thiamine. One possibility may be to maintain the intrinsic ROS status of cancer cells. Unchecked, excess ROS can lead to cellular apoptosis and necrosis and has been exploited as a chemotherapeutic approach (Simon, Haj-Yehia *et al.* 2000, Trachootham, Alexandre *et al.* 2009). Free thiamine has direct antioxidant properties as well as TPP being essential for glutathione production (Lukienko, Mel'nichenko *et al.* 2000, Martin, Singleton *et al.* 2003, Schmid, Stopper *et al.* 2008). Therefore, the contribution of thiamine and TPP in regulating cancer cell oxidative stress may be a contributing factor in cancer cell prosurvival responses.

In conclusion, these findings demonstrate that the genes involved in dictating thiamine homeostasis, such as SLC19A2, SLC25A19, and TPK-1 were significantly upregulated in clinical tissues and breast cancer cell lines. Additionally, the thiamine transporters THTR1 and THTR2 exhibited predominately membrane localization in ERα(+) cell lines and intracellular localization in ERα(-) cell lines. The increase in thiamine homeostasis genes correlated with an increase in intracellular free thiamine levels but not with the coenzyme TPP. Clarification is still required to assess if the alterations in THTR1/2 cellular localization and levels of thiamine and TPP are consistent with clinical cancer tissues. In addition, further work is needed to determine

the impact of thiamine and thiamine pyrophosphate on cancer cell metabolism and oxidative stress. Once known, these findings may provide a molecular basis for dietary influences on malignant progression and provide for new therapeutic interventions.