ADAPTIVE REGULATION OF THE VITAMIN B1 TRANSPORTER, SLC19A3

by

REBECCA LEA SWEET

(Under the Direction of Jason Zastre)

ABSTRACT

Thiamine (vitamin B1) is an essential nutrient requiring continuous dietary intake to meet cellular demands in support of metabolism. The activated form of thiamine, thiamine pyrophosphate, serves as a cofactor for enzymes involved in the TCA cycle and the non-oxidative portion of the pentose phosphate pathway. Due to the hydrophilicity of thiamine, it does not easily diffuse across biological membranes and requires the activity of Solute Carrier (SLC) transporters to facilitate uptake into cells. Cells have altered nutrient and growth factor requirements in order to maintain cellular metabolism and conserve anabolic energy under conditions of physiological stress such as hypoxia and nutrient deprivation. In the absence of dietary changes, cells require adaptive regulatory processes to conserve intracellular thiamine levels. Therefore, the purpose of this research is to characterize the effects of hypoxia and thiamine deficiency on thiamine transporter expression and investigate the mechanism of adaptive transcriptional regulation. Using a RT-PCR array strategy to evaluate changes in transporter expression within a chronic hypoxia breast cancer cell line model, we have identified various SLC transporters that are differentially regulated. In particular, the thiamine transporter SLC19A3 was found to be significantly upregulated during chronic and acute hypoxia.

SLC19A3 was also upregulated after exposure to thiamine deficient conditions. Interestingly, SLC19A2 was not adaptively upregulated during either hypoxia or thiamine deficient conditions.

A decrease in the upregulation of SLC19A3 was found after HIF-1α shRNA treatment indicating the potential involvement of HIF-1α in SLC19A3 adaptive regulation to hypoxia. We report stabilization of HIF-1α protein levels and an increase in the expression of genes involved in glycolytic metabolism during thiamine deficiency. To further investigate the mechanism of SLC19A3 adaptive regulation, we performed *in silico* sequence analysis and identified four putative hypoxic response elements within the promoter region of SLC19A3. Luciferase assays were used to analyze the promoter activity of deletion and mutation promoter constructs of SLC19A3. We identified two putative hypoxic response elements in the promoter region of SLC19A3 that are involved in the adaptive regulation to hypoxic conditions. Overall, these results suggest a transcriptional congruency between hypoxia and thiamine deficiency centering on the stabilization of HIF-1α.

INDEX WORDS: Thiamine, hypoxia, HIF-1α, cellular metabolism, transport, glycolytic phenotype, thiamine deficiency

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DEDICATION

This dissertation is dedicated to my grandmother, Helen Bertola. She was one of the strongest women I have had the honor of knowing and she taught me that there is no shame in being myself. Here's to those that wish us well, Gram.

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While I will be the sole recipient of this degree, its completion would not have been possible without the constant love and support of family, friends, peers, and advisors.

My life outside of the lab played a major part in keeping me sane throughout graduate school. First and foremost I would like to thank my family, in particular, my parents, sister and boyfriend, Jason. They are a constant support system that was there to lend an ear when I needed to vent, a shoulder to cry on (which happened more so in the last year of graduate school that any other time in my entire life) and they always knew what to say to make me smile. They have played a critical role in me becoming who I am and accepting me for who I have become. I am proud to call them my family and am more grateful than words can express to have them in my life.

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I would also like to thank my committee. Serving as a graduate committee member is a major time commitment and not only have they given me their time but also guidance in my research endeavors.

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ABBREVIATIONS

5-FU: 5-fluorouracil

α-KG: Alpha-ketoglutarate

α-KGDH: Alpha-ketoglutarate dehydrogenase

ABC: ATP Binding Cassette

Ach: Acetylcholine

AD: Alzheimer's Disease

AThTP: Adenosine thiamine triphosphate

AGE: Advanced glycation end products

ATP: Adenosine triphosphate

BCAAs: Branched chain amino acids

BCKDC: Branched chain alpha-keto acid dehydrogenase complex

BCRP: Breast Cancer Resistance Protein

BDK: Branched-chain α-keto acid dehydrogenase kinase

BDP: Branched-chain α-keto acid dehydrogenase phosphatase

bHLH: Basic helix-loop-helix

C-NLS: C-terminus nuclear localization signal

DFO: Desferrioxamine mesylate

eNOS: Endothelial isoform of nitric oxide synthase

ER: Estrogen receptor

F6P: fructose 6-phosphate

FAD: Flavin adenine dinucleotide

FH: Fumarate hydratase

FIH-1: Factor inhibiting HIF-1

G3P: Glyceraldehyde 3-phosphate

GKLF: Gut-enriched Krupple-like factor

HER2: Human epidermal growth factor receptor 2

HIF: Hypoxia inducible factor

HPH: HIF-1α prolyl hydroxylase

HMECs: Primary human mammilary epithelial cells

^{1}H-NMR: Proton nuclear magnetic resonance

HRP: Horseradish peroxidase

IFN: Interferons

LDHA: Lactate dehydrogenase A

LLC: Lewis lung carcinoma

LT: Low thiamine

MCT4: Monocarboxylate transporter 4

MRP: Multidrug Resistance Protein

MSUD: Maple syrup urine disease

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NF: Normal-fat

N-NLS: N-terminus nuclear localization signal

NT: Normal thiamine

NF1: Nuclear factor 1

NF-Y: Nuclear factor Y

ODD: Oxygen-dependent domain

PAS: Per-Arnt-Sim

PDC: Pyruvate dehydrogenase complex

PDH: Pyruvate dehydrogenase

PDK1: Pyruvate dehydrogenase kinase 1

P-gp: P-glycoprotein

PHDs: Prolyl hydroxylases

PMSF: Phenylmethylsulfonyl fluoride

PPP: Pentose phosphate pathway

PTD: Pyrithiamine induced thiamine deficiency

pVHL: von Hippel Lindau tumor suppressor protein

qRT-PCR: Reverse transcriptase polymerase chain reaction

RDI: Recommended daily intake

SD: Standard deviation

SDH: Succinate dehydrogenase

PAGE: Polyacrylamide gel electrophoresis

SLC: Solute Carrier

SP1: Stimulating protein 1

SP3: Stimulating protein 3

TAD-C: C-terminal trans-activation domain

TAD-N: N-terminal trans-activation domain

TBP: Tata-box-binding protein

TBS-T: Tris buffered saline-tween 20

TCA: Tricarboxylic acid cycle

TD: Thiamine deficiency

TDP: Thiamine diphosphate

TGF-α: Transforming growth factor alpha

TKT: Transketolase

TKTL: Transketolase-like protein

TMP: Thiamine monophosphate

TMPase: thiamine monophosphatase

TPK-1: Thiamine phosphokinase 1

TPPase: thiamine pyro-phosphatase

TRMA: Thiamine-responsive megaloblastic anemia

TSPAN1: Tetraspanin-1

TTP: Thiamine triphosphate

VEGF: Vascular endothelial growth factor

WE: Wernicke's encephalopathy

WKS: Wernicke Korsakoff Syndrome

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

In order to support growth and normal cellular function, cells are required to meet specific energy and biosynthetic demands. This intricate network of cellular metabolism has the potential to undergo homeostatic regulation to alter gene expression, protein synthesis, and energy production. Maintaining cellular homeostasis during physiological stresses involves alterations in metabolism requiring an increased uptake of nutrients to support changes in energy demands. Micronutrients are comprised of vitamins and minerals required by all living organisms in order to support all physiological functions. Many micronutrients require the functional expression of transporters belonging to the Solute Carrier (SLC) family to facilitate intracellular uptake. However, there is limited understanding of how micronutrient transporters are adaptively regulated to maintain cellular metabolism during metabolic stresses. Lack of such knowledge is an important issue since micronutrients are critical for cellular metabolism. Further investigation of micronutrient homeostasis would not only establish a mechanistic comprehension into the adaptive regulation of micronutrient transporters, but would provide insights into potential strategies that may modulate nutrient availability in various pathologies.

1.1. CELLULAR METABOLISM

1.1.1. Metabolic Pathways

In order to maintain normal cellular function, cells require a constant supply of energy. Energy is generated through catabolic processes that breakdown molecules in an organized and dynamic metabolic system. Large molecules such as polysaccharides and proteins are broken down into smaller units of simple sugars and amino acids, respectively, ultimately aiding in energy generation to sustain the cell. Chemical energy is most commonly produced in the form of adenosine triphosphate (ATP). The three main pathways acting in series to generate cellular energy are glycolysis, the tricarboxylic acid cycle (TCA), and oxidative phosphorylation (Figure 1.1) (Michal and Schomburg 2013).

Glycolysis is the central ATP producing pathway converting one glucose molecule to two pyruvate molecules. Glycolysis is the first pathway in the series, however it is not the major generator for cellular energy. Glycolysis yields only a net gain of 2 ATP molecules through the breakdown of glucose without the use of molecular oxygen. The glycolysis end product pyruvate is then converted to acetyl-CoA, linking glycolysis to the TCA cycle. The TCA cycle generates nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) by oxidizing acetyl groups to CO_2. Although the TCA does not directly use molecular oxygen, it is required for NADH to generate NAD+ through the loss of its electrons allowing the cycle to continue. Electrons from NADH and $FADH_2$ are donated to the electron transport chain. Electrons are passed along a series of reactions falling to lower energy states and the energy released is used to pump hydrogen ions across the mitochondrial membrane. The resulting proton gradient is

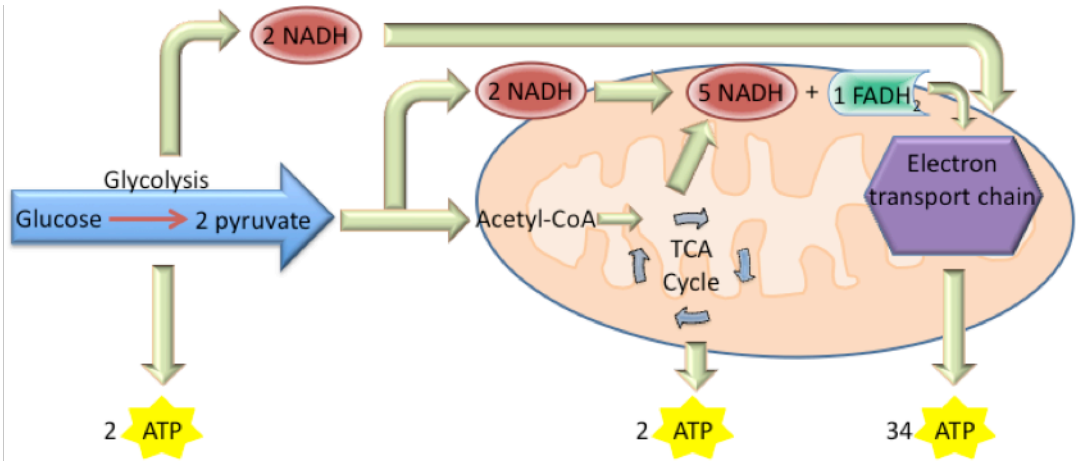


Figure 1.1. Cellular metabolism. Once inside the cell, glucose can undergo glycolysis resulting in the formation of two pyruvate molecules. Pyruvate is converted to acetyl-coA linking glycolysis to the TCA cycle. Within the TCA cycle, 3 NADH and 1 $FADH_2$ molecules are produced. NADH and FADH can serve as electron donors for the electron transport chain, ultimately producing 38 ATP.

used to drive the production of 38 ATP. At the end of the chain, electrons eventually combine with O_2 and produce water.

Intermediate products of glycolysis and TCA are used both as sources of metabolic energy and to produce many of the small molecules used as precursors for biosynthesis. The set of metabolic pathways that results in the biosynthesis of large molecules such as proteins is referred to as anabolism. Anabolism requires the input of energy resulting in cellular energy expenditure. Cells possess a metabolic balance in order to determine which type of metabolism is necessary to meet their immediate requirements. This balance comes in the form of an elaborate network of control mechanisms that serve to regulate and coordinate different metabolic processes depending on the state of the cell (Berg *et al.* 2002).

Another metabolic pathway in cells that primarily functions as an anabolic pathway, rather than catabolic, is the pentose phosphate pathway (PPP) (Figure 1.2). There are two distinct phases of the pathway, the oxidative and the non-oxidative (Wood 1985). The oxidative branch serves to generate NADPH, which can be used in bio-reductive synthesis reactions such as fatty acid synthesis. The non-oxidative portion results in the synthesis of 5 carbon sugars such as ribose-5-phosphate, which is used in the synthesis of nucleotides and nucleic acids.

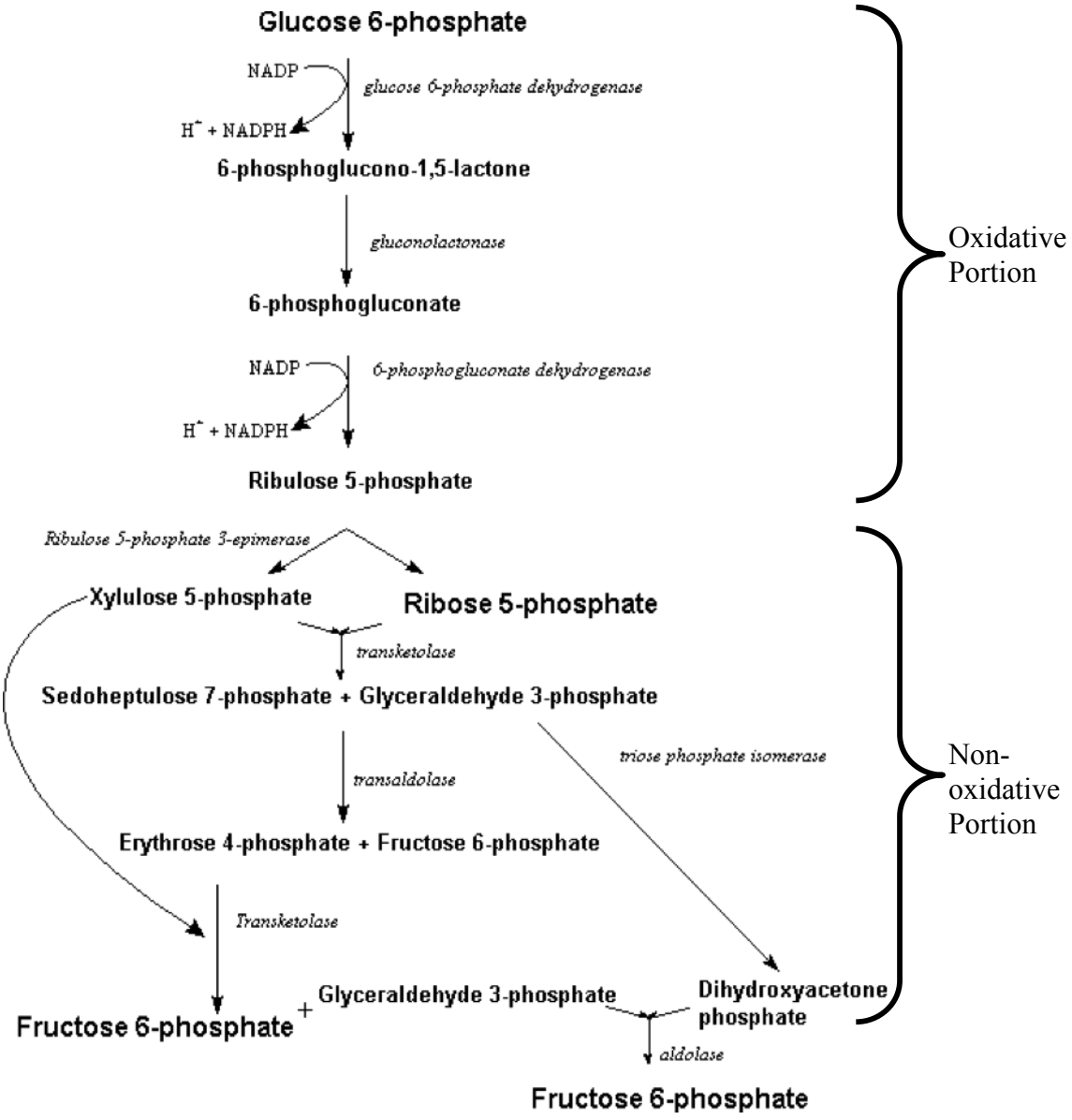


Figure 1.2. Pentose phosphate pathway. Glucose 6-phosphate enters the pentose phosphate pathway and shuttles through the oxidative phase, ultimately producing NADPH. The reactions in the non-oxidative portion are reversible. Transketolase and transaldolase function to convert by products of the pathway into substrates for glycolysis, linking the pentose phosphate pathway with glycolysis. (Figure taken from www.uic.edu).

1.1.2. Metabolic Stress

Physiological stresses can induce alterations in cellular metabolism aiding in cellular adaptation and survival. The pathways that make up cellular metabolism are dynamic and the cell can exploit different pathways to benefit the state of the cell or aid in cell survival. For instance, hypoxia as a result of stroke, tumors, sleep apnea or neonatal complications during pregnancy have been reported to induce a shift in cellular metabolism towards a reliance on glycolysis (Silverstein and Johnston 1984, Graham *et al.* 1992, Kamba *et al.* 1997, Kim *et al.* 2007). Changes in diet such as caloric restriction without malnutrition have been suggested to cause a metabolic shift toward increased biosynthesis of fatty acids, nucleotide precursors, and increased protein synthesis (Lee *et al.* 1999). In addition, minor conditions such as the presence of a fever can cause alterations in cellular metabolism in order to support increased energy expenditure during the immune response (Romanyukha *et al.* 2006).

1.1.3. Cellular Responses to Metabolic Stress

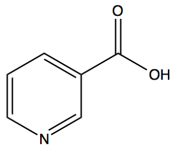
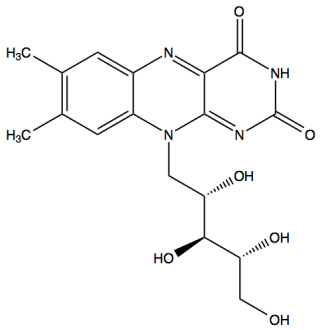
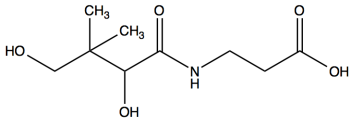
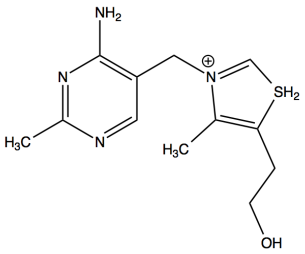
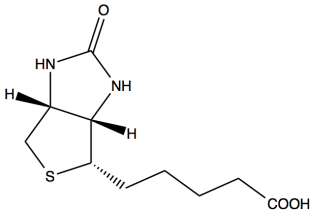
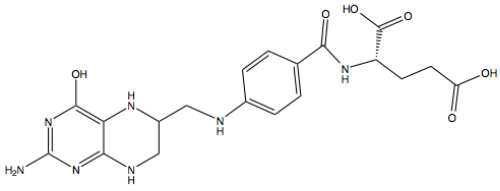
Metabolic programming in response to physiological stressors commonly affects genes involved in angiogenesis, cell survival, glucose metabolism and invasion. It is important to note that multiple genes are commonly affected to adapt to different physiological stresses. Exposure to low oxygen conditions, termed hypoxia, is a common stress that results in multiple gene expression changes. The glucose transporter GLUT1 is upregulated under hypoxic conditions increasing glucose uptake to support a glycolytic shift in metabolism (Bashan *et al.* 1992). Under low oxygen conditions, increases in the expression of pyruvate dehydrogenase kinase 1 (PDK1) results in the phosphorylation

and inhibition of pyruvate dehydrogenase (PDH) (Kim *et al.* 2006). Inactivation of PDH blocks the conversion of pyruvate to acetyl-CoA, which is required for entry into the TCA cycle. The build up of pyruvate can be inter-converted to lactate through the induction of lactate dehydrogenase A (LDHA) followed by removal from the cell through an increased expression of the monocarboxylate transporter (MCT4) (Koukourakis *et al.* 2006, Ullah *et al.* 2006).

1.1.4. Micronutrients

Coordinated supply of nutrients, such as glucose and micronutrients functioning as enzyme cofactors is required to support cellular metabolism. An enzyme cofactor (coenzyme) is the non-protein component of an enzyme that is required for an enzymes biological activity. The majority of coenzymes are water-soluble vitamins such as niacin, folic acid, pantothenic acid, pyridoxine, biotin, riboflavin and thiamine (Table 1.1). In general, vitamins are small organic molecules that are essential for normal cellular growth and function. Vitamins function in a wide range of biological activities, playing an integral role in maintaining cellular homeostasis. They are involved in many facets of metabolism including fatty acid synthesis (biotin), methyltransferase reactions (vitamin B12), and precursors for activated electron carriers (niacin for NAD, riboflavin for FAD) (Decker and Byerrum 1954, Wakil and Gibson 1960, Nishizuka and Hayaishi 1963, Weissbach and Taylor 1970). Folate and biotin are also reported to contribute to gene silencing through their involvement in DNA methylation and biotinylation of histones, respectively (Oommen *et al.* 2005).

Table 1.1. Essential vitamins and their role as coenzymes.

Vitamin	Structure	Coenzyme	Function
Niacin		NAD+	Oxidation or hydrogen transfer
Riboflavin		Flavin adenine dinucleotide (FAD)	Oxidation or hydrogen transfer
Pantothenic acid		Coenzyme A (CoA)	Acetyl group carrier
Thiamine		Thiamine pyro-phosphate (TPP)	Aldehyde group transfer
Biotin		D-(+)-Biotin	Carbon dioxide transfer
Folic Acid		Tetra-hydrofolate	One carbon transfer

Most vitamins have a recommended daily allowance (RDA) in the United States decided by the Food and Drug Administration. The general public commonly utilizes multivitamins to support optimal ingestion of nutrients. Supplementation of vitamins has also been used in the treatment of various pathologies. Niacin supplementation has been shown to be helpful for the management of high cholesterol and folic acid for cardiovascular disease (Jacobsen 1998, Yvan-Charvet *et al.* 2010, Jardine *et al.* 2012). The vitamins C, E, and β-carotene are well known for their antioxidant roles (Burton and Ingold 1981, Doba *et al.* 1985, Sies *et al.* 1992).

Alterations in the requirement for micronutrients are common during physiological stresses. Pregnancy requires an altered demand for micronutrients that are critical for normal embryonic development. Folate supplementation is especially important to support nucleic acid synthesis during fetal development, which involves a high level of cell division (Scholl and Johnson 2000). Suboptimal folate levels during gestation have been linked to neural tube defects and impaired cellular growth of the fetus and placenta (Scholl and Johnson 2000, Szostak-Wegierek 2000). Alterations in micronutrient requirements during strenuous physical activity are also common. In order to support the increased energy requirement of cells during exercise, maintaining optimal levels of antioxidant nutrients such as vitamins A and C is important (Maughan 1999). Iron has many physiological functions, including a role in the transport of oxygen, which is important for increased oxygen delivery to muscles during exercise (Maughan 1999). An alteration in the requirement of micronutrients during physiological stresses may also require adaptive responses in transport and activation mechanisms. However, very little is

known about the regulatory mechanisms controlling micronutrient availability and activation.

In particular, vitamin B1 (thiamine) is an essential cofactor for enzymes involved in glycolysis, the citric acid cycle and the non-oxidative portion of the pentose phosphate pathway (Horecker and Smyrniotis 1953, Racker *et al.* 1953, Park and Gubler 1969). Given thiamine's integral involvement in cellular metabolism, our research focuses on thiamine homeostasis and the adaptive regulation of thiamine transporters under physiological stresses such as hypoxia and nutrient deprivation.

1.2. THIAMINE, VITAMIN B1

1.2.1. Thiamine

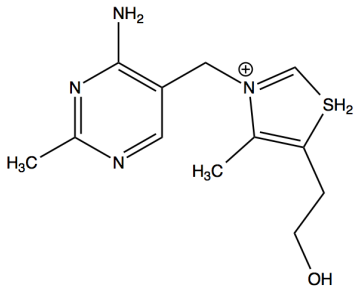
Thiamine is classified as an essential water-soluble vitamin used by all living organisms to support cellular metabolism. It is often referred to as the "thio-vitamin" due to its structure containing sulfur ($C_{12}H_{17}N_4OS$) depicted in Figure 1.3A. Structurally, thiamine is composed of a thiazole and pyrimidine ring joined together by a methylene bridge. In addition to the presence of a quaternary nitrogen, the thiazole ring contains both methyl and hydroxyethyl side chains. Due to its hydrophilicity, thiamine is soluble in polar solvents such as water, methanol and glycerol.

1.2.2. Discovery and history

Pathologies pertaining to overall poor nutrition were first documented in the 1880's, however, vitamins were unknown substances at that time. In the early 1900's observations made by Christiaan Eijkman, a military doctor in the Dutch Indies, lead to

the discovery of vitamins awarding him the Nobel Prize in Physiology and Medicine in 1929. Thiamine was the first water-soluble vitamin isolated and crystallized by two Dutch chemists Drs. Jansen and Donath in 1926. It was described as a "yellowish white powder with a salty, slightly nutty taste" and was given the name aneurin (anti-neuritic) vitamin due to the detrimental neurological defects caused by its deficiency (Carpenter 2000). It was not until 1934 that the United States chemist Dr. Williams determined its structure and renamed the vitamin to thiamine (Williams *et al.* 1934).

A.



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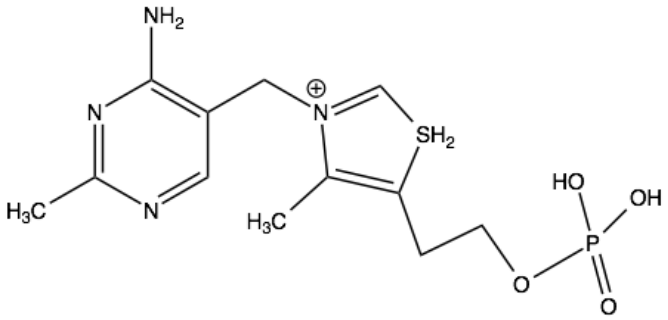

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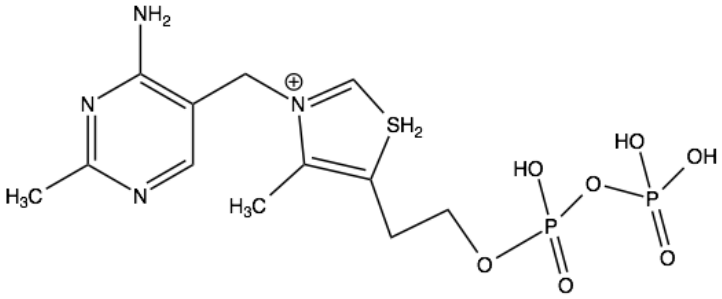

D.

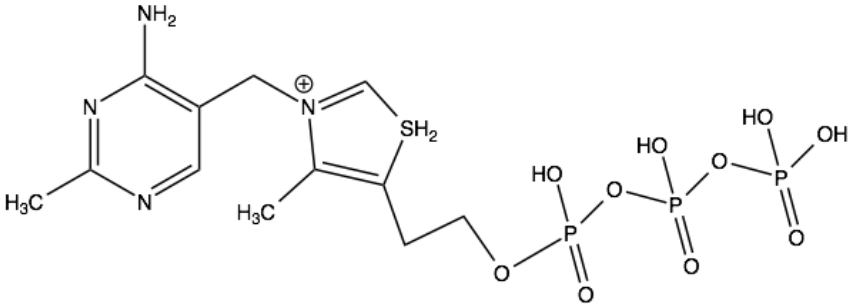

Figure 1.3. Structures of thiamine. (A) Thiamine, (B) thiamine monophosphate, (C) thiamine diphosphate, and (D) thiamine triphosphate.

1.2.3. Sources

Thiamine is an essential vitamin and is not synthesized by mammals; therefore, it must be obtained from exogenous sources. The two main sources of thiamine are through dietary intake and bacterial production (Sriram *et al.* 2012).

1.2.3.1. Dietary intake

Mammals and other complex eukaryotes lack thiamine biosynthetic genes and therefore, it is obligatory that thiamine be obtained from dietary sources. Thiamine is found naturally in many foods including breads, fish, meat, eggs, legumes and milk, as well as being used in the fortification of many processed foods (Table 1.2). For example, more thiamine is found in whole grains than in refined grains and therefore it is often supplemented in processed flour.

The recommended daily intake (RDI) of thiamine differs slightly based on gender and age (Table 1.2) but generally the RDI for adult men and women is approximately 1 mg/day (Pitkin *et al.* 2000). No adverse effects were reported in healthy patients who were given 1.2 mg/day and no intolerable upper intake level has been established (Smithline *et al.* 2012). However, in patients given doses up to 7.5 mg/day minor side effects such as nausea and indigestion have been reported (Meador *et al.* 1993). Many over-the-counter vitamin supplements contain a significantly large quantity of thiamine representing 100 to 6,600% of the RDI (Table 1.2).

Table 1.2: Amount and percent daily value (DV) of thiamine found in food and supplements

Dietary Source	Thiamine (mg)	% DV**
Natural*		
Pork, fresh (3 oz)	0.6	40
Fish (1/2 fillet)	0.3	20
Black Beans (1 cup)	0.4	27
Lima Beans (1 cup)	0.3	20
Potatoes (1 cup)	0.3	20
Okra (1 cup)	0.2	13
Chicken (1 cup)	0.2	13
Peas (1 cup)	0.2	13
Sunflower seeds (1 cup)	0.7	47
Pistachios (1 oz)	0.2	13
Pecans	0.2	13
Fortified*		
General Mills, Total Raisin Bran (1 cup)	1.6	107
General Mills, Total Corn Flakes (1.3 cups)	1.5	100
Bread crumbs (1 cup)	1.2	80
White Rice (1 cup)	1.1	73
Sandwich, with cold cuts (6" sandwich)	1.0	67
Cornmeal (1 cup)	0.9	60
Supplements		
Centrum		
Adult	1.5	100
Child (≥ 4yrs)	1.5	100
One A Day		
Women's 50+	4.5	300
Women	1.5	100
Girl Teen	2.3	153
Men's 50+	4.5	300
Men	1.2	80
Solaray		
Boy Teen	3.8	250
B Complex	7.5	500
Nature's Way		
Vitamin B1	100	6667
Nature Made		
Vegan B Complex	25	1667
B Complex	15	1000

*Values obtained from USDA **The % DV is the percentage of the RDI that each food/supplement contains. The RDI is determined based on the estimated average requirement of thiamine in that only a small percentage (~2-3%) of individuals may experience deficiency (Pitkin *et al.* 2000).

1.2.3.2. Bacterial Production

Thiamine biosynthesis occurs in bacteria, plants, fungi and some protozoans (Webb *et al.* 2007, Begley *et al.* 2008). Bacteria express thiamine biosynthetic genes allowing production and utilization of thiamine when thiamine cannot be obtained externally. The thiazole and pyrimidine rings are individually synthesized and then combined by thiamine-phosphate synthase to form thiamine monophosphate (TMP) (Figure 1.3B). While the biosynthetic pathways are slightly different between organisms, most bacteria and some eukaryotes form thiamine through the hydrolysis of TMP.

When thiamine is present in the growth medium of bacteria, it is imported from the environment resulting in the repression of thiamine biosynthetic genes. In microbes, thiamine plays a regulatory role in the intracellular uptake and expression of biosynthetic genes through riboswitches (Maundrell 1990, Nishimura *et al.* 1992, Praekelt *et al.* 1994, Webb *et al.* 1996). Thiamine biosynthesis is regulated through the binding of thiamine or thiamine derivatives directly to the mRNA encoding genes of the biosynthetic pathway, blocking their translation to enzymes (Bocobza and Aharoni 2008). These complexes create distinct structures that sequester ribosome-binding sites under conditions of sufficient or excess thiamine, resulting in negative feedback regulation for thiamine biosynthesis (Winkler *et al.* 2002).

A recent study has shown the human gut microbiota can be classified into three different enterotypes (1, 2 and 3) (Arumugam *et al.* 2011). Enterotypes are primarily driven by the composition of microbiota species and cannot be explained by geographical region, body mass, dietary intake, age or gender. Both free thiamine and the diphosphorylated form of thiamine (TPP) are produced by the microbiota of the large

intestine (Guerrant and Dutcher 1932, Najjar and Holt Jr 1943, Arumugam *et al.* 2011). Enterotype 2, which is enriched in *Prevotella* and *Desulfovibrio*, characteristically contains enzymes that are involved in the biosynthesis of thiamine pyrophosphate (TPP). It was previously assumed that the microbiota generated TPP was not imported by human colonocytes due to its size and charge and the fact that colonocytes have little or no surface alkaline phosphatase activity for conversion to thiamine (Czernobilsky and Tsou 1968, Barrow *et al.* 1989). However, Nabokina, et al. have recently identified a colonic uptake system for TPP in human colonocytes, suggesting TPP generated by microbiota can be absorbed and may participate in host thiamine homeostasis (Nabokina *et al.* 2013).

1.3. ABSORPTION

With exogenous sources of thiamine being vital for cell maintenance, intestinal absorption plays a critical role in maintaining thiamine homeostasis in the body. The quaternary nitrogen and overall hydrophilicity of thiamine necessitates a carrier-mediated process for absorption and cellular uptake. Two solute carrier transporters have been shown to be involved in the intracellular uptake of thiamine, SLC19A2 and SLC19A3 (Figure 1.4) (Fleming *et al.* 1999, Eudy *et al.* 2000). Another transporter, SLC25A19 has been shown to be involved in the transport of diphosphorylated thiamine across the mitochondrial membrane (Kang and Samuels 2008). Intestinal absorption of thiamine has also been described to occur by passive diffusion mechanisms at high concentrations and via members of the organic cation transporter family (Hoyumpa 1982, Zielinska-Dawidziak *et al.* 2008, Lemos *et al.* 2012).

1.3.1. SLC19 family

There are three members that constitute the SLC19 family: a folate transporter (SLC19A1, RFC1), and two thiamine transporters (SLC19A2, THTR1 and SLC19A3, THTR2) (Ganapathy *et al.* 2004). RFC1 is overexpressed in some cancers and has been reported to transport methotrexate, an antineoplastic and immunosuppressive agent, in addition to reduced folate (Dixon *et al.* 1994, Odin *et al.* 2003). Therefore, RFC1 has received significant attention as a potential target to enhance cellular uptake of chemotherapeutic agents into cells.

Sequence homology of the proteins encoded by the SLC19A family is shown in Figure 1.4A with RFC1 and THTR1 being 39% similar, RFC1 and THTR2 are 42% similar and THTR1 and THTR2 are 53% similar (Ganapathy *et al.* 2004). Although all three transporters share a high degree of amino acid sequence similarity, they have high substrate specificity. RFC1 does not transport thiamine and THTR1/2 has not been shown to transport reduced folate or other organic anions (Rajgopal *et al.* 2001, Subramanian *et al.* 2003). RFC1 transports substrates existing as anions at physiological pH while substrates for THTR1 and THTR2 exist as cations at physiological pH. The main functional similarity shared by all SLC19A family members is their pH responsiveness (Dutta *et al.* 1999, Rajgopal *et al.* 2001, Rajgopal *et al.* 2001). However, RFC1 has a higher transport capacity for its substrates at a low pH (5.5) while both THTR1 and THTR2 have a high thiamine transport capacity at pH's of 8 and 7.5, respectively (Dutta *et al.* 1999, Rajgopal *et al.* 2001, Rajgopal *et al.* 2001). The transport mechanism for folate influx through RFC1 is stimulated by an inwardly directed H^+ gradient with folate/H^+ symport or folate/OH^- antiport.

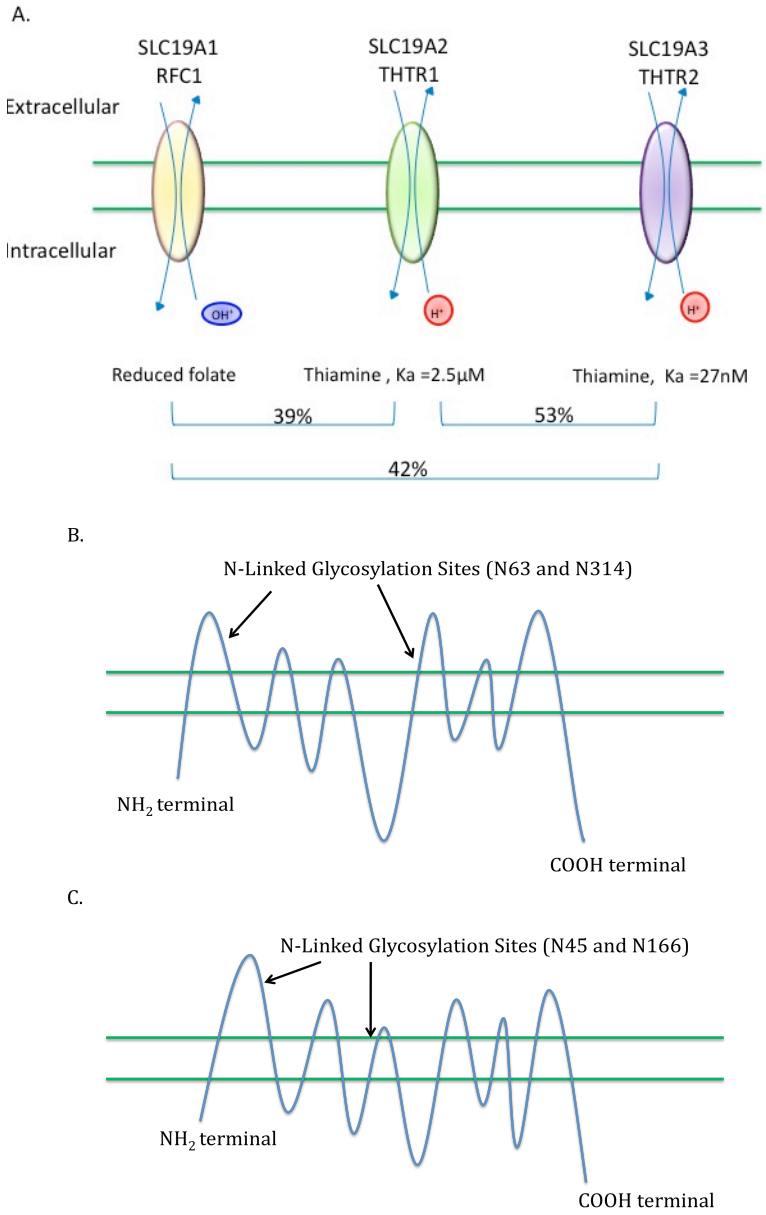

Figure 1.4. SLC19A2 and SLC19A3. (A) Members of the SLC19 family. Percentages represent sequence homology between the transporters. (B) SLC19A2 containing 12 transmembrane domains, 2 N-linked glycosylation sites and cytosolic NH_2 and COOH terminal ends. (C) SLC19A3 containing 12 transmembrane domains, 2 N-linked glycosylation sites and cytosolic NH_2 and COOH terminal ends.

Alternatively, both THTR1 and THTR2 transport thiamine by an outwardly directed H^+ gradient with thiamine/H^+ as their transport mechanisms (Figure 1.4A).

The transporters of the SLC19 family are ubiquitously expressed with differing levels dependent on tissue type. The expression of these transporters is generally high in absorptive tissues such as the intestine, kidney and placenta (Said *et al.* 1999, Said *et al.* 2004). All three transporters are expressed at the plasma membrane with RFC1 also expressed in the mitochondrial membrane (Marchant *et al.* 2002, Boulware *et al.* 2003, Subramanian *et al.* 2003).

Knockout mice models have been created using a C57Bl/6 background for SLC19A2 and SLC19A3. The SLC19A2$^{-/-}$ mice (THTR1-deficient) exhibit a generally nor mal phenotype aside from male infertility. However, on a thiamine free diet mice exhibit premature death, sensorineural deafness, megaloblastosis and diabetes mellitus with reduced insulin secretion (Oishi *et al.* 2002). SLC19A3$^{-/-}$ mice exhibit a similar phenotype in addition to demonstrating impaired thiamine uptake, lethargy, cachexia, hepatic necrosis, liver and kidney inflammation, and nephrosclerosis (Reidling *et al.* 2010).

1.3.1.1. SLC19A2

The thiamine transporter SLC19A2 is located on human chromosome 1q23 and the gene is 22,095 bases (Reidling *et al.* 2002). The 5'-regulatory region has been cloned with identification of its minimal promoter region reported to be -149 to +15 with +1 indicating the location of the transcriptional start site (Subramanian *et al.* 2003). Multiple putative *cis*-regulatory elements were reported within the minimal promoter region with a

role for gut-enriched Krupple-like factor (GKLF), nuclear factor-1 (NF1) and stimulating protein-1 (SP1) (Reidling and Said 2005).

The SLC19A2 gene encodes a 497 amino acid protein (THTR1) containing 12 transmembrane domains (Figure 1.4B) (Ganapathy *et al.* 2004). THTR1 is localized to the plasma membrane and relies on intact microtubules for intracellular trafficking (Subramanian *et al.* 2003). THTR1 protein contains two known N-glycosylation sites, which are involved in membrane targeting but not in transport function (Figure 1.4B) (Diaz *et al.* 1999, Dutta *et al.* 1999, Fleming *et al.* 1999, Labay *et al.* 1999, Balamurugan and Said 2002). Studies have shown THTR1 expression at both the apical and basolateral membrane domains in polarized epithelial cells (Subramanian *et al.* 2003). In blood brain barrier endothelial cells, THTR1 is exclusively localized to the luminal/blood side (Kevelam *et al.* 2013). THTR1 is a high capacity transporter that specifically transports thiamine with low affinity (Km= 2.5μM) (Dutta *et al.* 1999) (Liu *et al.* 2003).

1.3.1.2. SLC19A3

The SLC19A3 gene is located on human chromosome 2q37 and is 32,820 bases in size. The 5'-regulatory region of the SLC19A3 gene is 2,016 bp and its minimal promoter region is reported to be -77 to +59 with +1 indicating the location of the transcriptional start site (Nabokina and Said 2004). Within the minimal promoter region, a number of putative *cis*-regulatory elements have been reported with a critical role for SP1 (Nabokina and Said 2004).

The gene encodes a 496 amino acid protein serving as a second thiamine transporter, THTR2. THTR2 contains 12 transmembrane domains with two N-

glycosylation sites (Figure 1.4C). Thiamine transporter localization has been extensively studied with an emphasis on intestinal and renal epithelial cells due to the importance of acquiring thiamine from the diet. THTR2 is primarily localized in the plasma membrane and is reported to be restricted to the luminal apical side of the membrane in polarized epithelial cells (Boulware *et al.* 2003, Said *et al.* 2004, Ashokkumar *et al.* 2006). THTR2 is a low capacity transporter that specifically transports thiamine with high affinity (Km=27 nM) (Dutta *et al.* 1999) (Liu *et al.* 2003).

1.3.2. Mitochondrial transporter (SLC25A19)

The SLC25A19 gene is located on chromosome 17q25.3 and is 16,500 bases in size. Three mRNA splice variants differing in their 5' ends have been identified. The 5'-regulatory region of the gene is 1,080 bp and its minimal promoter region is reported to be -131 to +20 with +1 indicating the location of the transcriptional start site (Nabokina *et al.* 2013). Within the minimal promoter region, there is an essential role for nuclear factor Y (NF-Y) in controlling SLC25A19 basal activity (Nabokina *et al.* 2013).

SLC25A19 encodes a 320 amino acid protein that functions as a TPP transporter. SLC25A19 (DNC, TPC, MUP1, MCPHA, THMD3, THMD4) was initially reported as a mitochondrial deoxynucleotide carrier responsible for the uptake of deoxynucleotides into the matrix of the mitochondria (Dolce *et al.* 2001). However, homology comparisons to yeast TPP transporter and transport assays have clearly identified TPC as a TPP transporter, not a deoxynucleotide carrier (Lindhurst *et al.* 2006, Kang and Samuels 2008).

1.3.3. TPP transport (SLC44A4)

SLC44A4 is most commonly located on chromosome 6p21.3 and is 15,855 bases in size. The SLC44A4 gene encodes a 710 amino acid protein (hTTPT) that was recently identified as the human colonic thiamine pyrophosphate transporter (Nabokina *et al.* 2013). Uptake of TPP was reported to be temperature- and energy-dependent, and Na^{+-} independent. hTTPT is highly specific for TPP with an apparent Km of 0.17 μM. hTPPT is expressed in the colon but not the small intestine, however, this finding is very recent and more work needs to be done to fully elucidate tissue localization of this new TTP transporter.

1.4. PHYSIOLOGICAL FUNCTION OF THIAMINE

1.4.1. Activation

1.4.1.1. Biologically active forms: TMP, TPP, TTP, adenosine triphosphate

Upon transport into the cell, thiamine is converted by thiamine pyrophosphokinase-1 (TPK1) to the active co-enzyme thiamine pyrophosphate (diphosphate) (TPP) (Figure 1.5) (Nosaka *et al.* 2001). Phosphorylation of thiamine by TPK1 has been shown to be a significant driving force for thiamine uptake along with binding to apo-enzymes (Bettendorff *et al.* 1995). Human TPK1 exists as a homodimer and is expressed ubiquitously with high levels in the kidney, small intestine, and testis (Bettendorff *et al.* 1995, Bellyei *et al.* 2005). In addition to TPP, three other phosphorylated forms have been observed intracellularly in humans, thiamine monophosphate (TMP), thiamine triphosphate (TTP), and adenosine thiamine triphosphate (AThTP) (Gangolf *et al.* 2010).

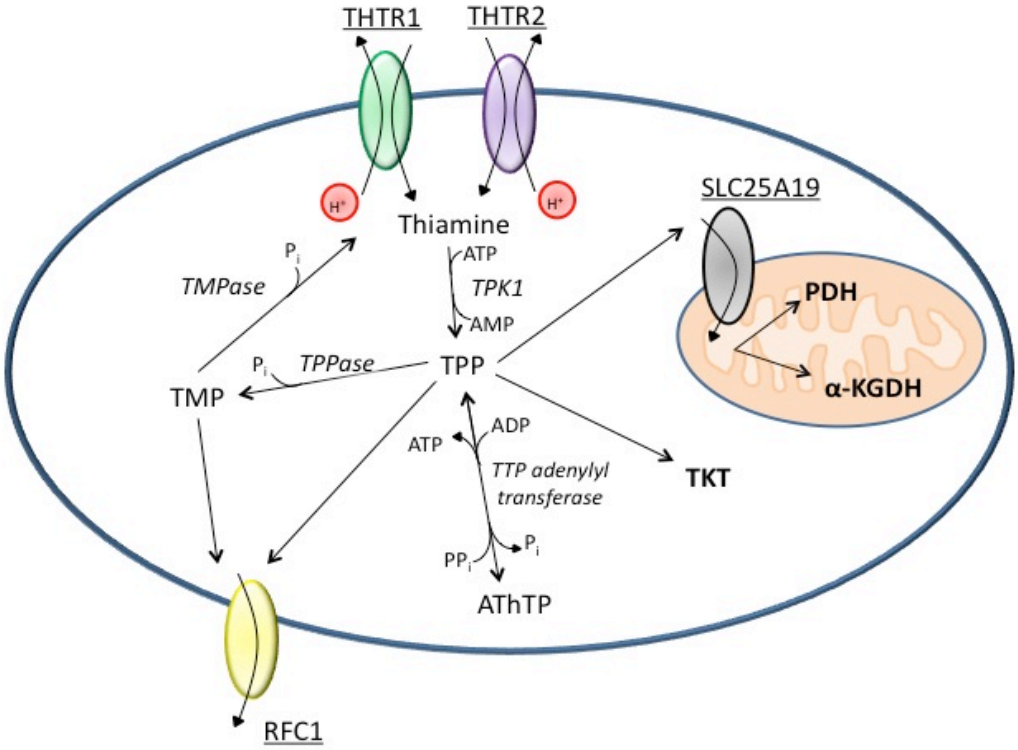

Figure 1.5. Activation of thiamine once transported into the cell by THTR1 and THTR2. Thiamine is activated to TPP by TPK1. TPP can then be transported into the mitochondria by SLC25A19 to serve as a coenzyme for PDH and α-KGDH, used as a coenzyme for TKT, be converted to AThTP by TTP adenylyl transferase, be exported from the cell through RFC1 or be converted to TMP by TPPase. TMP can be converted back to free thiamine through TMPase or transported out of the cell by RFC1.

Although the physiological functions of TMP, TTP, and AThTP have not been ascertained, TPP is the only known thiamine phosphorylate functioning as an enzyme cofactor. Figure 1.5 shows the activation pathways of thiamine once it is transported into the cell. TPP can be hydrolyzed by thiamine pyro-phosphatase (TPPase) to TMP, which is then hydrolyzed to form thiamine via thiamine monophosphatase (TMPase) (Rindi and Laforenza 2000). RFC1 has been shown to efflux the mono and diphosphate ester of thiamine (Zhao *et al.* 2001). It is unclear if the dephosphorylation and efflux of thiamine phosphorylates is to regulate intracellular thiamine levels to control cofactor and non-cofactor functions of thiamine phosphorylates.

1.4.1.2. Thiamine distribution

It is hypothesized that humans are highly sensitive to thiamine deficiency due to extremely limited thiamine storage ability compared to other mammals (Gangolf *et al.* 2010). Relative tissue levels of thiamine and its derivatives have a high inter-patient variability, which is thought to result from external factors, such as nutrition, disease, and age, as well as lifestyle (Gangolf *et al.* 2010). Distribution of thiamine and its derivatives differs between organs with the greatest concentrations in skeletal muscle, heart, brain, liver and kidneys (Gangolf *et al.* 2010). The concentration of thiamine in human plasma ranges from 10-20 nM with reports of only free thiamine and TMP in plasma while TPP and TTP are primarily found within erythrocytes (Kimura and Itokawa 1983).

1.5. THIAMINE DEPENDENT ENZYMES

Thiamine is critical for the activity of four key enzymes in cellular metabolism, pyruvate dehydrogenase (PDH), alpha-ketoglutarate dehydrogenase (α-KGDH) in the tricarboxylic acid (TCA) cycle, transketolase (TKT) within the pentose phosphate pathway (PPP), and branched chain alpha-keto acid dehydrogenase complex (BCKDC) involved in amino acid catabolism.

1.5.1. Transketolase (TKT)

TKT is the rate-limiting step of the non-oxidative branch of the PPP, is central to the oxidative branch, and is critical in the interchange of metabolites between glycolysis and the pentose shunt (Figure 1.6) (Novello and McLean 1968, Kauffman 1972). Glucose 6-phosphate enters the PPP and is converted to the nucleotide pentose sugar R5P through the non-thiamine-dependent oxidative branch. If not utilized for *de novo* nucleotide synthesis, R5P continues into the non-oxidative branch of the PPP where TKT ultimately converts R5P into fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate (G3P), which re-enters the Embden-Meyerhoff pathway. This creates a reversible link between glycolysis and the PPP through the action of transketolase and transaldolase catalyzing three successive reactions. Ultimately, excess R5P formed in the PPP or any ribose obtained through the diet can be converted into glycolytic intermediates (Berg *et al.* 2002).

Although the TKT reaction is generally considered to be the result of a single TKT gene, there are two additional TKT isoforms found in the human genome, termed TKT-like 1 (TKTL1) and TKT-like 2 (TKTL2). Homology comparisons of TKTL1 with

TKT have noted a deletion of 38 amino acids within the cofactor and catalytic domain, suggesting that TKTL1 is incapable of binding to TPP and carrying out the TKT reaction (Mitschke *et al.* 2010, Maslova *et al.* 2012). Schneider *et al.* engineered a 38 amino acid deletion pseudo-TKTL1 (TKTΔ38) mutant from TKT as a model of TKTL1 to elucidate this issue (Schneider *et al.* 2012). No conventional TKT activity of the TKTΔ38 mutant was detectable using a coupled spectrophotometric assay for the conversion of known physiological substrates. Moreover, circular dichroism and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy indicated that the TKTΔ38 mutant had no associated TPP. Using the same TKTΔ38 mutant system, Meshalkina *et al.* confirmed the lack of TKT activity in solution and were unable to detect TPP after acid or heat denaturation extraction methods (Meshalkina *et al.* 2012). Since TKT functions as a homodimer, it is unclear if the TKTΔ38 mutation alters dimer formation. Even if TKTL1 is enzymatically inactive as a homodimer, the expression of TKTL1 in mammalian systems may influence overall TKT activity through heterodimer formation with other TKT isoforms. The lack of TPP binding to the TKTΔ38 mutant does not preclude other thiamine derivatives from binding. The diphosphate group added to thiamine does not participate in the catalytic activity and functions primarily to anchor the cofactor into the apo-enzyme. Other thiamine derivatives with unknown function have been found intracellularly and they may be able to bind within the condensed catalytic site of TKTL1 (Gangolf *et al.* 2010). Thus, further work is needed to fully understand the biochemistry of TKTL1 in mammalian systems.

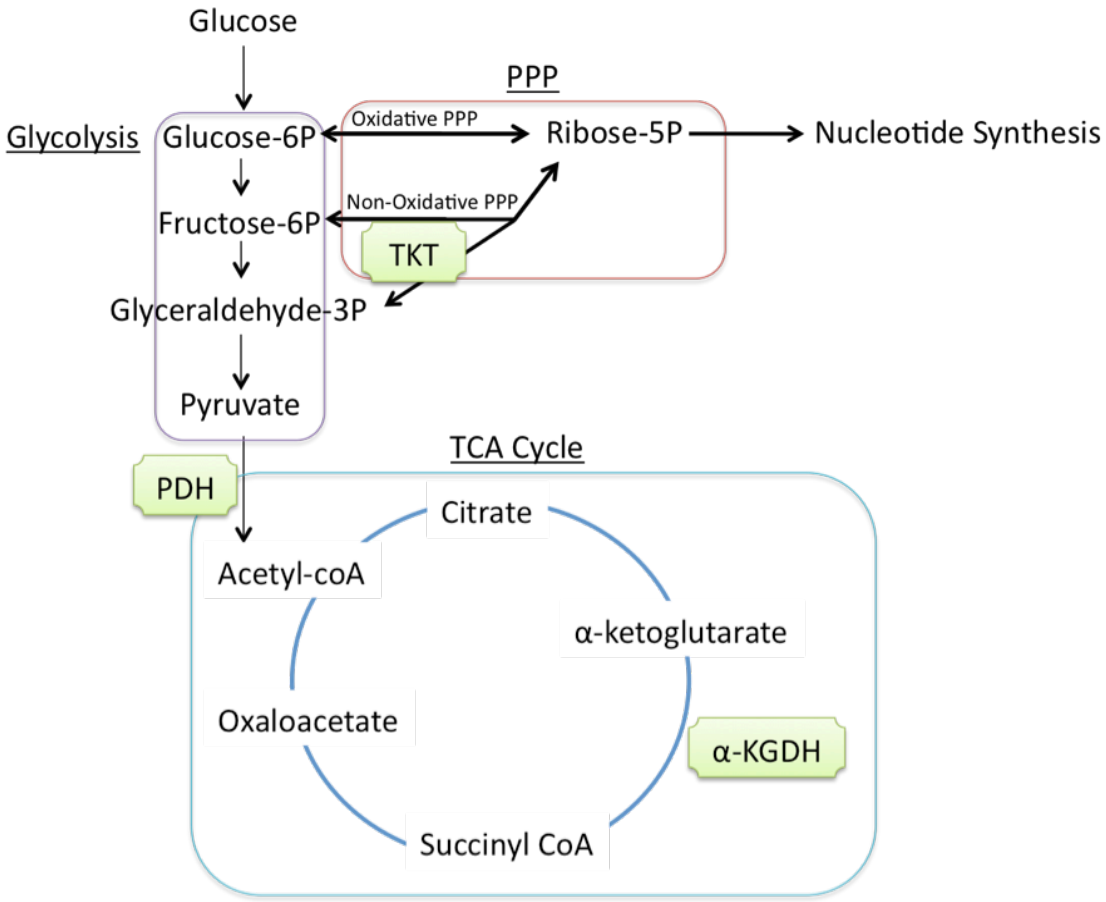

Figure 1.6. Involvement of thiamine in cellular metabolism. The activated form of thiamine, TPP, can be used as a cofactor for enzymes involved the TCA cycle (PDH) and (α-KGDH) and the non-oxidative portion of the pentose phosphate pathway PPP (TKT).

1.5.2. Pyruvate Dehydrogenase (PDH)

The conversion of pyruvate to acetyl-CoA takes place through a series of reactions mediated by the thiamine dependent enzyme PDH (Figure 1.6). Located within the mitochondrial matrix, the pyruvate dehydrogenase complex (PDC) is a multi-component enzyme complex consisting of three subunits (E1, E2, and E3).

E1 has two catalytic sites requiring TPP and Mg^{2+} as cofactors and is activated by the formation of a hydrogen bond between TPP and a glutamate residue in PDH. This leads to the formation of a reactive C2-carbanion. The TPP bound within PDH undergoes an electrophilic addition with the ketone of pyruvate. The resulting intermediate, β-alkoxide, contains a hydroxyethyl derivative covalently bound to TPP that serves to decarboxylate pyruvate. The resulting enol forms a stabilized 1,3-dipole with a positively charged nitrogen atom and undergoes a reductive acetylation with lipoamide-E2 (E2). Lastly, the remaining acetyl group is transferred to CoA, forming acetyl-CoA. The decarboxylation and reductive acetylation is considered to be the rate-limiting step for the conversion of pyruvate to acetyl-CoA.

As a result of its location at the junction between glycolysis and the TCA cycle, PDH activity functions as a critical gatekeeper for the continued metabolism of glucose (Figure 1.6). The activity of PDH is tightly regulated through phosphorylation by PDH kinase (PDK) at three sites on the E1 alpha subunit: Ser^{232}, Ser^{293}, Ser^{300} (Patel and Roche 1990). PDK is a family of four isoenzymes (PDK1, 2, 3 and 4) that function to inhibit PDH activity through ATP-dependent phosphorylation (Kolobova *et al.* 2001, Korotchkina and Patel 2001, Holness and Sugden 2003). Phosphorylation at either serine site results in inhibition of the complex in vitro (Korotchkina and Patel 1995). Regulation

of PDK activity is 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 (Roche and Hiromasa 2007). Another regulator of PDH phosphorylation is the thiamine cofactor TPP, which when bound to PDH, reduces the rate and extent of PDK-mediated phosphorylation (Kolobova *et al.* 2001). PDC is subject to a continuous cycle of phosphorylation/dephosphorylation allowing for tight regulation of its activity. PDH phosphatase is a heterodimer consisting of catalytic and regulatory subunits, serving to reverse the phosphorylation of the E1 subunit rendering it active. PDH phosphatase is competitively inhibited by acetyl-CoA, ATP and NADH while stimulated by insulin, PEP and AMP (Pettit *et al.* 1975, Newman *et al.* 1985).

1.5.3. Alpha Ketoglutarate Dehydrogenase (α-KGDH)

α-KGDH complex consists of three subunits: a thiamine pyrophosphate-dependent dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) (Koike *et al.* 1974, Koike and Koike 1976). While the E1 and E2 subunits are unique to α-KGDH, E3 is identical to that in PDC (Perham 1991, Berg and De Kok 1997). In addition, the reaction catalyzed by α-KGDH is chemically similar to that of PDC resulting in the production of NADH and CO_2.

Located in the mitochondria, α-KGDH acts as a site of control through the TCA cycle and is responsible for the conversion of α-KG and NAD+ to succinyl-CoA and NADH (Figures 1.1 and 1.6). As with most enzymes involved in the TCA cycle, α-KGDH is highly regulated and feedback inhibition of α-KGDH is catalyzed by the end products of the reaction (Garland 1964, Smith *et al.* 1974, Lawlis and Roche 1981). Other

points of regulation for α-KGDH include calcium and the ATP/ADP ratio. Micromolar levels of calcium and ADP were found to lower the Km while ATP increased the Km for α-ketoglutarate (McCormack and Denton 1979, Denton and McCormack 1980).

In the oxidative direction, glutamine derived α-KG entering the TCA cycle is a key carbon backbone for the synthesis of amino acids and nucleotides (DeBerardinis *et al.* 2007). Alternatively, glutamine derived α-KG can undergo reductive decarboxylation through the TCA cycle, forming citrate for lipid biosynthesis (Metallo *et al.* 2011, Mullen *et al.* 2011). This reverse TCA carbon flow would bypass the requirement for α-KGDH activity, allowing the cell to utilize α-KG unabated by changes in thiamine availability. It is unclear how α-KGDH activity is regulated to allow α-KG to move in the reverse direction but it is suggested to be triggered during hypoxic stress (Metallo *et al.* 2011, Wise *et al.* 2011).

1.5.4. Branched chain α-ketoacid dehydrogenase complex (BCKDC)

Valine, isoleucine, and leucine are essential branched chain amino acids (BCAAs) that can serve as an energy source as well as precursors for amino acid and protein synthesis (Harper *et al.* 1984, Platell *et al.* 2000). The metabolism of BCAAs involves transamination to the α-keto acid followed by irreversible oxidative decarboxylation by the thiamine-dependent BCKDC to form an acyl-CoA derivative (Harper *et al.* 1984). The continued catabolic breakdown of BCAA produces acetyl-CoA (from leucine) and succinyl-CoA (from valine and isoleucine) that enter the TCA cycle (Harper *et al.* 1984, Harris *et al.* 2005). BCKDC is a multi-component enzyme consisting of three subunits (E1, E2, and E3) and is located in the mitochondria (Harper *et al.* 1984). Similar to PDH,

BCKDC activity is regulated through reversible phosphorylation by branched-chain α-keto acid dehydrogenase kinase (BDK) and phosphatase (BDP) (Harris *et al.* 2004).

Loss-of-function mutations and BCKDC deficiencies are associated with accumulation of neurotoxic α-keto acids, referred to as maple syrup urine disease that is characterized by a sweet urine odor (Chuang *et al.* 2006). In hypermetabolic states such as exercise, sepsis, trauma, and cancer, the release of BCAAs from muscle protein provides a pool of amino acids for the synthesis of priority proteins and/or an important source of oxidative energy (Shimomura *et al.* 2004, Baracos and Mackenzie 2006, De Bandt and Cynober 2006).

1.6. THIAMINE DEFICIENCY

Mammals lack the ability to synthesize thiamine *de novo*, therefore they may enter a state of deficiency when the diet is devoid of thiamine or when thiamine uptake is impaired. The body does not instantaneously become thiamine deficient; rather it is a gradual process due to minimal thiamine stores in its phosphorylated form or thiamine bound to cofactors. Clinical symptoms of TD started to develop after only two weeks in humans receiving 0.15mg of thiamine per day (McCandless and Schenker 1968). This supports the reported time frame of depletion of thiamine reserves from the body (Williams *et al.* 1940). Serum thiamine levels are not a reliable indicator of thiamine status because minimal amounts of thiamine can reside inside cells. Currently, the preferred clinical test measuring thiamine levels uses high performance liquid chromatography to measure thiamine diphosphate in erythrocyte hemolysates. Once determined to be thiamine deficient, patients are commonly treated with thiamine

supplementation. 2010 guidelines recommend thiamine administered by IV at 200 mg 3 times a day in order to ensure physiological availability (Galvin *et al.* 2010).

1.6.1. Molecular and Metabolic Effects of TD

Under thiamine deficient conditions, the activities of thiamine-dependent enzymes for transketolation, decarboxylation and dehydrogenation decrease. These enzymes are intimately involved in cellular metabolism and a reduction in their activity can lead to the blockage of metabolic pathways or metabolite build up. Decreases in thiamine dependent enzyme activity in the brain are reported to be region specific with reduced α-KGDH playing a major role in TD-induced metabolic effects (Gibson *et al.* 1984, Butterworth *et al.* 1986, Butterworth and Héroux 1989).

A decrease in the oxidative decarboxylation activity of PDH and α-KGDH results in decreased glucose utilization and decreased ATP production, leading to the cellular accumulation of pyruvate and lactate (Aikawa *et al.* 1984, Oriot *et al.* 1991, Navarro *et al.* 2005). Areas of the brain exhibiting decreased glucose utilization in addition to lactic acidosis coincide with histological lesions caused by TD (McCandless and Schenker 1968, Hakim 1984). In addition, increases in extracellular glutamate have been reported in regions of the brain that are vulnerable to TD such as the thalamus, brain stem and the cerebellum (Hazell *et al.* 1993, Langlais and Zhang 1993). Altered glycolytic metabolism as a result of TD has also been demonstrated through a decreased incorporation of ^{14}C-glucose into glutamate, adding to the reduced energy status in TD animals (Aikawa *et al.* 1984). These findings provide evidence for glutamate-mediated excitotoxicity in TD corresponding with lesions in the brain (Armstrong-James *et al.* 1988).

A reduction in glutathione concentration in erythrocytes and heart occurs under TD conditions, causing a decrease in antioxidant activity for free radical scavenging (Hsu and Chow 1960). An increase in production and accumulation of reactive oxygen species (ROS) has been reported in TD brain as well as induction of the endothelial isoform of nitric oxide synthase (eNOS) (Raghavendra Rao *et al.* 1996, Langlais *et al.* 1997). Targeted disruption of the eNOS gene has been shown to reduce the damage caused by TD induced necrotic lesions (Calingasan and Gibson 2000).

TD has been linked to the induction of pro-inflammatory responses such as increased microglial reactivity (Ke *et al.* 2006, Vemuganti *et al.* 2006, Karuppagounder *et al.* 2007). Microarray analysis has shown an upregulation of pro-inflammatory genes including cytokines, chemokines, interferons (IFN), IFN-inducible proteins and transcription factors in vulnerable regions of the brain following TD exposure (Vemuganti *et al.* 2006). It is believed the induction of pro-inflammatory genes may be a result of oxidative impairment under thiamine deficient conditions.

1.6.2. Clinical Manifestations of Thiamine Deficiency

Lack of thiamine in the body can result from malnutrition, a diet high in anti-thiamine factors, or by impaired nutritional status associated with chronic disease (Butterworth 2006). Thiamine deficiency commonly presents acutely but its effects can be extremely detrimental. The clinical manifestations of thiamine deficiency affect multiple organ systems including cardiovascular, metabolic, neurologic, and mucotaneous. Symptoms of TD include congestive heart failure, metabolic acidosis, confusion, ataxia and seizures (Kitamura *et al.* 1993, Ozawa *et al.* 2001, Hanninen *et al.*

2006). Well-known disorders that result from thiamine deficiency include beriberi and Wernicke Korsakoff Syndrome (WKS).

1.6.2.1. Beriberi

Nutritional TD often manifests as either wet or dry Beriberi. Wet beriberi primarily involves cardiovascular complications characterized by extremely low systemic vascular resistance, heart failure and lactic acidosis (Bakker and Leunissen 1995). It is hypothesized that wet beriberi primarily develops as a result of ATP depletion in cardiac myocytes (Bakker and Leunissen 1995, DiNicolantonio *et al.* 2013). Under conditions of thiamine deficiency, a reduction in PDH activity causes a decrease in pyruvate conversion to acetyl-CoA for use in the TCA cycle. Decreased function of the TCA cycle in myocytes subsequently leads to decreased ATP production (Sica 2007). This can cause cellular acidosis and an increase in intracellular free fatty acid levels (Bakker and Leunissen 1995). To accommodate for the lack of ATP formation, there is an increase in glycolysis and the use of body fat resources in order to fulfill cellular energy requirements. There is also an accumulation of adenosine monophosphate, which is converted to adenosine and released into plasma. Excess levels of adenosine in plasma can induce systemic vasodilation, flushing and headaches, which are consistent with symptoms of acute TD (Bakker and Leunissen 1995). The entire process disturbs the normal function of the cardiovascular system and consequently there is an increase in ventricular filling pressures as well as oxygen consumption (Leslie and Gheorghiade 1996). Rising ventricular pressure damages vessels and causes decreased peripheral vascular resistance, increased cardiac output and venous congestion. Overall, patients

with wet beriberi exhibit cardiac failure, systemic vasodilation, lactic acidosis, edema, and fluid retention (Bakker and Leunissen 1995, Wilcox and Schreiner 1999).

Dry beriberi typically causes damage to the peripheral nervous systems and is commonly characterized by peripheral neuropathy (Zak *et al.* 1991). The impairment of sensory and motor nerve conduction velocities affects distal and proximal limbs (Zak *et al.* 1991). Dry beriberi can lead to loss of muscle strength and ultimately paralysis if gone untreated. Patients with dry beriberi have neurologic symptoms in the central and peripheral nervous systems, which manifests as WKS or a peripheral neuropathy, respectively.

1.6.2.2. Wernicke Korsakoff Syndrome

WKS primarily presents as Wernicke's encephalopathy (WE), characterized by ataxia, ophthalmoplegia and mental changes. Investigation of brain tissue from patients presenting with WE demonstrated selective damage to areas of the brain including the thalamus, brain stem, mammillary bodies and midbrain (Harper and Butterworth 1997). Thiamine administration has been shown to reverse WE during the early stages, however, most patients develop Korsakoff syndrome, an amnestic syndrome commonly requiring institutionalization (Caine *et al.* 1997, Kopelman *et al.* 2009). WKS can develop secondary to alcohol abuse or dry beriberi. It has been reported to develop in 1.5% of the general population, however, its occurrence in chronic alcoholism ranges from 35-80% (Thompson 1987, Cook *et al.* 1998). Both WKS and WE are associated with various manifestations of peripheral neuropathy including focal lesions within the thalamus and mammillary bodies (Sullivan and Pfefferbaum 2009).

1.7. TD ASSOCIATED PATHOLOGIES

1.7.1. TRMA

Mutations in the thiamine transporter SLC19A2 (mapped to chromosome 1q23.2-23.3) cause thiamine-responsive megaloblastic anemia (TRMA or Rogers syndrome) (Raz *et al.* 2000, Neufeld *et al.* 2001). TRMA is an early onset autosomal recessive disorder where mutations within the SLC19A2 gene cause pre-mature translation termination resulting in decreased thiamine transport activity. A common mutant of SLC19A2 (D93H) results in proper translocation to the plasma membrane, however, it lacks functional transport ability (Baron *et al.* 2003). Since TRMA only affects SLC19A2, patients are effectively treated with thiamine supplementation due to functionality of SLC19A3 (Ganapathy *et al.* 2004). Mutations in SLC19A2 have also been reported to be associated with diabetes mellitus and deafness (Labay *et al.* 1999).

1.7.2. Diabetes

Diabetic patients commonly develop TD, which may be due in part with poor enteral thiamine absorption and/or decreased renal uptake (Thornalley 2005, Page *et al.* 2011). Dietary intake of thiamine does not correlate with urinary thiamine excretion in clinical diabetes patients, and it is suggested that impaired tubular re-uptake is responsible (Adaikalakoteswari *et al.* 2012). In both experimental and clinical studies increased renal clearance of thiamine was linked to the decreased reuptake of thiamine from the glomular filtrate (Babaei-Jadidi *et al.* 2003, Thornalley *et al.* 2007). In an experimental diabetes model, high glucose concentrations resulted in a 37% decrease in apical to basolateral transport of thiamine across cell monolayers corresponding with a reduction in both THTR1 and THTR2 mRNA and protein levels (Larkin *et al.* 2012). This correlates with clinical

data where patients with type 1 or 2 diabetes were found to have increased renal clearance compared to their healthy counterparts (Thornalley *et al.* 2007, Adaikalakoteswari *et al.* 2012).

High dose thiamine treatment is common in order to avoid potential detrimental side effects associated with decreased plasma thiamine levels. Rats treated with excess thiamine or thiamine mimetic supplementation demonstrated reduced diabetic neuropathy. The beneficial effects of supplementation are thought to be through the activation of TKT (Hammes *et al.* 2003). Thiamine supplementation has also been demonstrated to reduce urinary microalbumin, reverse hyperglycemia-induced endothelial dysfunction and prevent retinopathy in rats (Thornalley 2005, Page *et al.* 2011, vinh quoc Luong and Nguyen 2012). Additionally, high dose thiamine therapy has been used in clinical trials in diabetic patients for the treatment and prevention of microalbuminurea and nephropathies with no reported adverse effects (Rabbani *et al.* 2009, Rabbani and Thornalley 2011).

1.7.3. Alzheimer's Disease

Thiamine deficiency manifests itself in a region specific manner in the brain causing irreversible cognitive impairment, similar to that of Alzheimer's Disease (AD). Therefore, it has been postulated that thiamine may play a role in the pathogenesis of AD (Mastrogiacomo *et al.* 1993). In pre-AD patients, activities of α-KGDH and PDH are negatively correlated with the clinical dementia rating score (Morris 1993). It has been established that patients with AD exhibit reduced thiamine levels and a reduction in thiamine-dependent enzyme activity in the brain and peripheral tissues (Butterworth and Besnard 1990, Gibson *et al.* 2000, Bubber *et al.* 2005, Nguyen 2011). A common

characteristic in the brains of patients with AD is the disturbance of normal functioning glucose metabolism. It is hypothesized that reductions in thiamine-dependent enzymes underlie the decline in glucose metabolism observed in AD patients (Ott *et al.* 1999, Gibson and Blass 2007, Kroner 2009, Takeda *et al.* 2010, Gibson 2013).

AD is a condition characterized by a decline in cognition and the build-up of abnormal clusters of protein fragments (plaques) and twisted strands of proteins around nerve cells (tangles). Advanced glycation end product (AGE) accumulation has been suggested to be involved in increased plaque formation (Loske *et al.* 2000). AGEs are a heterogeneous group of chemical modifications that are reported to cause damage to cells by a number of processes including neurotoxicity, oxidative stress and apoptosis (Loske *et al.* 1998). Dietary TD has been shown to increase AGE formation as assessed by increased plasma and tissue concentration of AGEs (Shangari *et al.* 2005, Depeint *et al.* 2007, Karuppagounder *et al.* 2009). The activation of thiamine dependent TKT by benfotiamine supplementation has been reported to reduce the formation of AGEs (Hammes *et al.* 2003).

Synthesis of the neurotransmitter acetylcholine (ACh) is decreased in AD, contributing to characteristic cognitive deficits (Francis *et al.* 1985, Perry 1988, Nguyen 2011). ACh is synthesized from choline and acetyl-CoA, requiring functional PDH activity to provide sufficient acetyl-CoA. There is an impairment of ACh synthesis under TD conditions as a result of decreased thiamine dependent enzyme activity (Cheney *et al.* 1969, Vorhees *et al.* 1977, Ruenwongsa and Pattanavibag 1984). Other studies have also implied the importance of thiamine in the presynaptic release of ACh (Meador *et al.* 1993). Very little is known about the effects of thiamine supplementation in AD patients

since few studies have been conducted reporting varying results (Blass *et al.* 1988, Butterfield *et al.* 2002, Maruszak *et al.* 2014).

1.7.4. Alcoholism

Thiamine and other B vitamins are preferentially depleted by high rates of alcohol metabolism (Singleton and Martin 2001, Martin *et al.* 2003). Deficiencies can also occur in alcoholics as a result of poor diet, liver disease and gastrointestinal disorders (Hoyumpa 1980, Martin *et al.* 2003, Subramanya *et al.* 2011). Physiological consequences of thiamine deficiency in alcoholism involve impaired enzyme utilization of TPK1 and TKT, and reduced intestinal and renal transport capacity (Thomson *et al.* 1970, Abe and Itokawa 1977, Hoyumpa *et al.* 1978, Hoyumpa 1980, Subramanian *et al.* 2010). Chronic alcohol consumption is also associated with down-regulation of thiamine transporters and decreased levels of thiamine-dependent enzymes within the brain. Specifically, reduced levels of TKT gene expression and protein as well as reduced α-KGDH have been reported (Hazell and Butterworth 2009).

Thiamine deficiency can cause metabolic and oxidative stresses that are associated with neurologic impairment in alcohol dependency (Pannunzio *et al.* 2000, Singleton and Martin 2001). Altered thiamine homeostasis in chronic alcoholics can lead to cognitive dysfunction and neuoropathic damage. Alcoholic brain disease is commonly characterized by a progressive loss of central and peripheral white matter that is suggested to be linked to alcoholism-related thiamine deficiency (He *et al.* 2007, Mellion *et al.* 2011, Laureno 2012).

1.7.5. Cancer

It has been hypothesized that a Western diet, characterized in part by excess thiamine supplementation, may be a factor for increased cancer incidence compared to other countries (Boros 1999). Thiamine is commonly supplemented in processed foods and readily consumed in over-the-counter vitamin and nutritional supplements in Western countries with generally high cancer incidences. In contrast, Asian and African countries principally consume food that is high in thiaminase, a natural thiamine-degrading enzyme, which may reduce thiamine exposure (Boros 1999). Although no direct studies have evaluated this hypothesis, several have attempted to correlate the intake of thiamine and other nutritional components with the risk of cancer. However, like so many other nutritional correlations with cancer incidence, the dietary intake of thiamine and cancer risk has provided conflicting results. Using nutritional questionnaires and a calculated average daily intake level, patients with prostate cancer consume less thiamine than those without cancer suggesting a negative association with cancer risk (Kaul *et al.* 1987). A 2008 study examined the relationship between the intake of B vitamins and incidence of breast, endometrial, ovarian, colorectal, and lung cancer in women (Kabat *et al.* 2008). No correlation was found between intake of the B vitamins, including thiamine, riboflavin, niacin, and folate, and the incidence of cancer. Interestingly, reduced thiamine administration increased aberrant crypt foci in the colons of rats fed a sucrose-based diet (Bruce *et al.* 2003). Patients with severe malnutrition have exhibited Baker's cyst, osteosarcoma, and submandibular gland cysts that were cured without recurrence after thiamine administration, suggesting a role of thiamine deficiency in tumor development (Lee *et al.* 2005).

A limited number of studies and case reports have determined the overall thiamine status in cancer patients. Clinically, thiamine status is quantified biochemically using a TKT assay of whole blood samples (Chamberlain *et al.* 1996). This assay involves measuring the increase in the activity of the thiamine-dependent enzyme TKT after added TPP. If deficient in thiamine, exogenous TPP will stimulate TKT activity, termed the TPP effect. Basu *et al*. demonstrated that patients with advanced cancer exhibit a greater TPP stimulating effect, suggestive of a reduced thiamine status (Basu *et al.* 1974, Basu and Dickerson 1976). Similarly, increased TPP effect was characterized in patients with B-chronic lymphocytic leukemia, Burkett's lymphoma, and acute myelomonocytic leukemia (Van Zaanen and Van Der Lelie 1992, Seligmann *et al.* 2001). Using an HPLC assay to directly quantify TPP levels in whole blood, Tsao *et al*. demonstrated a significant decrease of TPP in patients with advanced stages of non-small cell lung cancer (Tsao *et al.* 2007). Although the reason for a decrease in thiamine status in the blood is unclear, one study noted that cancer patients had a higher level of thiamine urinary excretion (Basu and Dickerson 1976). The authors suggested that the decrease in thiamine status might not be due to reduced dietary intake of thiamine, but an inability to activate thiamine to TPP (Basu *et al.* 1974). However, thiamine status is primarily assayed biochemically in whole blood and limited studies have quantified thiamine/TPP directly in cancer tissue. The reductions in peripheral thiamine/TPP may be a consequence of extensive accumulation and/or utilization by cancer cells. Trebukhina *et al*. demonstrated that tumor growth resulted in a depletion of tissue vitamin stores and an increase in the TPP-stimulating effect in blood (Trebukhina *et al.* 1985). During tumor growth, cancer cells maintained a constant level of TPP while host liver tissue exhibited a

perpetual decline (Trebukhina *et al.* 1982). In post-surgical or autopsy tissues, a 2.5-fold increase in thiamine levels was found in colon adenocarcinomas relative to un-invaded control tissue (Baker *et al.* 1981). Overall these studies strongly suggest a preferential accumulation of thiamine into cancer cells that may be responsible for the alteration in peripheral thiamine status during malignancy.

Aside from the disease itself, chemotherapeutic drugs such as 5-fluorouracil (5-FU) and ifosfamide have been associated with inducing a thiamine-deficient state in patients (Aksoy *et al.* 1980, Buesa *et al.* 2003). In most cases, patients exhibit neurological impairment similar to the sequelae observed in thiamine deficiency conditions such as Wernicke's encephalopathy (Buesa *et al.* 2003, Hamadani and Awan 2006, Ames *et al.* 2010). How these drugs that are structurally unrelated to thiamine are capable of inducing a deficiency is unknown and may involve distinct mechanisms. No change in thiamine or TPP levels was found in patients receiving ifosfamide treatment, suggesting that ifosfamide or a metabolite may inhibit a thiamine-dependent pathway (Buesa *et al.* 2003, Losa *et al.* 2005). Accumulation of thiamine was found to increase in cancer cells and rat hepatocytes when treated with 5-FU and doxifluridine (Heier and Dornish 1989). The enhancement in thiamine uptake by 5-FU in cancer cells has been associated with an increase in intracellular TPP while free thiamine remained constant (Heier and Dornish 1989). Treatment of rats for three consecutive days with 5-FU was found to result in an increase in the TPP-stimulating effect on whole blood TKT, and a decrease in liver thiamine stores (Basu *et al.* 1979). Thus, 5-FU appears to decrease peripheral thiamine levels by increasing cellular accumulation and conversion to TPP. Although the mechanism for either drug still needs continued research, high-dose

thiamine is capable of reversing the symptoms of thiamine deficiency (Aksoy *et al.* 1980, Buesa *et al.* 2003, Hamadani and Awan 2006).

Self-supplementation vitamin preparations containing levels of thiamine greater than the RDI are readily accessible and considered to be safe and harmless for patients (Table 1.4). Although the use of thiamine to treat deficiency-related symptoms attributed to the disease or therapy is warranted, this is currently done with limited comprehension of the role thiamine may contribute towards malignant progression. In light of our knowledge regarding alterations of thiamine homeostasis in cancer, the impact of thiamine supplementation on cancer growth has received minimal research attention. In 2001, Comin-Anduix *et al*. evaluated the effect of increasing thiamine supplementation in multiples of the RDI on an Ehrlich ascites tumor-mouse model (Comín Anduix *et al.* 2001). Their findings indicated a statistically significant stimulatory effect of thiamine supplementation on tumor growth compared to non-supplemented controls. Moderate doses of 12.5 to 37.5 times the RDI had the greatest stimulatory effect, peaking at approximately 250% greater tumor cell proliferation with 25 times the RDI. Interestingly, at values above 75 times the RDI, no change was found in tumor cell proliferation, and a slight decrease was found at 2,500 times the RDI. This observation suggests that there is a specific range in which thiamine supports proliferation. A recent study explored the relationship between a high-fat diet and thiamine levels on the tumor latency in the Tg(MMTVneu) spontaneous breast cancer-tumor mouse model (Daily *et al.* 2012). In this study a normal-fat (NF) diet contained 10% of the calories from fat while the high-fat diet contained 60%. Low thiamine (LT) levels were defined as 2 mg of thiamine per 4,057 kcal and normal thiamine (NT) levels as 6 mg per 4,057 kcal. Tumor latency was

significantly longer (295 days) in animals given a NF/LT diet compared with animals on NF/NT (225 days). Interestingly, the delay in tumor latency from LT was abolished when given a high-fat diet. This demonstrates an important interplay of dietary constituents on tumor progression that needs further characterization. Although more research is needed to confirm and evaluate the role of thiamine on disease progression, these studies have significant clinical implications. First, patients requiring thiamine to treat either chemotherapy or disease-associated deficiency should receive high-dose thiamine to avoid enhancing tumor growth. Second, self-supplementation of thiamine by cancer patients should be avoided as the low-to-moderate levels of thiamine may contribute to disease exacerbation.

The importance of thiamine in cancer cell proliferation is highlighted by studies using the thiamine-degrading enzyme thiaminase. Liu *et al*. demonstrated that the addition of thiaminase into cell culture media containing thiamine had a significant growth inhibitory effect on breast cancer cells (Liu *et al.* 2010). Thiaminase reduced ATP levels in cancer cells, demonstrating thiamine's key role in support of cancer cell bioenergetics. Moreover, a pegylated version of thiaminase was capable of delaying tumor growth and prolonging survival in an RS4 leukemia xenograft model (Daily *et al.* 2011). Thiamine's key role in cancer cell metabolism and survival is further demonstrated by studies using the thiamine analog oxythiamine, which functions as an anti-coenzyme and is capable of reducing *in vivo* and *in vitro* tumor cell growth (Boros *et al.* 1997, Rais *et al.* 1999, Ramos-Montoya *et al.* 2006). Inhibition of TKT by oxythiamine reduces DNA and RNA synthesis through reductions in ribose 5-phosphate (R5P) synthesis, the pentose carbon backbone of all nucleotides. Oxythiamine also has

been shown to induce apoptosis in rat PC-12 cells via mitochondria-dependent caspase 3-mediated signaling pathways (Chornyy *et al.* 2007). The effect on nucleotide synthesis is highlighted by the prominent G1 cell cycle arrest induced by oxythiamine in Ehrlich's tumor cells (Rais *et al.* 1999). Yang *et al*. demonstrated that oxythiamine decreases cell migration and invasion *in vitro*, as well as reduced lung metastases in mice with Lewis lung carcinoma (LLC) (Yang *et al.* 2010). Interestingly, oxythiamine did not reduce the proliferation of LLC cells at concentrations that reduced migration and invasion. The effect of oxythiamine was attributed to a dose-dependent reduction in MMP-2 and MMP-9 activity and expression. This finding suggests that thiamine-dependent pathways have other repercussions on cancer progression in addition to effects on cellular proliferation.

1.8. RATIONALE AND GOAL

Pathological conditions resulting in cellular exposure to nutritional and metabolic stresses require adaptations to maintain cell growth and survival. Transcriptional regulation can induce alterations in gene expression patterns effecting cellular dependence on metabolic pathways within the glycolytic network. An adequate supply and cellular pool of enzyme cofactors is essential to support altered glycolytic metabolism. Thiamine, a water-soluble micronutrient, plays an essential role in normal cellular functions, growth and development. Its activated form serves as a critical enzymatic cofactor for enzymes involved in glycolysis, the citric acid cycle, and the non-oxidative portion of the pentose phosphate pathway. Therefore, the overall goal of this research is to investigate the adaptive regulation of thiamine homeostasis during exposure to physiological stressors such as limited oxygen availability and nutrient deprivation.

1.9. RESEARCH OBJECTIVES

1) Determine the effect of hypoxic stress on micronutrient transporter expression and thiamine transporter function.
2) Determine the effect of nutrient deprivation on the expression of genes involved in cellular metabolism.
3) Investigate the adaptive regulation of the thiamine transporters during thiamine deficiency.
4) Establish a metabolic congruency between thiamine deficiency and pseudo-hypoxia.
5) Investigate the role of HIF-1α in the regulation of thiamine transporter expression.

CHAPTER 2

COMMON METHODOLOGY

2.1. CHEMICALS

Thiamine hydrochloride and pyrithiamine hydrobromide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Desferrioxamine mesylate (DFO) were purchased from Calbiochem (La Jolla, CA, USA). Radiolabeled ^{3}H-Thiamine hydrochloride with a specific activity of 20 Ci/mmol was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). YC1 was purchased from Tocris Bioscience (Minneapolis, MN, USA).

2.2. CELL CULTURE REAGENTS

All cell culture grade reagents including trypsin/EDTA, penicillin/streptomycin, RPMI 1640 media and thiamine deficient RPMI 1640 (TD Media) was custom formulated by were obtained from Mediatech (Manassas, VA, USA) unless otherwise stated. Fetal bovine serum (FBS) and dialyzed FBS (10 kDa cut off) was purchased from Atlanta Biologicals (Atlanta, GA, USA) or PAA Laboratories (Dartmouth, MA). Cell culture treated flasks, plates and dishes were from Greiner Bio-one (Monroe, NC). Thiamine deficient RPMI 1640 (TD media) was custom formulated by Omega Scientific (Tarzana, CA).

2.3. CELL LINES

All cells lines used in this work were purchased from ATCC (Manassas, VA) unless otherwise noted.

2.3.1. MCF10A

MCF10A cells were isolated from the mammary gland of a female having fibrocystic disease (ATCC). The cells are epithelial in morphology and depicted in Figure 2.1. The non-tumorigenic breast epithelial cell line MCF10A was maintained in DMEM:F12 (Mediatech) supplemented with 5% horse serum, 1% penicillin/streptomycin, 20 ng/ml EGF (Peprotech), 0.5 microgram/ml Hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma Aldrich) and 10 microgram/ml insulin (Sigma Aldrich).

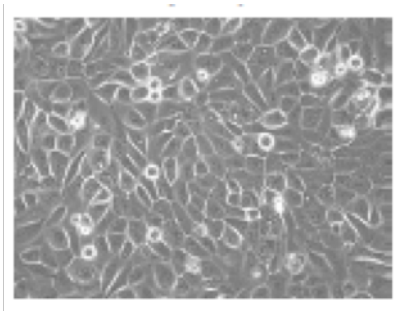

Figure 2.1. MCF10A cells. Nontumorigenic immortalized MCF10A breast cells. Picture reproduced from (Botlagunta *et al.* 2010).

2.3.2. MCF12A

MCF12A (ATCC® CRL-10782™) is a non-tumorigenic breast epithelial cell line taken from a patient exhibiting fibrocystic breast disease. MCF12A cells were maintained in DMEM:F12 (Mediatech) supplemented with 5% horse serum, 1% penicillin/streptomycin, 20 ng/ml EGF (Peprotech), 0.5 microgram/ml Hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma Aldrich) and 10 microgram/ml insulin (Sigma Aldrich).

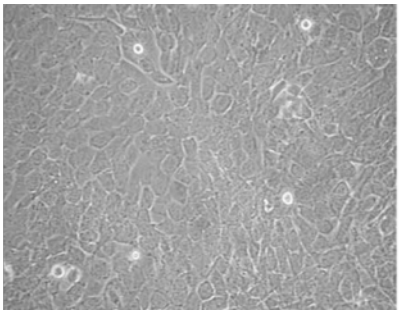

Figure 2.2. MCF12A cells. Nontumorigenic MCF12A breast epithelial cell line. Figure reproduced from (Patel *et al.* 2005).

2.3.3. Primary human mammilary epithelial cells (HMEC)

Primary human mammilary epithelial cells (HMECs) isolated from adult female breast tissue were obtained from Lonza (Walkersville, MD, USA). HMECs were cultured according to the supplier protocol using mammary epithelial cell growth media containing bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, and gentamicin sulfate amphotericin-B (Lonza).

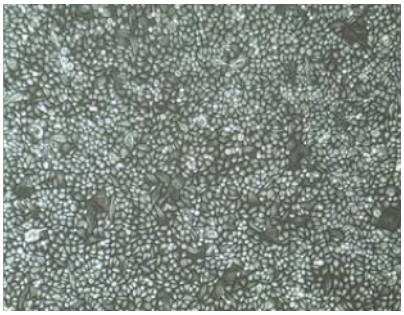

Figure 2.3. HMEC cells. Primary HMEC breast epithelial cells. Figure reproduced from lifelinecelltech.com.

2.3.4. BT474

BT474 (ATCC® HTB-20™) are estrogen receptor positive (ER+) breast cancer cells, isolated from a primary site invasive ductal carcinoma. They were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin.

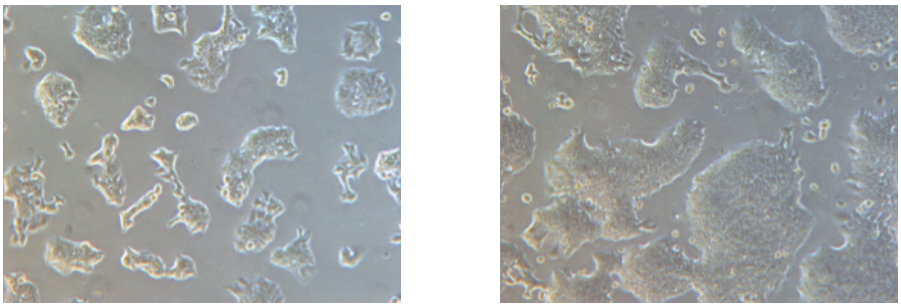

Figure 2.4. BT474 cells. BT474 cells at low density (left) and high density (right). Pictures generated by Rebecca Sweet.

2.3.5. MCF7

MCF7 (ATCC® HTB-22™) is an estrogen receptor positive (ER+) breast cancer cell line. The cells were derived from a metastatic site via pleural effusion from a female presenting with adenocarcinoma. MCF7 cells were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin.

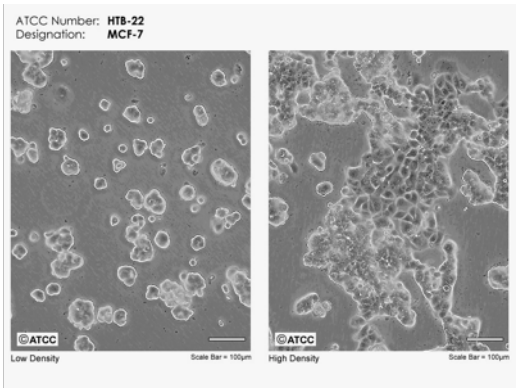

Figure 2.5. MCF7 cells. MCF7 cells at low density (left) and high density (right). Pictures reproduced from ATCC.org.

2.3.6. BT-20

BT-20 (ATCC® HTB-19™) is an estrogen receptor negative (ER-) breast cancer cell line. BT-20 is a tumorigenic cell line that was first harvested by E.Y. Lasfargues and L. Ozzello in 1958 in cells that were spilling out of a tumor when it was cut into thin slices. BT-20 cells were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin.

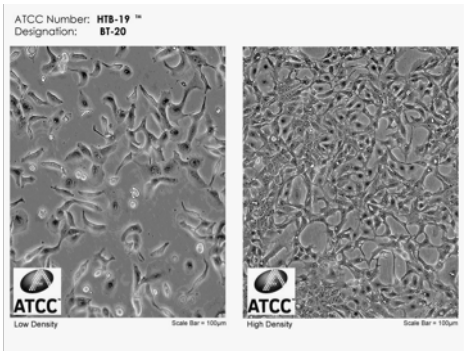

Figure 2.6. BT20 cells. BT20 cells ay low density (left) and high density (right). Picture reproduced from ATCC.org.

2.3.7. MDA-MB-231

MDA-MB-231 (ATCC® HTB-26™) is an estrogen receptor negative (ER-) breast cancer cell line. MDA-MB-231 cells were derived from a metastatic site via pleural effusion. The MDA-MB-231 cell line was obtained from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin.

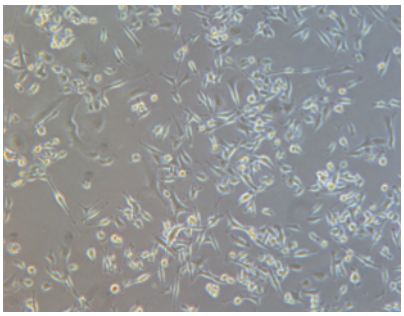

Figure 2.7. MDA-MB-231 cells. MDA-MB-231 cells at low density. Picture taken by Rebecca Sweet.

2.3.8. SK-N-BE

SK-N-BE (ATCC® CRL-2271™) are a neuroblastoma cell line derived from a metastatic site (bone marrow). The cells are neuroblast in morphology with some cells having long processes and others being epithelioid like. They also grow as a mixture of adherent and suspension.

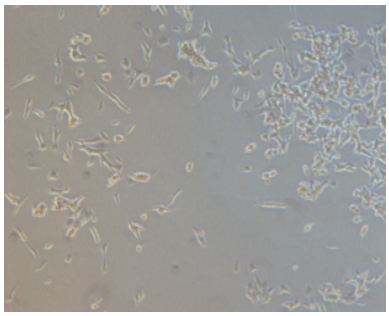

Figure 2.8. SK-N-BE cells. SK-N-BE cells at low density. Picture taken by Rebecca Sweet.

2.4. CULTURING AND TREATMENTS

Cells were routinely cultured at 37°C and 5% CO_2, designated as normoxic conditions.

2.4.1. Hypoxic Treatments

All hypoxic treatments and chronic hypoxic culturing were conducted at 37^0C with 5% CO_2 and 1% O_2 using an incubator outfitted with a ProOX oxygen controller (Biospherix, Lacona, NY) supplying nitrogen gas. Oxygen levels were monitored daily using a Bacharach Fyrite® Gas Analyzer (Bacharach, Inc, Pittsburgh, PA**)** and the ProOX

oxygen controller was calibrated weekly using 100% N_2 for 0% O_2 and atmospheric air for 21% O_2. Cell culture media for all hypoxia experiments and media changes were pre-equilibrated in the hypoxic environment for a minimum of 24h before use. For acute 1% O_2 hypoxic exposure, cells were allowed to grow until approximately 60% confluency in normoxic conditions. After which, media was removed and hypoxia conditioned media was added. Cells were then placed in the hypoxic incubator and maintained at 1% O_2 for 24, 48 and 72h. Long term culturing of cells at 1% O_2 were maintained in the hypoxic incubator with media changes every 2 days and cultures split once cells reached approximately 75% confluency.

2.4.2. Desferrioxamine (DFO) Treatments

Cells treated with the chemical hypoxic mimetic DFO were media changed with fresh media in addition to 250 μM DFO. Treated cells were then cultured for 24, 48 and 72 h under normoxic culture conditions.

2.4.3. Thiamine Deficiency Treatments

Thiamine deficiency treatment conditions were slightly different due to a change in source of the custom made media. Thiamine deficient media and conditions are described in detail for each corresponding chapter.

2.5. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (qRT-PCR)

RNA was isolated using the EZNA Total RNA Kit I (Omega Bio-tek, Norcross, GA) and reverse transcribed (1 µg) to cDNA using qScript cDNA Synthesis Kit as per manufacturers protocol (Quanta Biosciences Inc., Gaithersburg, MD). qRT-PCR was performed using a LightCycler® 480 II (Roche Applied Science, Indianapolis, IN). Primers for each gene were designed using the Roche Universal ProbeLibrary website to correspond with a specific 8-9 nucleotide hydrolysis probe labeled at the 5' end with fluorescein (FAM) (Table 2.1). The Tata-box-binding protein (TBP) reference assay kit supplied by Roche Applied Science was used as a housekeeping gene. Changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method for relative quantification and expressed as the fold change relative to normoxic or untreated groups (Schmittgen and Livak 2008).

Table 2.1. Primers used for qRT-PCR. Primers used for qRT-PCR are shown with their sequences and their associated probe numbers.

Gene	Accession Number	Primers	Probe
SLC19A2	NM_006996	F-GAC ACC CCA GCT TCT AAC CA R-AAG GAG ACG GTC TGG CTT G	72
SLC19A3	NM_025243	F-AAT GGG GCC GTA GAA GCT AT R-TTT CAC ATA ACC CAC TGC AAA	5
SLC25A19	NM_021734	F-ATA GGC TAT GGA GCT GTC CAA T R-AAT TCC CGG GCG TCA TAC	34
GLUT1	NM_006516.2	F-GCC CAT GTA TGT GGG TGA A R-AGT CCA GGC CGA ACA CCT	81
ALDOA	NM_001127617.2	F-TGC CAG TAT GTG ACC GAG AA R-GCC TTC CAG GTA GAT GTG GT	66
VEGF	NM_001025367.2	F-CAG ACT CGC GTT GCA AGA R-GAG AGA TCT GGT TCC CGA AA	12

2.6. IMMUNOBLOT ANALYSIS

2.6.1. Whole Cell Lysate Extraction

Cells were washed in ice cold phosphate-buffered saline and lysed using 50mM Tris, 250mM NaCl, 1mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Calbiochem). Whole cell lysates were centrifuged at 16,000g for 20 min at 4^0C and the supernatant was collected. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

2.6.2. Nuclear Extraction

For isolation of nuclear lysates, cells were first lysed with 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA pH=7.9 and protease inhibitor cocktail on ice for 15 min. After which, 10% NP-40 was added at a ratio of 62.5 µL/mL of lysate then mixed and set on ice for an additional 5 min. Lysates were then centrifuged at 16,000xg for 10 min at 4°C and the supernatant was discarded. The pellet was resuspended in 20 mM Hepes, 0.4 M NaCl, 1.0 mM EDTA and 1.0 mM EGTA pH=7.9 containing protease inhibitor cocktail and incubated on ice for 40 min, vortexing every 10 min for 30 sec. The sample was then centrifuged at 16,000xg for 5 min at 4°C and the resulting nuclear supernatant collected. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific).

2.6.3. Western Blotting

Samples (50μg) were electrophoresed on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (Hybond-P, GE Healthcare, Piscataway, NJ). The membrane was blocked with 5% milk (Carnation) in Tris buffered saline-tween 20 (TBS-T) for 1h. The membrane was then immunoblotted for HIF-1a (mouse anti-human, BD Biosciences, San Jose, CA) at 1:1000 in 0.5% BSA/TBS-T for 3h. Blot was washed 3 times for 10 min each with TBS-T then blotted with 1:10,000 goat anti-mouse horseradish peroxidase (HRP) secondary antibody (Millipore, Billerica, MA) for 1h. Beta actin was used for loading control (rabbit anti-human, Thermo Scientific, Rockford, IL) and probed at 1:1000 in 0.5% BSA/TBS-T for 3h, washed 3 times for 10 min with TBS-T then 1:20,000 goat anti-rabbit-HRP secondary antibody (Millipore, Billerica, MA) for 1h. Blots were visualized using Supersignal West Pico (Thermo Scientific, Rockford, IL) and captured with a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA).

2.7. ANTIBODIES

The rabbit monoclonal anti-HIF-1α antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). The rabbit monoclonal anti-SP1 and the rabbit monoclonal anti-SP3 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse monoclonal anti-P84/N5-5E10 antibody was kindly provided by GeneTex (Irvine, CA, USA).

2.7.1. HIF-1α

HIF-1α is a transcription factor that is rapidly degraded under normoxic conditions. In the absence of oxygen, HIF-1α protein is stabilized and it forms a heterodimer with HIF-1β and translocates to the nucleus, turning on HIF-1α target genes. HIF-1α sometimes demonstrates double banding during immunoblot analysis yielding a band at 120 kDa. The epitope recognized by this antibody (A300-286A, Bethyl Laboratories) maps to a region between residues 775 and 826 on the C-terminus of HIF-1α protein. For Western blotting, the primary antibody was used at 1:500 with a goat-anti-rabbit-HRP secondary antibody at 1:15,000.

2.7.2. SP1

SP1 transcription factor is a C2H2-type zinc finger-containing DNA binding proteins that controls the expression of numerous genes implicated in a variety of cell events (Xu *et al.* 2000, Ossipova *et al.* 2002, Kaluz *et al.* 2003). SP1 can bind to GC-rich motifs with high specificity and affinity and plays a crucial role in activation/repression of transcription initiation in response to stimuli, including various environmental stimuli through interactions with the nearby transcriptional factors or components of the basal transcriptional machinery. SP1 has highly regulated post-translational modifications including glycosylation, phosphorylation, acetylation and proteolytic cleavage. The SP1 antibody (H-225) (Santa Cruz) corresponds to the amino acids 121-345 near the N-terminus of SP1 of human origin. For Western blotting, the primary antibody was used at 1:500 with a goat-anti-rabbit-HRP secondary antibody at 1:10,000.

2.7.3. SP3

SP3 transcription factor contains a DNA-binding domain comprised of three zinc fingers at the C-terminus and binds to GC boxes in the promoter region of genes. Multiple isoforms of SP3 exist due to alternative splicing. The SP3 (Genetex) antibody corresponds to the carboxy terminus of SP3. For Western blotting, the primary antibody was used at 1:200 with a goat-anti-rabbit-HRP secondary antibody at 1:10,000.

2.7.4. THTR1

Thiamine transporter THTR1 is a 498 AA protein serving to transport thiamine into the cell. The protein contains 12 transmembrane domains with both C-terminal and N-terminal domains located in the cytoplasm. The THTR1 antibody (Alpha Diagnostics) corresponds to the N-terminal cytoplasmic domain. For Western blotting, the THTR1 primary antibody was used at 1:500 with a goat anti-rabbit-HRP secondary antibody at 1:15,000.

2.7.5. THTR2

Thiamine transporter THTR2 is a 496 AA acid protein that transports thiamine into the cell. The protein contains 12 transmembrane domains with both C-terminal and N-terminal domains located in the cytoplasm. For Western blotting, the THTR2 primary antibody was used at 1:500 with a goat anti-rabbit-HRP secondary antibody at 1:15,000.

2.7.6. β-Actin

Actins are a group of highly conserved proteins in eukaryotic cells playing a role in cell motility, integrity and structure. β-Actin is one of six actin isoforms and is one of the two non-muscle cytoskeletal actins. The monoclonal anti-β-Actin (A5316, Sigma Aldrich) recognizes an epitope located on the N-terminal end of the *β*-isoform of actin. For Western blotting, the β-Actin primary antibody was used at 1:500 with a goat anti-mouse-HRP secondary antibody at 1:25,000.

2.7.7. p84

p84 is an 84 kDa nuclear matrix protein used as a nuclear specific marker. The p84 antibody (GTX70220, GeneTex) recognizes amino acids 15-374 of the protein. For Western blotting, the primary antibody was used at 1:500 with a goat-anti-mouse-HRP secondary antibody at 1:15,000.

2.8. STATISTICAL ANALYSIS

All results are expressed as mean +/- standard deviation (SD) with a minimum of n=3 independent experiments. Comparisons between groups were done using a two-tailed two sample *t*-test with a significance level of $p<0.05$.

CHAPTER 3

HYPOXIA INDUCED UP-REGULATION AND FUNCTION OF THE THIAMINE TRANSPORTER, SLC19A3 [1]

[1] Rebecca Sweet, Amber Paul, and Jason Zastre. (2010). *Cancer Biology & Therapy*. 10:1101-1111. Reprinted here with permission of publisher.

3.1. 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 numerous 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. This study investigated 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 was found to be significantly upregulated. Similarly, upregulation was also observed in breast cancer cell lines compared to human mammary epithelial cells. These findings demonstrate an adaptive response by breast cancer cells to increase cellular availability of thiamine. Alterations in gene expression also occur under conditions of physiological stresses. Adaptive responses within hypoxic tumor microenvironments require the altered expression of Solute Carrier (SLC) transporters to maintain nutrient uptake in support of cellular metabolism and biosynthesis. Using a real time PCR array strategy to further characterize changes in transporter expression within a chronic hypoxia breast cancer cell line model (BT474), we have found a 31 fold increase in the expression of the thiamine transporter, SLC19A3. Thus, investigations into the expression changes of the thiamine transporters, SLC19A2 and SLC19A3, and the role of hypoxia inducible factor-1 alpha (HIF-1α) regulating their expression was conducted. Chronic culturing of BT474 and MCF7 estrogen receptor positive cells in 1% O_2 up to

142 days consistently demonstrated a high level of SLC19A3 expression with a mean of approximately 40 and 8 fold, respectively, with no change in SLC19A2. A corresponding 2 fold increase in thiamine uptake over 15 min was measured in chronic hypoxic BT474 cells compared to normoxia. Acute 1% O_2 exposure of BT474 and MCF7 cells up to 72 h demonstrated a 7.5 and 2.5 fold increase, respectively, in SLC19A3 expression. The chemical hypoxia mimetic desferrioxamine, resulted in an approximately 70 fold increase in SLC19A3 expression. Stable shRNA knockdown of HIF-1α reduced hypoxia mediated SLC19A3 up-regulation by approximately 3 fold compared to scrambled construct. In conclusion, SLC19A3 transporter expression was observed to be upregulated under acute, chronic and DFO induced hypoxia. The attenuated increase in SLC19A3 expression after HIF-1α knockdown suggests a role for HIF-1α mediated pathways regulating SLC19A3 gene expression.

3.2. INTRODUCTION

For the purposes of this dissertation, additional background information on hypoxia has been included in the introduction.

Cells require oxygen to support aerobic metabolism and act as an electron acceptor in organic and inorganic reactions. Oxygen levels in ambient air is 21% while in most mammalian tissues O_2 levels range from 2-13% due to tissue distribution and diffusion limitations (Wolfle *et al.* 1983, Jiang *et al.* 1996, Stamler *et al.* 1997). A condition of reduced oxygen in the range of 0.2-2% O_2 is termed hypoxia. The resulting hypoxic microenvironment can be described as chronic, arising from the limited diffusion

of oxygen to cells distal from the vessel or acute, developing from perfusion limitations of oxygen delivery to adjacent cells (Harris 2002).

3.2.1. Hypoxia Inducible Factors

Due to the heavy reliance on oxygen to support basic cellular function and metabolism, specific molecular mechanisms allow the detection of low oxygen conditions. The initial reaction to oxygen deprivation is a state of defense where protein translation and ion channel activity are arrested in order to preserve ATP (Hochachka 1986, Boutilier 2001). Cells are able to reduce their oxygen demand in order to balance the limited supply in hypoxic environments. Low oxygen conditions activate the master transcriptional factor HIF-1α. HIF-1α is a member of the hypoxia-inducible transcription factor subfamily of the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) protein family (Semenza 2000). In addition to HIF-1α, the family also contains HIF-2α and HIF-3α. The structure, regulation, and function of all three are closely related, however, HIF-2α and HIF-3α expression are tissue specific, supporting the hypothesis of each serving specialized roles (Semenza 2000). HIF-1α and HIF-2α have been suggested to play similar functions in response to hypoxia and have been shown to regulate both unique and common genes (Grabmaier *et al.* 2004, Warnecke *et al.* 2004, Hu *et al.* 2007). HIF-1α specifically regulates glycolytic genes and HIF-2α exclusively regulates cyclin D1, transforming growth factor alpha (TGF-α) and transcription factor Oct-4 (Grabmaier *et al.* 2004, Rankin *et al.* 2005, Covello *et al.* 2006, Hu *et al.* 2007). HIF-3α has been suggested to be a negative regulator of hypoxia induced gene expression (Fleming *et al*.

2001). For the purpose of this dissertation, we have focused our efforts on HIF-1α due to our interest in genes involved in glycolytic metabolism.

3.2.1.1. Structure

HIF-1α is an 826 amino acid protein (120 kDa) containing two main domains: the PAS domain and the bHLH domain (Figure 3.1) (Semenza 2001). The PAS domain is located on the N-terminus and contains two internal homology units, A and B repeats serving to interact with proteins. The bHLH domain is also located on the N-terminus and is required for both protein dimerization and DNA binding.

The C-terminal half of HIF-1α contains two transactivation domains, TAD-N and TAD-C (Pugh *et al.* 1997). In addition to the transactivation domains, the C-terminus contains a 3 C-NLS critical for the nuclear translocation of HIF-1α while N-NLS is reported to be less important (Kallio *et al.* 1998).

3.2.2. Regulation of HIF-1

HIF-1α is constitutively expressed, however, it is degraded under normoxic conditions. HIF-1α is stabilized under hypoxic conditions and forms a heterodimer with HIF-1β (ARNT). They are collectively termed HIF-1 and regulation primarily involves degradation of the HIF-1α subunit under normoxia and can occur at multiple levels including protein stabilization, post-translational modifications, nuclear translocation, dimerization, transcriptional activation and interaction with other proteins (Zagórska and Dulak 2004).

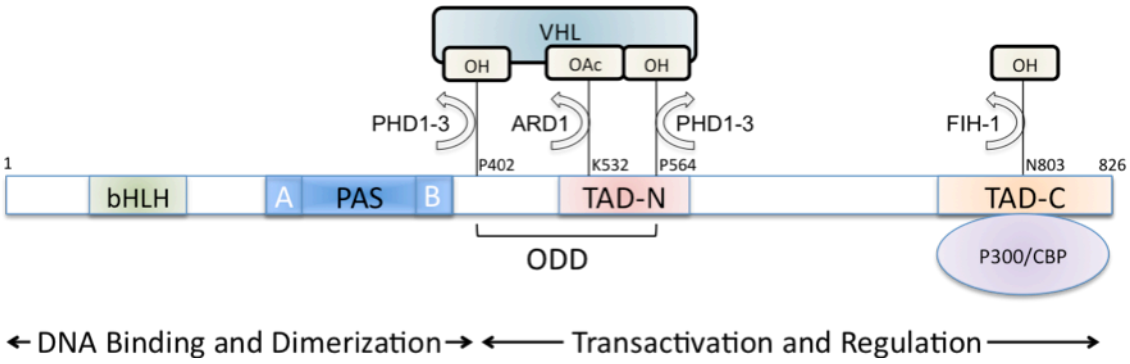

Figure 3.1. HIF-1α functional domains and regulatory regions. HIF-1α is made up of two main domains, basic helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains with TAD-N and TAD-C serving as transactivation domains. In normoxic conditions, alterations in the oxygen dependent domain (ODD) facilitate HIF-1α degradation. Proline residues P402 and P564 are hydroxylated by prolyl hydroxylase proteins 1-3 (PHD1-3) which is required for the binding of VHL. In addition, the acetylation of lysine residue K532 by ARD1 acetyltransferase aids in the binding of VHL. Another level of regulation under normoxic conditions is through the hydroxylation of the asaparagine residue N803 by the enzyme FIH-1. This hydroxylation blocks the binding of cofactors p300 and CBP which ultimately blocks HIF-1 mediated gene transcription. Under hypoxic conditions, PHD activity decreases resulting in less hydroxylation of P402 and P564 and VHL is unable to bind allowing HIF-1α stabilization. When N803 is not hydroxylated, cofactors p300 and CBP are able to bind allowing for the transcriptional activation of HIF-1α target genes. (Figure adapted from (Semenza 2003)).

3.2.2.1. Oxygen Dependent Degradation

Oxygen allows prolyl hydroxylases (PHDs) to act as a mediator notifying the cell of low oxygen conditions by serving as a low affinity substrate. When oxygen tension drops, PHDs are inactivated, resulting in the stabilization of HIF-1α (Stiehl *et al.* 2006). Under normoxic conditions and in the presence of iron, PHDs hydroxylate proline residues located in the ODD domain of HIF-1α (Figures 3.1 and 3.2) (Epstein *et al.* 2001). This allows for the subsequent binding of the β-domain of the von Hippel Lindau tumor suppressor protein (pVHL) to the ODD domain of HIF-1α (Bonicalzi *et al.* 2001). pVHL then acts as a substrate recognition component for the E3 ubiquitin ligase protein ultimately resulting in ubiquitination of both N- and C-terminus.

Hydroxylation of asparagine (Asn 803) on the TAD-C domain serves as another level of regulation (Figure 3.1) (Lando *et al.* 2002). Factor inhibiting HIF-1 (FIH-1), a asparingyl hydroxylase that modifies HIF-1α, is a member of the Fe(II)- and 2-oxoglutarate-dependent superfamily of deoxygenases (Lando *et al.* 2002). Asparagine hydroxylation of the TAD-C domain reduces interactions with cofactors such as p300/CBP resulting in the silencing of transactivation domains.

Both the ODD and TAD-C domains have been shown to regulate the activity of HIF-1α. Replacement of the proline residue with alanine resulted in a stable protein under normoxic conditions with low activity while replacement of the asparagine with an alanine residue resulted in an unstable protein (Lando *et al.* 2002). Hydroxylation of either the proline residue in the ODD or the asparagine residue in the TAD-C results in decreased overall activity compared to when neither residue is hydroxylated.

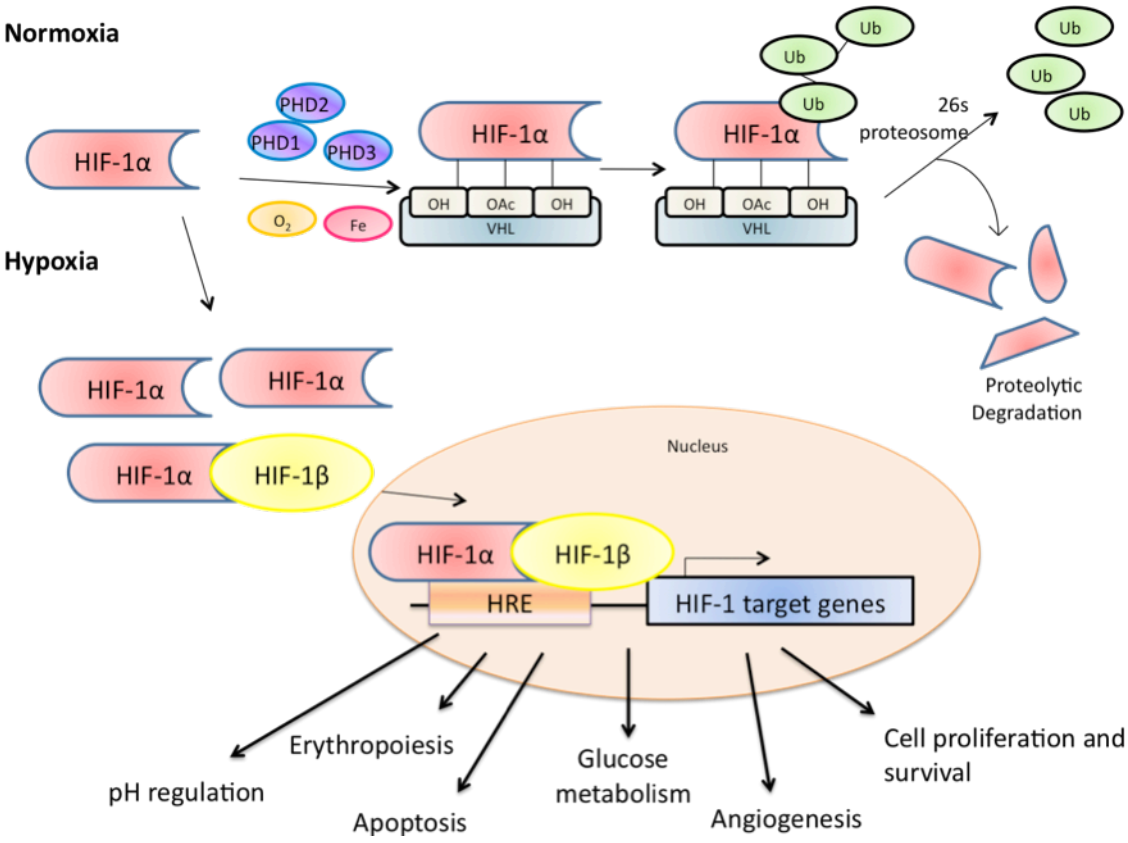

Figure 3.2. HIF-1α stabilization and HIF-1 target genes. Under normoxic conditions HIF-1α is hydroxylated by prolyl hydroxylases 1-3 (PHD1-3) allowing for the subsequent binding of the von Hippel Lindau factor (VHL). VHL serves as an E3 ubiquitin ligase and ubiquitination of HIF-1α results in proteolytic degradation. In the absence of oxygen, PHDs are inactive resulting in the stabiliation of HIF-1α protein. HIF-1α forms a heterodimer with HIF-1β forming the HIF-1 complex and translocates to the nucleus. The HIF1 complex binds hypoxic responsive elements (HREs) in the promoter region of HIF-1α target genes resulting in the activation of over 100 different genes involved in multiple cellular processes.

3.2.2.2. Oxygen Independent Degradation

Although HIF-1α was initially identified due to its effects during hypoxia, other contributing factors can also cause HIF-1α protein stabilization during normoxic conditions. Dysfunctions of enzymes and metabolic intermediates have both been shown to stabilize HIF-1α independent of low oxygen, termed pseudo-hypoxia (Lu *et al.* 2002).

3.2.2.2.1. Inhibitors of Prolyl Hydroxylases

Prolyl hydroxylases aid in coordinating the supply of HIF-1α protein levels during normoxic conditions and require an iron to catalyze the hydroxylation of HIF-1α. With the reliance of prolyl hydroxylases on iron, transition metals such as cobalt and nickel have been suggested to inhibit prolyl hydroxylases by substituting the ferrous ion coordinated by PHDs (Epstein *et al.* 2001). The iron chelator, desferrioxamine is also commonly used to mimic hypoxic conditions due to its ability to inhibit PHDs by the removal of iron from their catalytic domains (Wang and Semenza 1993).

3.2.2.2.2. Dysfunction of VHL

The main function of VHL is to act as a component of ubiquitin ligase that targets HIF-1α under normoxic conditions. Mutations in the VHL gene result in dysfunction of the VHL protein causing von Hippel-Lindau syndrome (Friedrich 1999). VHL syndrome typically exhibits aberrant HIF-1α stabilization and serves as a hereditary predisposition to the development of some cancers. Mutations in VHL have been reported at multiple sites that can result in tissue-specific risks of cancer (Hoffman *et al.* 2001). VHL syndrome has been linked to the development of retinoblastoma, central nervous systems

hemangioblastomas, clear cell renal carcinomas and pheochromocytomas (Hoffman *et al.* 2001).

3.2.3. Metabolic Intermediates

Changes in enzymes such as succinate dehydrogenase (SDH), fumarate hydratase (FH), and pyruvate have been shown to stabilize HIF-1α. SDH is located at the crossroads between the TCA cycle and the respiratory chain in the mitochondria. Mutations in the subunits of SDH have resulted in HIF-1α stabilization under normoxic conditions and the creation of a pseudo-hypoxic state. Ultimately, HIF-1α stabilization results in abnormal gene regulation promoting cellular proliferation, angiogenesis and alterations in glycolytic metabolism (Brière *et al.* 2005, Selak *et al.* 2005, Hao *et al.* 2009). A decrease in FH activity leads to an accumulation of fumarate that has also been found to coincide with HIF-1α stabilization (Isaacs *et al.* 2005). Excess fumarate is hypothesized to act as a competitive inhibitor of HIF-1α prolyl hydroxylase (HPH) competing with its co-substrate α-KG (Isaacs *et al.* 2005).

In addition, stoichiometric changes in pyruvate have been shown to stabilize HIF-1α and mediate gene expression (Lu *et al.* 2002, McFate *et al.* 2008, Sun *et al.* 2010). Pyruvate is formed within the Embden-Meyerhof pathway and is converted to acetyl-CoA by the thiamine-dependent enzyme PDH. Pyruvate has been suggested to stimulate HIF-1α protein accumulation through inhibition of HIF-1α degradation, however the exact mechanism remains undetermined (Lu *et al.* 2002). Ethyl pyruvate, a derivative of pyruvate has been shown to increase the stability of HIF-1α protein under normoxic conditions through the inhibition of its VHL factor mediated degradation (Kim *et al.*

2010). Pyruvate can also be interconverted to lactate via lactate dehydrogenase (LDHA). In addition to a buildup of pyruvate, excess lactate levels have also demonstrated HIF-1α protein stabilization (Lu *et al.* 2002). However, it is suggested lactate must first be converted back to pyruvate in order to stimulate HIF-1α protein accumulation (Lu *et al.* 2002).

3.2.4. HIF Binding to DNA

Once stabilized, HIF-1α forms a heterodimer with HIF-1β to bind hypoxic response elements in target genes. The majority of bHLH transcription factors have been shown to bind E-boxes located on the major groove of the DNA double helix (Ellenberger *et al.* 1994). While the direct association in unknown, both HIF-1α and HIF-1β have been suggested to associate with the major groove of the DNA binding helix in order to enhance transcription of HIF target genes (Bergeron *et al.* 1999).

3.2.5. HIF and Disease

Oxygen homeostasis is fundamental to all living cells and an imbalance in oxygen delivery can have detrimental effects. The HIF pathway is a key player responsible for maintaining a tight regulation on oxygen usage and is involved in the development of many pathologies. Specifically, hypoxia and the HIF pathway have been linked to heart disease, cerebro-vascular disease, developmental diseases and cancer (Semenza 2000, Ke and Costa 2006).

Positive effects of HIF-1α activation have been shown to be associated with ischemic, hypoxic and inflammatory conditions. Atherosclerosis is the blockage of

arteries by plaques that can ultimately lead to tissue ischemia. Acute ischemia and early infarction in the human heart have been reported to induce HIF-1α mRNA and protein expression (Lee *et al.* 2000). HIF-1α induces vascular endothelial growth factor expression to promote angiogenesis resulting in an increase in oxygen delivery to the ischemic region (Figure 3.2) (Lee *et al.* 2000). Induction of HIF-1α and the expression of HIF inducible genes have also been demonstrated in the ischemic retina, myocardial ischemia (sheep model), and cerebral ischemia (rat model) (Martin *et al.* 1998, Bergeron *et al.* 1999, Ozaki *et al.* 1999, Grimm *et al.* 2002).

Hypoxia also plays a critical role in embryonic development. During the first 10-12 weeks of pregnancy, human embryos develop under hypoxic conditions (Semenza 2000). The cells in the fetus and placenta exhibit a proliferative phenotype that is controlled by oxygen levels and the induction of HIF-1 target genes (Genbacev *et al.* 1996, Genbacev *et al.* 1997). The attempted creation of HIF-1$\alpha^{-/-}$ mice resulted in severe cardiac and vascular malfunctions ultimately resulting in embryonic lethality (Kotch *et al.* 1999). The fetus and placenta are exposed to and connect with maternal blood after the first trimester, resulting in a more invasive phenotype demonstrating decreased HIF-1α expression (Semenza 2000). Intrauterine growth retardation is a leading cause of fetal and neonatal morbidity and mortality. It is suggested to result from decreased placental perfusion causing placental and fetal hypoxia (Khaliq *et al.* 1999). Hypoxia induces insulin-like growth factor binding protein 1 which inhibits insulin-like growth factors resulting in deregulated fetal growth (Tazuke *et al.* 1998).

Adaptation of cells to low oxygen conditions is critical in tumor development due to rapid proliferation and inadequate vasculature. HIF is commonly over-expressed in

human cancers and as such plays an important role in tumor progression (Graeber *et al.* 1996, Zhong *et al.* 1999, Zagzag *et al.* 2000). HIF-1α activity in cancer has been suggested to be increased both by physiologic and epigenetic mechanisms (Semenza 2003, Vogelstein and Kinzler 2004). HIF-1α levels are reported to be positively correlated with tumor progression and poor patient survival in brain, breast, stomach, colorectal carcinoma, ovarian, cervical and prostate tumors among others (Yoshimura *et al.* 2004, Generali *et al.* 2006, Winter *et al.* 2006, Maynard and Ohh 2007, Generali *et al.* 2009). Alternatively, loss of HIF-1α activity in a nude mouse xenograft model resulted in increased tumor latency and decreased vascular density (Jiang *et al.* 1997, Maxwell *et al.* 1997, Ryan *et al.* 1998). Activation of HIF target genes gives cancer cells a distinct survival advantage allowing them to alter their reliance on metabolism that requires substantial amounts of oxygen (Figure 3.2).

3.2.6. Downstream effects of HIF-1α Stabilization

When cells experience reduced oxygen tensions, HIF-1α protein is stabilized and can impact the expression of over 100 genes involved in metabolism, erythropoiesis, angiogenesis, glucose metabolism, cell proliferation and apoptosis (Ke and Costa 2006). Using HIF-1α as an indicator of hypoxia, approximately 43 of 77 breast cancer specimens exhibited a significant level of HIF-1α expression (Gruber *et al.* 2004). Tumor hypoxia and the expression of HIF-1α have been correlated with being a predictive marker for chemotherapy and radiotherapy failure (Generali *et al.* 2006, Moeller and Dewhirst 2006). In addition, hypoxia has been linked directly to more aggressive tumors, promoting tumor stem cell or dedifferentiated phenotype, metastasis, disease relapse and

ultimately poor patient prognosis (Schindl *et al.* 2002, Gruber *et al.* 2004, Dales *et al.* 2005, Liao *et al.* 2007).

3.2.7. Transporters and Metabolism

As cancer cells undergo the cell cycle, metabolic requirements are met by the facilitated uptake of nutrients and the removal of metabolites utilizing transporters from the SLC and ATP Binding Cassette (ABC) superfamilies. The role of ABC proteins such as P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP), and the Multidrug Resistance Proteins (MRP's) have been well characterized to impart chemo-resistance to tumor cells by limiting intracellular drug permeability (Huang *et al.* 2004, Schinkel and Jonker 2012). Unlike ABC proteins, the precise involvement of SLC transporters in cancer cell growth, differentiation, and drug sensitivity are not thoroughly investigated. Currently there are greater than 300 SLC genes that function to transport endogenous nutrients and hormones such as glucose, amino acids, nucleosides, steroids, vitamins and essential inorganic cations and anions, etc (He *et al.* 2009). Recent efforts in understanding the role of SLC transporters in cancer have begun to focus on their epigenetic regulation in cancer, utility as prognostic indicators, and non-drug related transport functions supporting tumor suppressive or pro-survival roles in cancer (Gupta *et al.* 2006, Gupta *et al.* 2006, Thangaraju *et al.* 2006, Muto *et al.* 2007, Kaira *et al.* 2008, Lee *et al.* 2008, Lockhart *et al.* 2008, Whitman *et al.* 2008, Wlcek *et al.* 2008, Santini *et al.* 2010).

The activation of glycolytic pathways and genes by HIF-α are an essential cellular adaptation within tumor hypoxic microenvironments to maintain cellular

homeostasis, energy production, and proliferation (Harris 2002). To support these metabolic pathways, hypoxia-induced alterations in the expression of several SLC transporters appears to be an essential response to ensure nutrient requirements are maintained. The SLC transporters GLUT1 and GLUT3 are upregulated to increase glucose uptake in support of the hypoxia induced glycolytic shift (Chen *et al.* 2001, Macheda *et al.* 2005). The monocarboxylate transporter MCT4 has also been shown to be upregulated after exposure to hypoxia through HIF-1α mediated pathways to facilitate the removal of intracellular lactate converted from the glycolytic end product, pyruvate (Ullah *et al.* 2006). In comparison, the equilibrative nucleoside transporter, ENT1 is repressed under hypoxia in cardiomyocyte and endothelial cell lines (Chaudary *et al.* 2004, Casanello *et al.* 2005, Eltzschig *et al.* 2005).

3.2.7.1. Thiamine Transporters and Cancer

Clinically, thiamine deficiencies have been reported in early and advanced breast cancer patients as measured by the "TPP effect" (Basu and Dickerson 1976). To compensate for reduced cellular levels of thiamine, adaptive responses attempt to increase intracellular thiamine through the upregulation of thiamine transporter expression (Reidling and Said 2005). Alternatively, a decrease in SLC19A3 expression was demonstrated in breast cancer compared to corresponding normal tissue using a cDNA array (Liu *et al.* 2004). Down-regulation of SLC19A3 was also found in gastric and colon cancer (Liu *et al.* 2009, Ikehata *et al.* 2012). The decrease in expression appears to involve epigenetic repression through hypermethylation and histone

deacetylation of the SLC19A3 promoter (Liu *et al.* 2009, Ikehata *et al.* 2012). However, no correlation with THTR2 protein expression in cancer has been reported to date.

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. Unfortunately, there is limited information concerning the expression and function of the vast majority of SLC transporters that may also be involved in the adaptive cell survival responses within hypoxic tumor microenvironments. We hypothesize high affinity micronutrient transporters are altered in expression and function under hypoxic stress to maintain cellular metabolism. Therefore the objective of this work was to determine differences in expression patterns of the thiamine transporters between normal and cancerous tissue/cells, localization of thiamine transporters and expression changes of SLC transporters in a chronic hypoxia breast cancer cell culture model. Using a real time PCR array strategy, radiolabeled transport assay, and shRNA gene knockdown approach, we have found that SLC19A3, a thiamine transporter, was notably upregulated within breast cancer cells exposed to hypoxic stress.

3.3. MATERIALS AND METHODS

3.3.1. Breast cancer cDNA array

Expression levels of SLC19A2 and SLC19A3 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, USA). The cDNA array consisted of 48 clinical samples normalized to β-actin [see supplier's home page

(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 Web site to correspond with a specific 8–9 nucleotide hydrolysis probe labeled at the 5′ end with fluorescein. Probes used were #72 for SLC19A2 and #5 for SLC19A3. The β-actin reference assay kit supplied by Roche Applied Science was used as the internal control gene. Gene expression was normalized to β-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).

3.3.2. Drug Transporter PCR Array (RT-PCR)

BT474 cells chronically cultured between 64-88 days in 1% O_2 were used for real time PCR array profiling using the 96 well drug transporter RT^2 Profiler PCR array system from SABiosciences (Frederick, MD). RNA was extracted using E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA) followed by on-column DNASE digestion. Isolated RNA (2mg) was converted to cDNA and diluted with SYBR green master mix as per the manufacturer's protocol and supplied reagents (SABiosciences). The array was assayed using a LightCycler® 480 II (Roche Applied Science, Indianapolis, IN) and data analyzed utilizing the provided web based portal (SABiosciences) and $2^{-\Delta\Delta Ct}$ method for relative quantification.

3.3.3. qRT-PCR

Primer–probe combinations for each gene are as described in Chapter 2. The Tata-box-binding protein (TBP) reference assay kit supplied by Roche Applied Science

was used as a housekeeping gene. Fold expression comparing HMECs with breast cancer cell lines was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

3.3.4. Western blotting

The protein expression of THTR1 and THTR2 was assessed between breast epithelial and cancer cells by Western blot. Whole cell lysates were prepared as described in Chapter 2. 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 (8 ml) of the cell-impermeable Sulfo-NHS-SS-biotin (0.25 mg/ml) was added to each flask and incubated at 4°C for 30 min with gentle shaking followed by addition of a quenching solution provided by the manufacturer. Cells were then scraped and centrifuged at 500g for 3 min. 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-s bursts at low power (Branson Sonifier 150). Cells were then incubated on ice for 30 min with occasional mixing and centrifuged at 10,000g for 2 min at 4°C. The resulting supernatant was added to a NeutrAvidin Agarose column and incubated for 60 min at room temperature with end-over-end mixing. The column was centrifuged for 1 min at 1000g, flow through was discarded, and the column was rinsed with wash buffer containing protease inhibitors. SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer containing 50 mM dithiothreitol was added to the column and incubated for 60 min at

room temperature with end-over-end mixing. The column was centrifuged for 2 min at 1000g 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 (50 μg) and cell surface protein fractions (50 μg) were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). The membrane was immunoblotted with a 1:500 dilution of THTR1 (Alpha Diagnostics, San Antonio, TX, USA) or THTR2 (Proteintech Group, Chicago, IL, USA) antibodies. β-Actin (1:1000) was used as housekeeping protein (Thermo Scientific). The blots were then probed with a 1:15,000 goat anti-rabbit-HRP secondary antibody (Millipore, Billerica, MA, USA). Densitometry was performed using Fluorchem SP software.

3.3.5. Immunofluorescence

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 10 min, then blocked and permeabilized with 1% horse serum and 0.2% Triton X-100 for 30 min. 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 1 h at room temperature. Phosphate-buffered saline containing 0.02% Tween-20 (PBS-T) was used to wash the cells three times for 15 min each. Cells were incubated with secondary antibody (1:1000) (goat anti-rabbit IgG) conjugated with Alexa 594 (Invitrogen, Molecular Probes) for 1 h and then washed 3 times with PBS-T for 15 min each. Cover slips were mounted using ProLong Gold anti-fade reagent with DAPI

(Invitrogen, Eugene, OR, USA) on glass slides. Immunostained cells were imaged with a Nikon TM Eclipse fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) 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 75 nM LysoTracker probe for 30 min at 37°C. Medium was then removed, and cells were fixed and co-stained for THTR1 as described above.

3.3.6. Thiamine transport assay

BT474 cells grown in normoxia and chronically at 1% O_2 were seeded into 100 mm culture dishes and allowed to grow in their respective conditions until cells reached approximately 75% confluency. Cells were then harvested using trypsin/EDTA, collected and centrifuged at 600 g for 5 min. The cell pellet was washed twice with 37°C transport buffer (Hanks Balanced Salt Solution with 10 mM HEPES pH = 7.4). Transport was initiated by resuspending the cell pellet with 5 nM ^{3}H-thiamine in 37°C transport buffer. After 1, 5, 10 and 15 min, a 200 µL aliquot was removed and cells separated from free ^{3}H-thiamine by layering onto 50 µL of an 85:15 mixture of silicone oil (fluid 550, Dow Corning, Midland, IL): liquid paraffin (VWR, West Chester, PA). Cells were rapidly centrifuged at 14,000 g for 30s. During the course of the transport assay, the cell suspension was routinely inverted to avoid sedimentation and was maintained in a 37°C water bath. The resulting aqueous and oil layer was aspirated and the cell pellet lysed

using 1% Triton-X 100 and an aliquot used for liquid scintillation counting (LS-6500, Beckman Coulter, Brea, CA). Thiamine uptake was normalized to total cellular protein, measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL) and reported as pmol/mg protein.

3.3.7. Transfection with HIF-1α shRNA

To evaluate the role of HIF-1α in mediating the hypoxia induced changes in SLC19A3 and SLC19A2 expression, HIF-1α gene knockdown strategies were utilized. A scrambled sequence and four different 29mer HIF-1α shRNA constructs in pGFP-V-RS vector targeted against accession #NM_001530 and NM_181054 were obtained from OriGene (Rockville, MD). BT474 cells were seeded into a 6 well plate and were individually transfected with 2 μg of each construct using FuGENE HD (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Transfected cells were selected in 0.25 μg/ml puromycin (Mediatech, Manassas, VA) and assessed for HIF-1α knockdown by western blot and qRT-PCR as described above. Constructs demonstrating the greatest level of knockdown for HIF-1α mRNA measured by qRT-PCR and HIF-1α protein by western blot compared to non-transfected and scrambled sequence controls were used for experimentation.

3.3.8. Statistical analysis.

See description in Chapter 2.

3.4. RESULTS

3.4.1. Model Validation

For the purposes of this dissertation, some figures herein have been used from a paper by Zastre, et al. that was published as a joint effort by our laboratory (Zastre *et al.* 2013). The figures included in this section (Figures 3.4, 3.5, 3.6 and 3.7) were generated solely by Rebecca Sweet and the full text of the article can be found in the Appendix (Appendix 1).

Since cancer cells require an increase in energy, reductive capacity, and anabolic precursors to support unregulated cell division, upregulating the genes that determine thiamine homeostasis may be a necessary cellular adaptation to support proliferation. The amplified carbon flux by cancer cells to increase biomass during cell division may necessitate a coordinated enhancement in cellular thiamine status facilitated through the upregulated expression and/or activity of transporters and activating enzymes that regulate thiamine homeostasis (Zastre *et al.* 2013). Therefore, it was of interest to establish if the cellular determinants regulating thiamine homeostasis differ between breast cancer and normal breast epithelia.

3.4.1.1. Characterization of Gene Expression Between Normal and Breast Cancer Tissue

Real time qPCR arrays containing cDNA produced from isolated normal and breast cancer tissues were utilized to compare the gene expression of SLC19A2 and SLC19A3. Results in Figure 3.3 display box and whisker plots with error bars set at the 10th and 90th percentiles comparing the expression in normal tissue to total tumor tissue. Since the loss of estrogen receptor alpha (ERα) expression and overexpression of human

epidermal growth factor receptor 2 (HER2) has been linked with disease progression, results were also separated based on ERα and HER2 expression stated in pathology reports provided by the supplier. In the 43 tumor tissue samples, 27 were ERα (+), 10 were ERα (−), 11 were 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.

3.4.1.2. Comparative Gene Expression between HMECs and Breast Cancer Cell Lines

Figure 3.4 displays the gene expression profile of thiamine transporters in several common breast cancer cell lines compared to primary HMECs. 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 HMECs 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. In comparison, the ERα (−) cells BT20 and MDA-MB-231 exhibited

increases in the fold expression for SLC19A2 but to a lower extent than the ERα (+) cell lines.

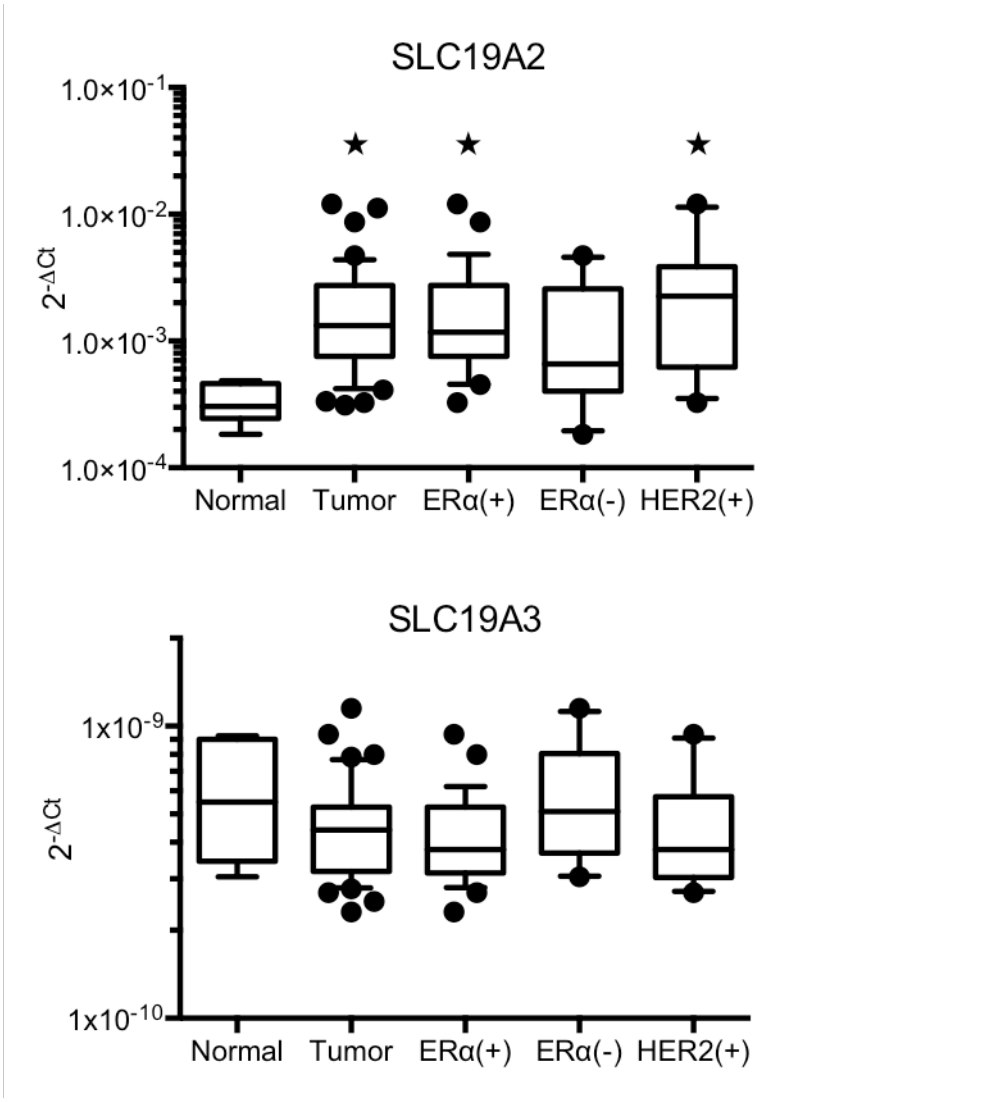

Figure 3.3. Expression of thiamine transporters SLC19A2 and SLC19A3, in breast cancer and normal tissues from 48 clinical samples. Gene expression levels were determined by qRTPCR using TissueScan Breast Cancer cDNA Array II. Results are expressed as the Log2 gene expression normalized to actin. Total tumor tissue was further differentiated by ER and HER2 status.

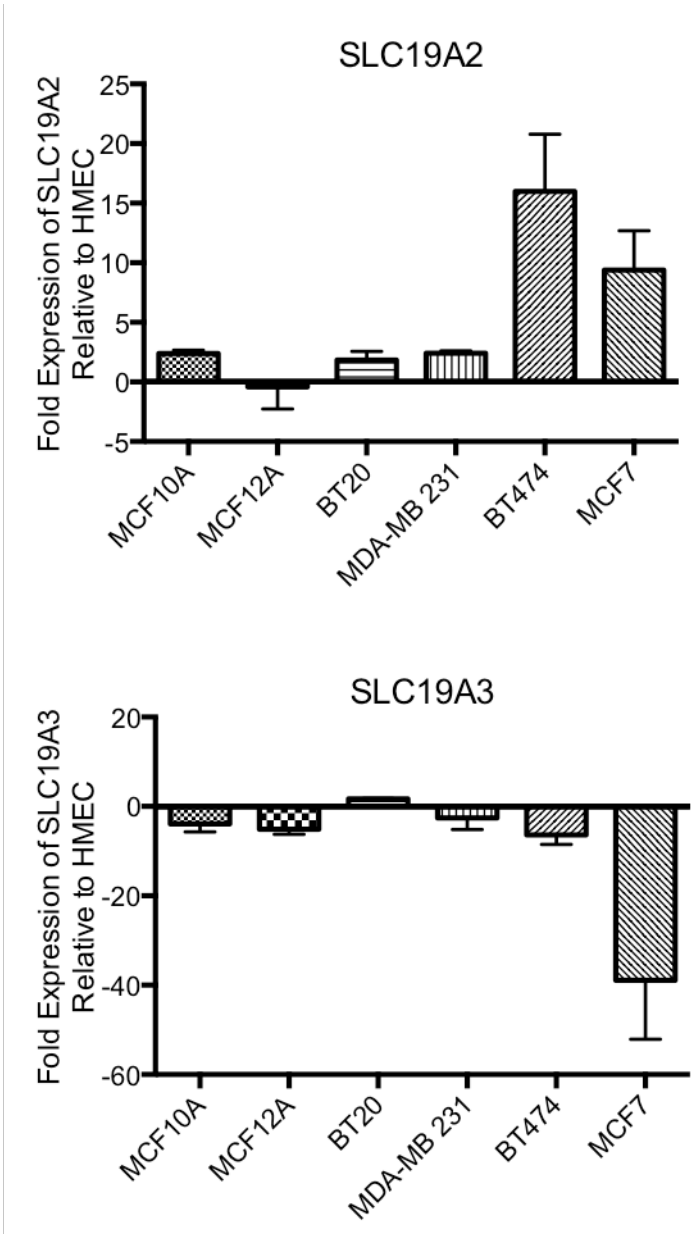

Figure 3.4. Expression of thiamine transporters SLC19A2 and SLC19A3 in immortalized non-tumorigenic breast epithelia and breast cancer cell lines compared to primary human mammary epithelial cells (HMECs). Gene expression levels were determined by qRTPCR using TBP as housekeeping gene. Results are expressed 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.

3.4.1.3. Protein expression of THTR1/2 in HMEC and breast cancer cells

To establish the relative expression of thiamine transporters at the protein level, whole cell lysates of all cell lines were evaluated using Western blotting. Results in Figure 3.5A show representative Western blots and relative density measurements of whole cell lysates for THTR1 and THTR2. 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 THTR1 in the nontransformed cell lines MCF12A and MCF10A was consistent with HMEC, except for a greater band intensity of THTR2 in MCF12A 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 3.5B 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 *et al.* 2003, Ashokkumar *et al.* 2006). Consistent with total SLC19A2 (THTR1) gene expression (Figure 3.4), 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 that no surface expression was detected for BT20 cells.

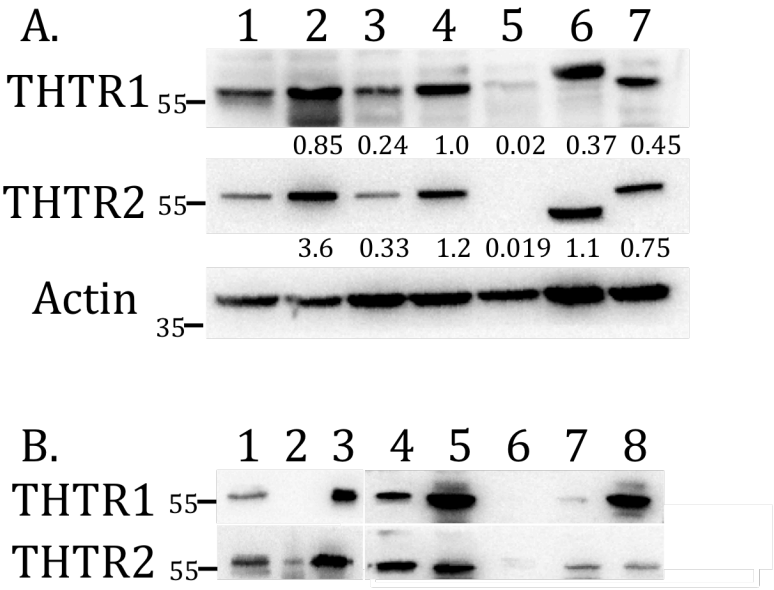

Figure 3.5. Representative Western blots of THTR1 and THTR2 in immortalized non-tumorigenic breast epithelia, breast cancer cell lines, and HMECs. (A) Whole cell lysate preparations of (1) HMEC, (2) MCF12A, (3) MCF10A, (4) BT474, (5) BT20, (6) MDA-MB-231, (7) MCF7 were immunofractionated on a 10% SDS-Page gel. Beta-actin was used as housekeeping protein. (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.

3.4.1.4. 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 (Figure 3.5B), THTR1 was readily detected on the plasma membrane for MCF12A, BT474 and MCF7 cells (Figure 3.6A). 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 intra-cytoplasmic staining. Also consistent with the membrane biotinylation studies (Figure 3.5B), limited to no cell surface staining for THTR1 was discernible for HMECs, BT20 and MDA-MB-231 cells (Figure 3.6A). In contrast to the cell surface localization (Figure 3.5B), THTR2 appeared to be predominantly confined intracellularly with minimal membrane localization discernible by immunofluorescence for all cell lines evaluated (Figure 3.6A).

To detect the identity of the cytoplasmic compartments in which THTR1 was localized, we performed colocalization analyses of THTR1 with markers for various organelles (viz., ER, mitochondria and lysosomes). Our results indicated that intracytoplasmic THTR1 in BT474 was predominantly localized in the lysosomes as judged by significant colocalization of THTR1 with a LysoTracker dye (Figure 3.6B).

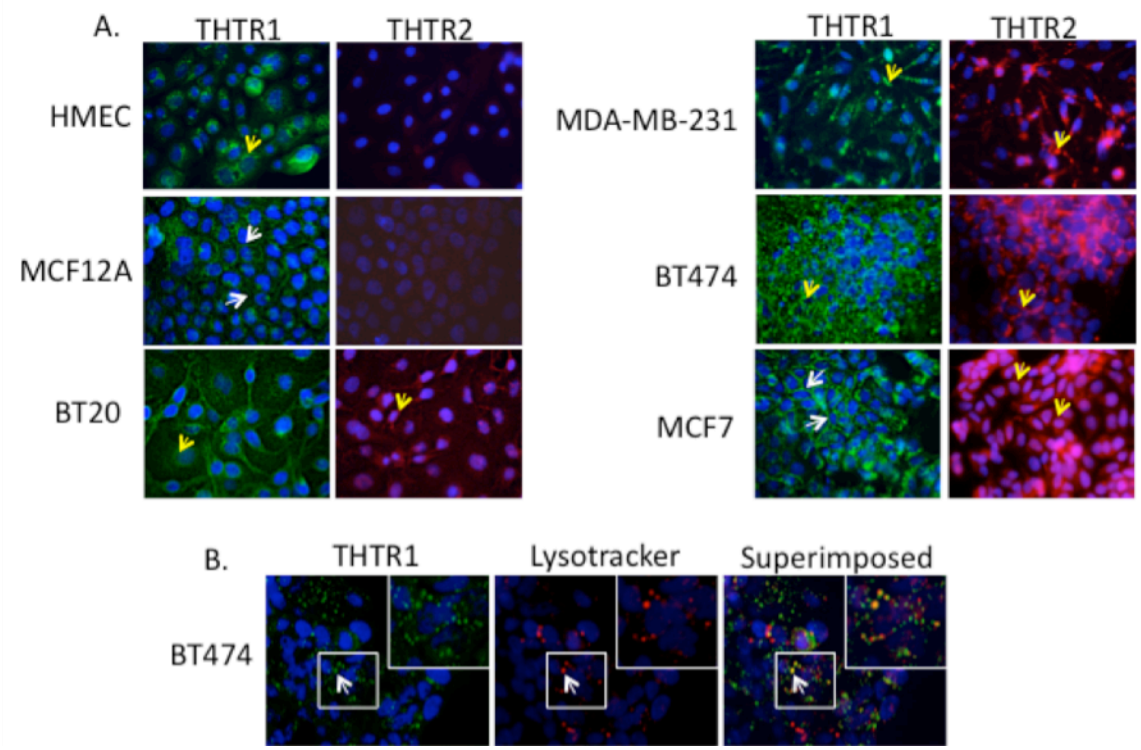

Figure 3.6. Cellular localization of THTR1 and THTR2 by Immunofluorescence. (A) Localization of THTR1 and THTR2 in breast cancer cell lines compared with HMECs and MCF12A cells. (B) Co-localization of Lysotracker dye with THTR1 in BT474 cells.

3.4.2. Expression profiling by RT-PCR array

Gene expression profiling heat map for BT474 cells chronically cultured in hypoxia compared to similar culture age BT474 cells grown under standard normoxic conditions are shown in Figure 3.7. The drug transporter array consists of 29 ABC and 55 SLC genes and provides a reasonable gene set for data mining changes in expression of transporters between normoxic and hypoxic cells. Transporters excluded from Figure 3.7 and Table 3.1 exhibited crossing points greater than the set threshold of 35 or exhibited non-specific melt curves. Interestingly, SLC19A3 (THTR2), a thiamine transporter was found to be up-regulated approximately 31 fold (Table 3.1). Known hypoxia responsive genes such as SLC2A1 (GLUT1) and SLC2A3 (GLUT3) were upregulated approximately 1.6 and 2.4 fold respectively in hypoxic BT474 cells. Similarly, SLC16A3 (MCT4) was up-regulated 5.4 fold compared to normoxic cells (Table 3.1). To highlight several transporters with altered regulation, the concentrative nucleoside transporter, SLC28A2, up-regulated 3.1 fold while the equilibrative nucleoside transporters, SLC29A1 (ENT1) and SLC29A2 (ENT2) were both down-regulated 1.8 fold (Table 3.1). The copper transporter SLC31A1 (CTR1) was down-regulated 2.2 fold and the breast cancer resistance transporter (ABCG2) was down-regulated 3.1 fold. Since SLC19A3 showed the highest fold change and due to the role of thiamine as an essential cofactor in glycolytic pathways, we chose to further investigate the expression of the thiamine transporters, SLC19A2 and SLC19A3 in hypoxia.

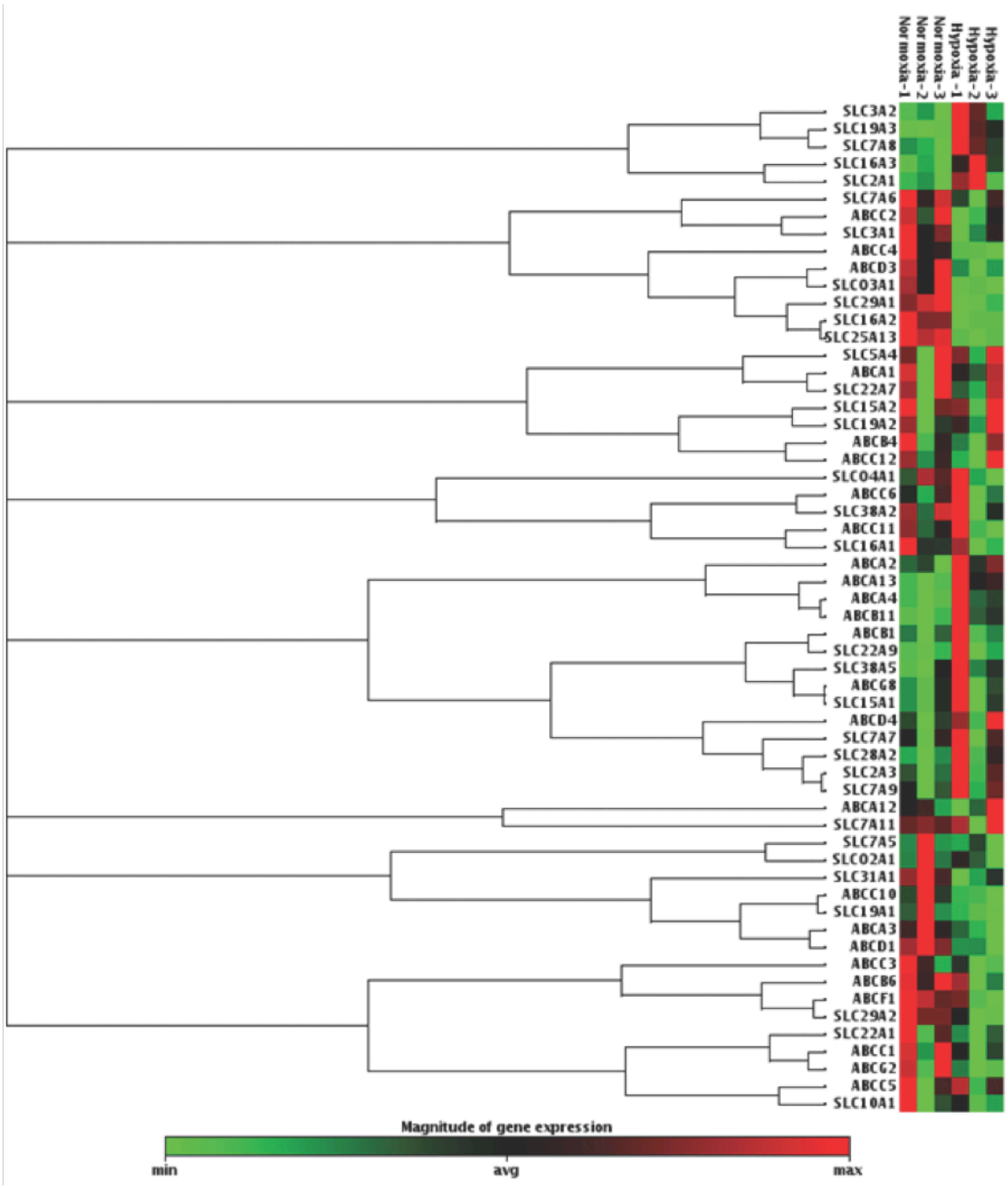

Figure 3.7. Drug transporter expression profiling by RT-PCR array of BT474 cells chronically cultured in 1% O_2 compared to normoxic cultures. BT474 cells cultured under hypoxic (1% O_2) conditions ranged from 64-88 days and compared to age matched normoxic cultures.

Table 3.1. Fold changes in transporter expression within BT474 cells exposed to chronic hypoxia compared to normoxia using RT-PCR drug transporter array.

Gene	Gene Bank Number	Protein	Common Name	Fold Change
ABCA1	NM_005502	ATP-binding cassette, sub-family A (ABC1), member 1	ABC-1, CERP	1.01
ABCA2	NM_001606	ATP-binding cassette, sub-family A (ABC1), member 2	ABC2	1.55
ABCA3	NM_001089	ATP-binding cassette, sub-family A (ABC1), member 3	ABC-C, ABC3	-1.52
ABCA4	NM_000350	ATP-binding cassette, sub-family A (ABC1), member 4	ABC10, ABCR, ARMD2, CORD3	4.96
ABCA12	NM_173076	ATP-binding cassette, sub-family A (ABC1), member 12		-1.03
ABCA13	NM_152701	ATP-binding cassette, sub-family A (ABC1), member 13		4.75
ABCB1	NM_000927	ATP-binding cassette, sub-family B (MDR/TAP), member 1	MDR1, P-gp	1.33
ABCB4	NM_000443	ATP-binding cassette, sub-family B (MDR/TAP), member 4	ABC21, GBD1, MDR2	-1.42
ABCB6	NM_005689	ATP-binding cassette, sub-family B (MDR/TAP), member 6	ABC, ABC14. PRP	-1.53
ABCB11	NM_003742	ATP-binding cassette, sub-family B (MDR/TAP), member 11	BSEP	3.53
ABCC1	NM_004996	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	ABC29, ABCC, MRP, MRP1	-1.25
ABCC2	NM_000392	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	MRP2, cMRP	-2.07
ABCC3	NM_003786	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	MLP2, MOAT-D, MRP3	-1.88
ABCC4	NM_005845	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	MOAT-B, MRP4	-3.39
ABCC5	NM_005688	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	MOAT-C, MRP5, SMRP	1.02
ABCC6	NM_001171	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	MOATE, MRP6, PXE, PXE1	-1.16
ABCC10	NM_033450	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	MRP7, SIMRP7	-1.65
ABCC11	NM_032583	ATP-binding cassette, sub-family C (CFTR/MRP), member 11	MRP8	-1.23
ABCC12	NM_033226	ATP-binding cassette, sub-family C (CFTR/MRP), member 12	MRP9	-1.66
ABCD1	NM_000033	ATP-binding cassette, sub-family D (ALD), member 1	ALD, ALDP, AMN	-1.85
ABCD3	NM_002858	ATP-binding cassette, sub-family D (ALD), member 3	PMP70, PXMP1	-1.93
ABCD4	NM_005050	ATP-binding cassette, sub-family D (ALD), member 4	ABC41	1.87
ABCF1	NM_001090	ATP-binding cassette, sub-family F (GCN20), member 1	ABC27, ABC50	-1.54
ABCG2	NM_004827	ATP-binding cassette, sub-family G (WHITE), member 2	BCRP, BCRP1, BMDP	-3.15
ABCG8	NM_022437	ATP-binding cassette, sub-family G (WHITE), member 8	GBD4	1.23
SLC2A1	NM_006516	Solute carrier family 2 (facilitated glucose transporter), member 1	GLUT1	1.58
SLC2A3	NM_006931	Solute carrier family 2 (facilitated glucose transporter), member 3	GLUT3	2.40
SLC3A1	NM_000341	Solute carrier family 3 (cystine, dibasic and neutral amino acid transporters), member 1	ATR1, CSNU1, D2H	-1.61
SLC3A2	NM_002394	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2		1.97
SLC5A4	NM_014227	Solute carrier family 5 (low affinity glucose cotransporter), member 4	SGLT3	1.22
SLC7A5	NM_003486	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	LAT1	-1.41
SLC7A6	NM_003983	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 6	LAT-2	-1.52
SLC7A7	NM_003982	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	LAT3	1.22
SLC7A8	NM_182728	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8	LAT2	1.34
SLC7A9	NM_014270	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 9	BAT1	1.62
SLC7A11	NM_014331	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	CCBR1	-1.20
SLC10A1	NM_003049	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	NTCP	-1.24

SLC15A1	NM_005073	Solute carrier family 15 (oligopeptide transporter), member 1	HPECT1, HPEPT1, PEPT1	1.25
SLC15A2	NM_021082	Solute carrier family 15 (H+/peptide transporter), member 2	PEPT2	1.08
SLC16A1	NM_003051	Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	MCT1	-1.69
SLC16A2	NM_006517	Solute carrier family 16, member 2 (monocarboxylic acid transporter 8)	MCT7	-3.30
SLC16A3	NM_004207	Solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	MCT3, MCT4	5.40
SLC19A1	NM_194255	Solute carrier family 19 (folate transporter), member 1	CHMD, FOLT, IFC1, REF(	-2.27
SLC19A2	NM_006996	Solute carrier family 19 (thiamine transporter), member 2	TC1, THT1, THTR1, TI	1.14
SLC19A3	NM_025243	Solute carrier family 19, member 3THTR2	THTR2	31.1
SLC22A1	NM_003057	Solute carrier family 22 (organic cation transporter), member 1	OCT1	-1.63
SLC22A7	NM_006672	Solute carrier family 22 (organic anion transporter), member 7	OAT2	-1.09
SLC22A9	NM_080866	Solute carrier family 22 (organic anion transporter), member 9	OAT4, OAT7	1.90
SLC25A13	NM_014251	Solute carrier family 25, member 13 (citrin)	CTLN2	-7.06
SLC28A2	NM_004212	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	CNT2, HCNT2	3.12
SLC29A1	NM_004955	Solute carrier family 29 (nucleoside transporters), member 1	ENT1	-1.84
SLC29A2	NM_001532	Solute carrier family 29 (nucleoside transporters), member 2	ENT2	-1.81
SLC31A1	NM_001859	Solute carrier family 31 (copper transporters), member 1	CTR1	-2.16
SLC38A2	NM_018976	Solute carrier family 38, member 2	SAT2, SNAT2	-1.15
SLC38A5	NM_033518	Solute carrier family 38, member 5	SN2	1.26
SLCO2A1	NM_005630	Solute carrier organic anion transporter family, member 2A1	PGT	-1.37
SLCO3A1	NM_013272	Solute carrier organic anion transporter family, member 3A1	OATP-D	-6.25
SLCO4A1	NM_016354	Solute carrier organic anion transporter family, member 4A1	OATP-E	-1.14

3.4.3. Thiamine transport in hypoxic versus normoxic BT474 cells

Thiamine transport assays were performed over 15 min to determine if the increase in SLC19A3 expression results in changes in thiamine transport in hypoxic BT474 cells. Thiamine transport within hypoxic and normoxic BT474 cells was linear over 15 min (Figure 3.8). An approximately 2 fold increase in thiamine uptake at 5, 10, and 15 min was found in BT474 cultured in chronic 1% O_2 compared to normoxic cultures (Figure 3.8).

3.4.4. Expression of SLC19A2 and SLC19A3 after acute 1% O_2 and DFO exposure

To determine the effect of acute hypoxia on the expression of the thiamine transporters, SLC19A2 and SLC19A3 mRNA expression levels were detected using qRT-PCR after 24, 48 and 72h exposure to 1% O_2. SLC19A3 expression levels increased up to 7.5 and 3 fold in BT474 and MCF7 cells, respectively, after 72h exposure (Figures 3.9A and 3.9B). SLC19A3 also increased in estrogen receptor negative cell lines BT20 and MDA-MB-231 after 1% O_2 up to 72h, however to a lesser extent than the estrogen receptor positive cell lines (Figures 3.9C and 3.9D). There was no change in SLC19A2 after hypoxic exposure in all of the breast cancer cell lines tested.

As a complementary approach, BT474 cells were treated with the chemical hypoxic mimetic, DFO (250mM). Figure 3.10A demonstrates that over the time course of exposure to either 1% O_2 or DFO, HIF-1α protein was detectable and persistent. After 72h exposure to DFO, SLC19A3 transporter expression was upregulated approximately 70 fold while no change in SLC19A2 transporter expression was observed compared to untreated cells (Figure 3.10B).

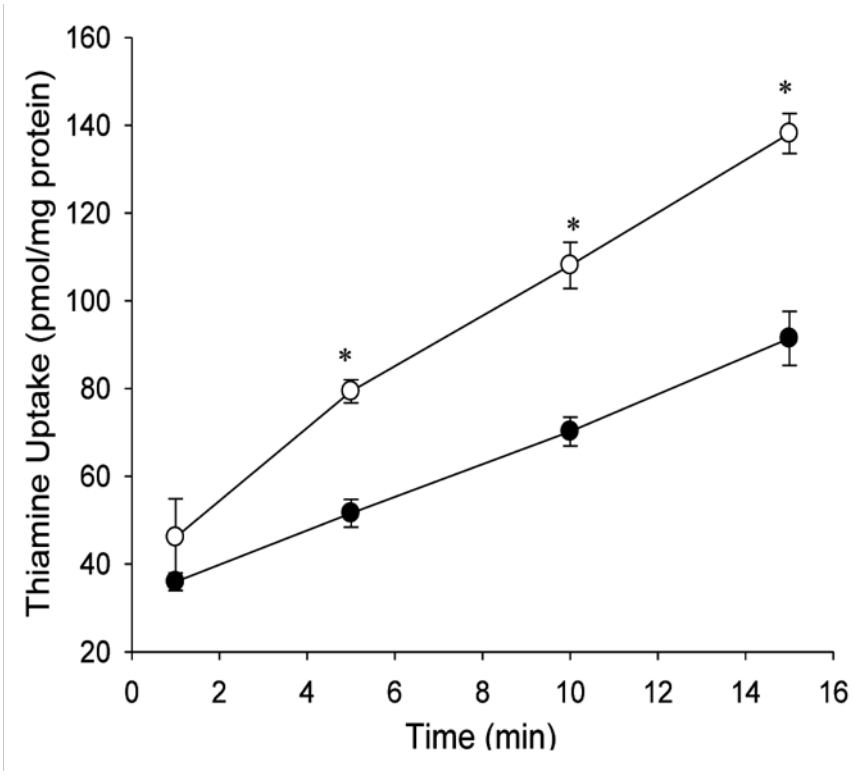

Figure 3.8. Thiamine transport after hypoxic exposure. Thiamine uptake into hypoxic versus normoxic BT474 cells. BT474 cells grown in normoxia (●) and chronically in 1% O_2 (○) were suspended with 5 nM 3H-thiamine in 37^0C transport buffer. At time points of 1, 5, 10 and 15 min, an aliquot was removed and uptake was stopped by centrifuging through a silicone oil:liquid paraffin layer. The resulting cell pellet was lysed and thiamine uptake determined by scintillation counting and normalized to total cellular protein. Results are

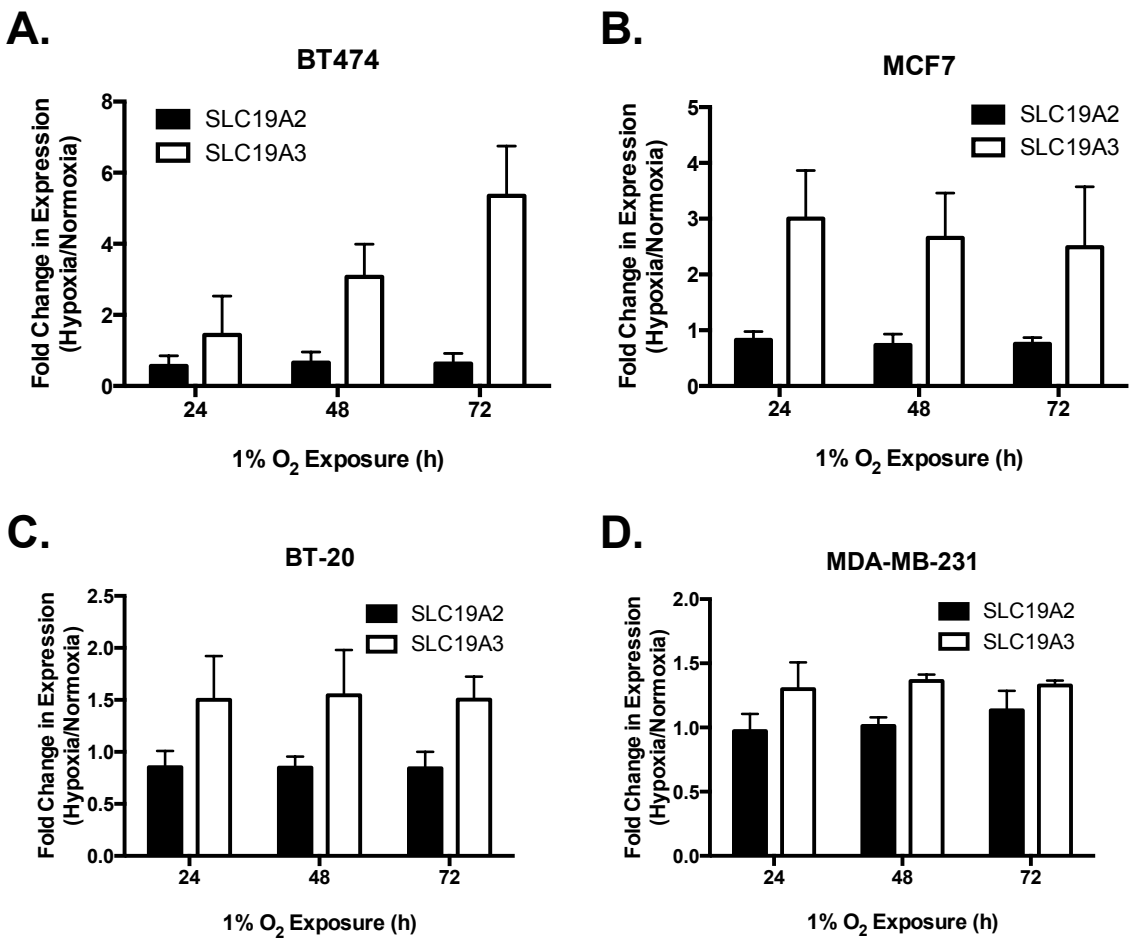

Figure 3.9. Expression of SLC19A2 and SLC19A3 after acute hypoxia. (A) BT474, (B) MCF7, (C) BT20 and (D) MDA-MB-231 cells were acutely exposed to 1% O_2 for 24, 48 and 72h. qRT-PCR was performed to determine the expression of SLC19A2 and SLC19A3 using TBP as the house keeping gene. Results are expressed as an average fold change +/- standard deviation compared to cells cultured under normoxic conditions with n=3 independent experiments.

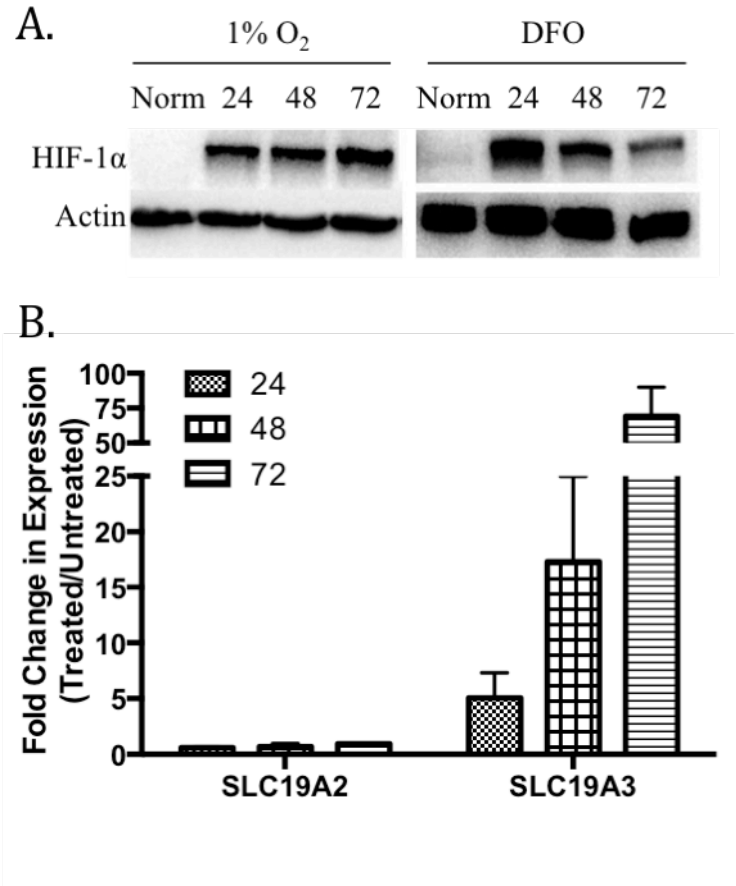

Figure 3.10. Expression of SLC19A2 and SLC19A3 after treatment with DFO. (A) Western blot showing HIF-1α protein expression after exposure for 1% O_2 and DFO up to 72 h. (B) mRNA expression levels of SLC19A2 and SLC19A3 in BT474 ells after treatment with DFO 24, 48, and 72 h.

3.4.5. Impact of thiamine depletion and excess on SLC19A2 and SLC19A3 expression

In case the glycolytic shift associated with hypoxia depleted the level of exogenous thiamine supplied in culture media, we supplemented media with 1mM thiamine and assessed expression changes of SLC19A2 and SLC19A3 after 72h of 1% O_2 exposure. Figure 3.11A shows that the fold change of SLC19A2 and SLC19A3 gene expression is comparable to that described in Figure 3.11A. In contrast, when BT474 cells were exposed to thiamine-deficient media for 72h under normoxic conditions no change in expression for both transporters was found compared to cells exposed to regular cell culture growth media.

3.4.6. Role of HIF-1a in SLC19A2 and SLC19A3 expression in hypoxia

The potential role of HIF-1α in mediating SLC19A3 expression changes in hypoxia was evaluated using HIF-1α knockdown strategies. BT474 cells were transfected with shRNA HIF-1α constructs and a scrambled sequence followed by puromycin selection to ensure consistent knockdown of HIF-1α during the course of hypoxia treatment. Cells with stable HIF-1α knockdown demonstrated approximately 70% knockdown at the mRNA level measured by qRT-PCR (data not shown). HIF-1α knockdown substantially reduced HIF-1α protein levels after exposure to 1% O_2 over 72h compared to scrambled shRNA sequence (Figure 3.12A). No significant change in SLC19A2 expression was found over 72h exposure to 1% O_2 between scrambled and HIF-1α knockdown (Figure 3.12B). However, a significant decrease in SLC19A3 expression of approximately 3 fold was observed after 1% O_2 exposure for 72h compared to scrambled (Figure 3.12C).

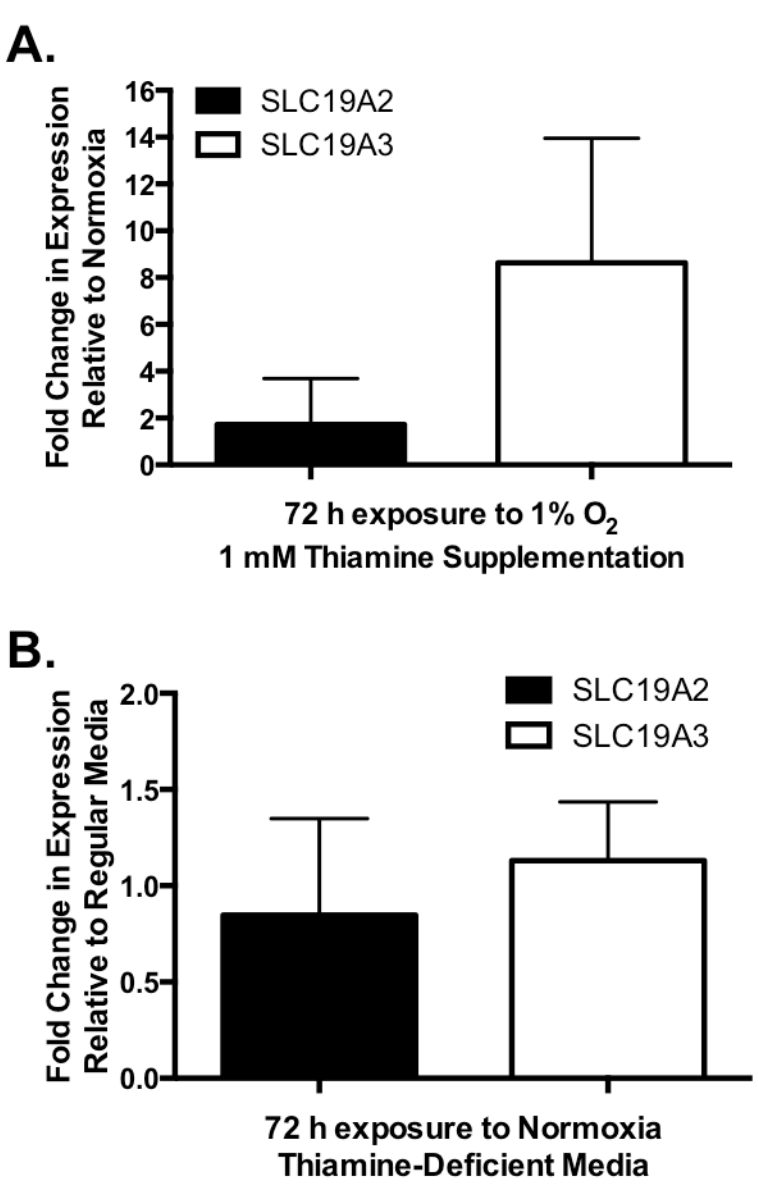

Figure 3.11. Impact of excess thiamine supplementation and depletion on SLC19A2 and SLC19A3 expression. (A) BT474 cells were treated with culture media supplemented with 1mM thiamine and exposed to hypoxic conditions (1% O_2) for 72h. qRT-PCR was performed to determine SLC19A2 and SLC19A3 expression and results are presented as fold change with +/- standard deviation relative to BT474 cells cultured under normoxic conditions for n=3 independent experiments. (B) BT474 cells were exposed to thiamine depleted culture media for 72h under normoxic conditions. qRT-PCR was performed to assess SLC19A2 and SLC19A3 expression and results are presented as an average fold change +/- standard deviation relative to BT474 cells cultured with regular culture media under normoxic conditions for n=3 independent experiments.

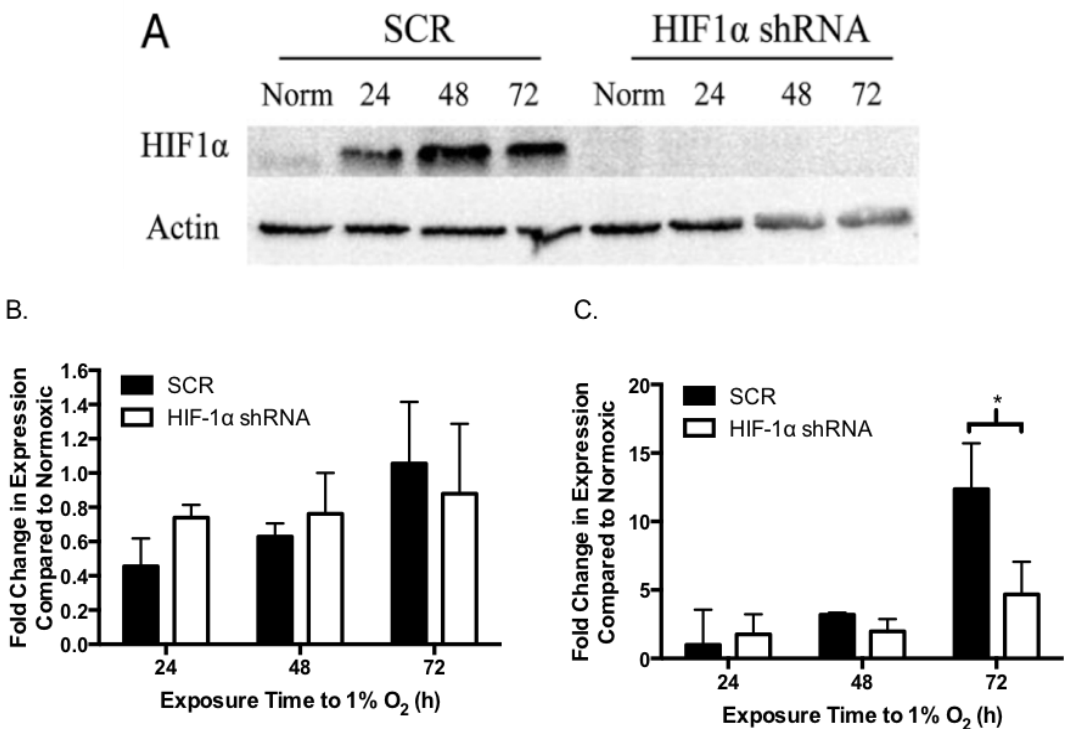

Figure 3.12. Role of HIF-1α in SLC19A2 and SLC19A3 expression. BT474 cells were transfected with 2mg HIF-1α shRNA or scrambled sequence using FuGENE HD. Transfected cells were selected using puromycin. (A) A representative Western blot for HIF-1a and b-actin as loading control comparing scrambled sequence to HIF-1a knockdown after 24, 48, and 72h exposure to 1% O_2. Expression of (B) SLC19A2 and (C) SLC19A3 was determined using qRT-PCR with TBP as the house keeping gene. Results are reported as fold change in expression +/- standard deviation compared to untreated normoxic BT474 cells for n=3 independent experiments. (*) Statistically significant differences $p < 0.05$.

3.5. 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. As established by Warburg, cancer cells are highly glycolytic under aerobic conditions providing them with a metabolic and growth advantage compared to normal cells (Warburg 1956). The glycolytic markers, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase have been found to increase in breast carcinomas (Isidoro *et al.* 2005). In addition, Akt and transketolase-like protein 1 (TKTL1) are also upregulated to support the glycolytic phenotype of breast cancer cells (Schmidt *et al.* 2010). This metabolically demanding process is supported in part by oncogene-mediated regulation of nutrient transport processes to increase cellular supply of carbon sources (Kim *et al.* 2004, Wise *et al.* 2008). This also may necessitate a coordinated enhancement in coenzyme supply to support enzyme activity.

Using cDNA qRT-PCR arrays, we have established that SLC19A2 is over-expressed in clinical breast cancer tissue samples. To further expand on these findings, we compared the gene and protein expression of thiamine transporters in several common breast cancer cell lines to primary HMECs. A similar trend of gene overexpression of SLC19A2 and down-regulation of SLC19A3 was observed in the majority of breast cancer cell lines tested compared with clinical samples. Our findings of a decrease in SLC19A3 in breast cancer tissue support previous reports in gastric and colon cancer (Liu *et al.* 2009, Ikehata *et al.* 2012). When compared to HMECs, gene and protein expression levels of the thiamine homeostasis genes were similar to the nontumorigenic

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 three of the four cancer cell lines evaluated, protein expression of THTR1 was greater than the non-tumor tissue control, consistent with the relative increase in gene expression. 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 *et al.* 2003, Liu *et al.* 2009, Ikehata *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 *et al.* 2003, Subramanian *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 and 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 colocalized with THTR1 at the plasma membrane that enhances THTR1 stability (Nabokina *et al.* 2011). Tetraspanin proteins are important in cell motility and invasion and are associated with acting as a tumor metastasis suppressor (Richardson *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 *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 three to five 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 *et al.* 2003, Subramanian *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 punctate 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 nontumorigenic 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 *et al.* 1999, Ashokkumar *et al.* 2006). Activation and/or inhibition of protein kinase pathways has been shown to modulate membrane trafficking and transport activity of other solute carrier transporters, such as OAT1 and OATP1A2 (Zielinska-Dawidziak *et al.* 2008, Zhou *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.

Under anaerobic conditions, both GLUT1 (SLC2A1) and GLUT3 (SLC2A3) are upregulated to support increased glucose cellular uptake and energy production via the glycolytic pathway (Chen *et al.* 2001, Macheda *et al.* 2005). An increased pyruvate dehydrogenase kinase activity under hypoxia inhibits pyruvate dehydrogenase and ultimately the conversion of pyruvate to acetyl-CoA, redirecting pyruvate away from the mitochondria toward lactate (Kim *et al.* 2006). An increase in the plasma membrane lactate transporter MCT4 (SLC16A3) has also been reported, which relieves the cell of accumulated lactic acid produced during glycolysis under hypoxic conditions (Ullah *et al.* 2006). Thus, the activation of glycolytic pathways and expression of SLC transporters appears to be a critical cellular adaptation to maintain cellular metabolism and homeostasis under hypoxic stress.

To further characterize transporter expression changes within hypoxic breast cancer cells, we utilized a chronic hypoxia cell culture model and performed a RT-PCR array assay to assess changes in transporter expression (Figure 3.8). BT474 cells were chosen since these cells originated from a primary site invasive ductal carcinoma (Lacroix and Leclercq 2004). The results in Table 3.1 have demonstrated that various transporters appear to be differentially regulated as a result of hypoxic stress. In agreement with previous reports, an increase in the hypoxia responsive transporter genes SLC2A1 (1.6 fold), SLC2A3 (2.4), and SLC16A3 (5.4) was observed in hypoxic BT474 cells (Table 3.1). Other transporters demonstrating an increase in expression included SLC28A2 (3.12), ABCB11 (3.53), ABCA2 (1.55), ABCA4 (4.96), ABCA13 (4.75), ABCB11 (3.53), SLC3A2 (1.97), and SLC22A9 (1.90). Alternatively, SLC29A1 (-1.84), SLC29A2 (-1.81), ABCG2 (-3.15), SLC31A1 (-2.16), ABCC2 (-2.07), ABCC3 (-1.88), ABCC4 (-3.39), SLC3A1 (-1.61), SLC7A6 (-1.52), SLC16A1 (-1.69), SLC16A2 (-3.30), SLCO3A1 (-6.25), and SLC19A1 (-2.27) showed a decrease in expression after hypoxic stress. Transport of anticancer drugs by SLC transporters can be a major contributing factor in the efficacy of chemotherapeutic drugs. Transporters demonstrating changes in our hypoxia model include SLC28A2, SLC29A1, SLC31A1, and SLC19A1 have also been shown to be essential for chemosensitivity of drugs such as gemcitabine, cisplatin, and methotrexate, respectively (Worm *et al.* 2001, Holzer *et al.* 2004, Oguri *et al.* 2007). Therefore, these findings suggest that regulatory changes in ABC and SLC transporter expression within hypoxic breast cancer cells may contribute to differences in chemosensitivity and chemoresistance within hypoxic cells. Furthermore, altered

expression during hypoxic stress may be an essential adaptation to maintain transport of nutrients, and cofactors to support energy production and biosynthesis.

Interestingly, the thiamine transporter SLC19A3 was found to be upregulated during chronic hypoxic culturing in the breast cancer cell line, BT474 (Table 3.1 and Figure 3.9). To establish if an increase in SLC19A3 expression occurs after acute hypoxia exposure, cells were treated for up to 72h in 1% O_2 or with DFO. Although it is unclear why there was a substantially different extent of SLC19A3 expression between 1% O_2 (7.5 fold in BT474 and 2.5 fold in MCF7) and DFO (70 fold), both treatments demonstrated no change in SLC19A2 (Figure 3.11A, 11B and 11C). The magnitude for DFO induced up-regulation (~70 fold) resembles the response observed within chronic hypoxia (~41 fold) (Figure 3.9). The mechanism of action for HIF-1α stabilization by the iron chelator DFO is through inhibition of iron dependent hydroxylase activity (Yuan *et al.* 2003). Since we did not perform dose dependent studies for DFO mediated SLC19A3 induction, it is plausible that we have induced a representative anoxic state through complete inhibition of hydroylase activity. Although the magnitude of HIF-1α stabilization is not evident in Figure 3.11C comparing 1% O_2 with DFO, the presence of limited oxygen levels (1%) implies we do not have anoxic conditions. Therefore, the chemical hypoxia mimetic DFO may be inducing a more exaggerated response, while exposure to the more relevant treatment of 1% O_2 requires a longer time to facilitate a higher level of expression.

Our findings, along with recent evidence have demonstrated a down regulation of SLC19A3 in breast and gastric cancers (Liu *et al.* 2003, Liu *et al.* 2009, Zastre *et al.* 2013). However, these studies compared whole tumor tissue to normal tissue and did not

address potential cellular heterogeneity within tumors due to microenvironment effects such as hypoxia. In contrast, our results suggest that under hypoxic stress, upregulation or re-establishment of SLC19A3 expression in breast cancer cells may be an important adaptive response. Our result not only demonstrated an increase in SLC19A3 transporter expression but an increase in overall thiamine transport which could potentially be supporting the glycolytic phenotype of hypoxic cancer cells (Figure 3.10). Clinically, thiamine deficiencies have been reported in advanced cancer patients and high dose thiamine supplementation is commonly used as a nutritional supplement (Basu and Dickerson 1976, Boros *et al.* 1998, Comín Anduix *et al.* 2001). However, increasing thiamine supplementation 12 – 25 times the recommended daily allowance to mice stimulated the growth of an Ehrlich's ascites tumor model (Comín Anduix *et al.* 2001). Therefore, the increase in SLC19A3 expression and the corresponding enhancement in thiamine transport under hypoxia may be a critical pro-survival response to supply thiamine in support of the PPP and maintenance of tumor cell metabolism and proliferation within hypoxic microenvironments.

Since the glycolytic shift associated with hypoxia may deplete the levels of exogenous thiamine supplied in culture media, we evaluated expression changes in BT474 cells supplemented with excess thiamine and conversely treated BT474 cells in thiamine deficient media. Culturing of HEK293 cells in thiamine depleted (TD) media has been reported to increase SLC19A2 and SLC19A3 expression (Ashokkumar *et al.* 2006). In contrast to HEK293 cells, no change in SLC19A2 or SLC19A3 expression was observed in BT474 cells when exposed to thiamine deficiency (Figure 3.12B) (Ashokkumar *et al.* 2006). A similar increase in the fold change for SLC19A3 was

observed with excess thiamine compared to regular media, further demonstrating that levels of exogenous thiamine in culture media does not appear to be involved in the observed increase in expression.

Although it is unclear as to the mechanism for the observed hypoxia mediated increase in SLC19A3 expression, under acute exposure to hypoxia or the chemical hypoxia mimetic DFO, we observed an increase in HIF-1α stabilization over 72 h of exposure (Figure 3.11C). To establish the role of the hypoxia responsive transcriptional factor HIF-1α in mediating the enhancement of SLC19A3 expression, we utilized HIF-1α shRNA knockdown strategies. An attenuated hypoxic response in SLC19A3 expression compared to scramble control group after hypoxia exposure was observed with no change in SLC19A2 (Figure 3.13B and 3.13C). Although these results do not elucidate the mechanism for hypoxia mediated regulation of SLC19A3, it suggests that HIF-1α mediated pathways may play a direct or indirect role in regulating SLC19A3 expression. Recently, it has been shown that basal expression of SLC19A3 is under the transcriptional control of SP1 and SP3 (Discher *et al.* 1998, Nabokina and Said 2004). Evidence suggests that hypoxia can increase SP1 expression, increase SP1 nuclear localization, and decrease expression of the dual transcriptional activator/repressor, SP3 (Discher *et al.* 1998, Xu *et al.* 2000, Szalad *et al.* 2009). Thus, it is feasible that HIF-1α may enhance the action of SP1/SP3 to increase transcription of SLC19A3. Additionally, hypermethylation of the SLC19A3 promoter has been demonstrated as a possible contributor in the down-regulation of SLC19A3 in gastric cancer (Liu *et al.* 2009). Hypoxic stress can induce global hypo- and hyper-methylation and alter histone acetylation to regulate hypoxia mediated gene expression (Shahrzad *et al.* 2007).

Therefore, alterations in the methylation status of the SLC19A3 promoter during hypoxia may also be a contributing factor in SLC19A3 gene expression under hypoxic conditions.

Compared to non-tumor tissue, there was an increase in THTR1 that correlated with a relative increase in gene expression. Alternatively, THTR2 protein expression was increased in cancer cell lines that contradicted the low levels of gene expression. In addition, we have demonstrated expression changes of ABC and SLC transporters in a chronic hypoxic breast cancer cell line compared to normoxia. We have identified a previously unrecognized thiamine transporter, SLC19A3 to be a hypoxia responsive gene. The critical importance of thiamine as a cofactor in glycolytic pathways that supply energy, biosynthetic intermediates, and reductive capacity suggests that increasing thiamine transport may facilitate a metabolic advantage for breast cancer cell survival within hypoxic microenvironments. Future work will need to further understand the mechanism for hypoxia-mediated expression of SLC19A3 and the functional role in supporting the glycolytic phenotype within hypoxic tumor microenvironments.

CHAPTER 4

HIF1-α MEDIATED GENE EXPRESSION INDUCED BY VITAMIN B1 DEFICIENCY [2]

[2] Sweet, Rebecca and Zastre, Jason. Accepted by *International Journal for Nutrition and Vitamin Research*. Reprinted here with permission of publisher.

4.1. ABSTRACT

It is well established that thiamine deficiency results in an excess of metabolic intermediates such as lactate and pyruvate, likely due to insufficient levels of cofactor for the function of thiamine-dependent enzymes. When in excess, both pyruvate and lactate can increase the stabilization of the hypoxia inducible factor 1-alpha (HIF-1α) transcription factor resulting in the trans-activation of HIF-1α regulated genes independent of low oxygen, termed pseudo-hypoxia. Therefore, the resulting dysfunction in cellular metabolism and accumulation of pyruvate and lactate during thiamine deficiency may facilitate a pseudo-hypoxic state. In order to investigate the possibility of a transcriptional relationship between hypoxia and thiamine deficiency, we measured alterations in metabolic intermediates, HIF-1α stabilization and gene expression. We found an increase in intracellular pyruvate and extracellular lactate levels after thiamine deficiency exposure to the neuroblastoma cell line SK-N-BE. Similar to cells exposed to hypoxia, there was a corresponding increase in HIF-1α stabilization and activation of target gene expression during thiamine deficiency, including GLUT1, VEGF and aldolase A. Both hypoxia and thiamine deficiency exposure resulted in an increase in the expression of the thiamine transporter SLC19A3. These results indicate thiamine deficiency induces HIF-1α mediated gene expression similar to that observed in hypoxic stress, and may provide evidence for a central transcriptional response associated with the clinical manifestations of thiamine deficiency.

4.2. INTRODUCTION

Vitamin B1 (thiamine) and in particular thiamine pyrophosphate (TPP), is an essential cofactor required to maintain cellular metabolism (Singleton and Martin 2001). The inability of mammals to synthesize thiamine *de novo* necessitates a continuous dietary intake to satisfy cellular metabolic requirements. Thiamine deficiency (TD) typically arises from malnutrition, consuming a diet high in anti-thiamine factors such as thiaminase, and in chronic diseases such as alcoholism, cancer, HIV, and gastrointestinal diseases (Sriram *et al.* 2012). The most well known disorders associated with thiamine deficiency include beriberi and Wernicke-Korsakoff syndrome (Donnino 2004). Regardless of etiology, the clinical manifestations of thiamine deficiency are commonly metabolic acidosis, encephalopathy, optic neuropathy, and peripheral neuropathy (Romanski and McMahon 1999, Kumar 2010).

There are a number of potential mechanisms for TD-induced encephalopathy, with most centralized on the consequences of reduced thiamine-dependent enzyme activity (Butterworth *et al.* 1986, Butterworth 1989). Biochemically, TD reduces the activities of pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (α-KGDH) (Gubler *et al.* 1974, Butterworth *et al.* 1986, Jankowska-Kulawy *et al.* 2010). This is consistent with the observed clinical manifestations in TD patients that demonstrate an increase in blood and urinary pyruvate levels and lactic acidosis (Bueding *et al.* 1941, Stotz and Bessey 1942). The accumulation of lactate and resulting tissue acidosis has been suggested to be a major contributor to neuronal cell death in TD related disorders (Pannunzio *et al.* 2000). Moreover, a build-up of intracellular pyruvate has been

shown to be pro-apoptotic and may further contribute to neural degeneration (Thangaraju *et al.* 2006).

As the end products of glycolytic metabolism, pyruvate and lactate have been shown to have a significant influence on cellular signaling that regulates cell metabolism. When in excess, both pyruvate and lactate can increase the stabilization of the hypoxia inducible factor 1-alpha (HIF-1α) transcription factor independent of low oxygen, termed pseudo-hypoxia (Lu *et al.* 2002). HIF-1α mediated pathways facilitate a Pasteur effect on cellular metabolism, increasing glycolysis and conversion to lactate (Seagroves *et al.* 2001). Aside from effects on metabolism, hypoxic signaling pathways may also induce apoptosis and necrosis (Shimizu *et al.* 1996). Comparable necrotic lesions were found in patients with hypoxia/ischemia and TD in the thalamus and mamillary bodies, suggesting congruency between the cellular response to hypoxic and TD stress (Vortmeyer and Colmant 1988, Vortmeyer *et al.* 1993). Therefore, the objective of this work was to determine whether thiamine deficiency stabilizes HIF-1α and induces expression of HIF-1α regulated genes.

4.3. MATERIALS AND METHODS

4.3.1. Materials

See Chapter 2 for cell culture reagent details. Fetal bovine serum (FBS) was purchased from PAA Laboratories (Dartmouth, MA). Thiamine hydrochloride and pyrithiamine hydrobromide were purchased from Sigma-Aldrich (St. Louis, MO). Thiamine deficient RPMI 1640 was custom formulated by Mediatech (Manassas, VA).

4.3.2. Cell Culture

The SK-N-BE neuroblastoma cell line was used throughout this study as a model for the effects of thiamine deficiency on neuronal cells (Bettendorff 1995, Bettendorff *et al.* 1995). The cells were obtained from ATCC (Manassas, VA) and maintained in TD medium supplemented with 30 nM thiamine hydrochloride, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and referred to as T30 medium.

4.3.3. Hypoxia Exposure

Hypoxic treatments were conducted at 37ºC in an atmosphere of 1% O_2 and 5% CO_2 in an incubator outfitted with a ProOX oxygen controller (Biospherix, Lacona, NY) supplying nitrogen gas. Oxygen levels were monitored daily using a Bacharach Fyrite® Gas Analyzer (Bacharach, Inc., Pittsburgh, PA) and the ProOX oxygen sensor calibrated weekly. All T30 media used for hypoxia experiments were pre-equilibrated in the hypoxia incubator at 1% O_2 for a minimum of 24 h before use. SK-N-BE cells were seeded into 100 mm culture dishes and allowed to grow until approximately 60% confluency in normoxic conditions. After which, medium was removed and hypoxia conditioned medium was added and cells placed in the hypoxia incubator for various times.

4.3.4. Thiamine Deficiency

Pyrithiamine hydrobromide was supplemented into thiamine deficient medium to aid in the induction of TD (Schwartz *et al.* 1975). Cells were cultured with thiamine deficient RPM1 1640 medium supplemented with 50 µM pyrithiamine hydrobromide,

10% FBS and 1% penicillin/streptomycin and referred to as PTD medium. Cells were grown until approximately 60% confluency in T30 medium at which time medium was removed and replaced with PTD medium for the indicated times.

4.3.5. Inhibition of HIF-1α mediated gene expression

To attenuate HIF-1α mediated responses, cells exposed to hypoxia and PTD were treated with the HIF-1α inhibitor YC1 and a dominant negative HIF-1α construct (Jiang *et al.* 1996, Chun *et al.* 2001). Cells were pre-treated with 25 μM YC1 for 24 h after which medium was removed and appropriate treatment medium was added in addition to 10 μM YC1. All treated samples were exposed to 10 μM YC1 for a total of 5 days. PTD treatments were started on the same day as the 10 μM YC1 and lasted 5 days while 1% O_2 treated samples were exposed to 10 μM YC1 3 days prior to starting the 48 h 1% O_2 treatment to ensure all cells were exposed to YC1 for the same time periods.

The dominant negative form of HIF-1α lacking a DNA-binding domain, transactivation domains, and an oxygen-dependent degradation domain of HIF-1α was cloned from human reference cDNA using previously reported primer sets (Chen *et al.* 2003). Cloned fragments were recovered and ligated into pcDNA3.1 (Invitrogen, Grand Island, NY) (HIF-1α-DN). Sequence was verified using the University of Georgia Genomics facility. Cells were grown to ~ 60% confluency, medium was replaced and the transfection complex of 2.5μg plasmid and 5μL Metefectene Pro (Biontex Laboratories, San Diego, CA) was added as per manufacturer's protocol. After 24 h incubation, medium was removed and treatment media was added.

4.3.6. Pyruvate and Lactate Assay

Pyruvate (intracellular) and lactate (intracellular and extracellular) were quantified in SK-N-BE cells using a pyruvate assay kit (Eton Bioscience, San Diego, CA) and an L-lactate Assay Kit (Eton Bioscience, San Diego, CA) after PTD treatment. Pyruvate and lactate were quantified as per manufacturer's protocol using whole cell lysates (WCL). Isolation of WCL was achieved using 1% NP-40, 0.5% sodium deoxy-cholate, 0.1% sodium dodecyl sulfate (SDS) in a pH=8.5 50 mM Tris, 250 mM NaCl, 1 mM EDTA buffer supplemented with protease inhibitor cocktail (EMD Biosciences, La Jolla, CA). Lysates were centrifuged at 16,000xg for 20 min at 4°C and the supernatant was collected. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Briefly, 50 µL of samples and standards were loaded into a 96 well plate followed by the addition of 50 µL of the supplied reaction reagent. The plate was then incubated at 37°C for 30 min and pyruvate was assayed using fluorescence measured at Ex=544nm and Em=590 nm and lactate was assayed by measuring the absorbance at ƛ=490 nm using a Spectramax M2E plate reader (Molecular Devices, Sunnyvale, California). Results were normalized to total protein measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL).

4.3.7. Real-time quantitative PCR (qRT-PCR)

RNA was isolated after either hypoxia or PTD treatments using the EZNA Total RNA Kit I (Omega Bio-tek, Norcross, GA) and reverse transcribed (1 µg) to cDNA using qScript cDNA Synthesis Kit as per manufacturers protocol (Quanta Biosciences Inc., Gaithersburg, MD). The fold change in gene expression comparing untreated and treated

SK-N-BE cells was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

4.3.8. Western blotting

The protein expression of HIF-1α was assessed in SK-N-BE cells after PTD and 1% O_2 exposure by Western blot. The membrane was immunoblotted for HIF-1α (rabbit anti-human, Bethyl Laboratories, Inc., Montgomery, TX) at 1:500 then blotted with 1:2,500 goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Millipore, Billerica, MA). Loading control was the nuclear specific P84/N5-5E10 (mouse anti-human, GeneTex, Irvine, CA) probed at 1:1,000 then blotted with 1:15,000 goat anti-mouse-HRP secondary antibody (Millipore, Billerica, MA)

4.3.9. Statistical Analysis

All experiments were performed with a minimum of three independent experiments. Statistical significance was evaluated between groups using unpaired Student t-test with a significance level of $p<0.05$ using Graphpad Prism 6.

4.4. RESULTS

4.4.1. Stabilization and nuclear localization of HIF-1α in SK-N-BE cells during thiamine deficiency

To determine the effect of thiamine deficiency on the stabilization of HIF-1α, nuclear lysates of SK-N-BE cells treated with PTD medium were analyzed by Western blot. Figure 4.1 demonstrates an increase in nuclear localization of HIF-1α after 1, 2 and

5 days of PTD treatment compared to T30 control. Double banding of HIF-1α was observed after some treatments. Unprocessed HIF-1α is ~95 kDa while the fully post-translationally modified form is ~116 kDa and depending on the treatment type it is common to see either a single or a double band (Wang and Semenza 1993).

4.4.2. Expression of known HIF-1α target genes and thiamine transporters after 1% O_2 exposure

qRT-PCR was used to determine the effect of 1% O_2 exposure on known HIF-1α target genes SLC2A1 (GLUT1), vascular endothelial growth factor (VEGF) and aldolase A. As seen in Figure 4.2A, expression of GLUT1, VEGF and aldolase A increased with time, peaking at 5.4, 18.6 and 2.9 fold, respectively after 48 h of 1% O_2 exposure. The effect of 1% O_2 exposure on the expression of the thiamine transporters SLC19A2 (THTR1) and SLC19A3 (THTR2) was determined using qRT-PCR. Figure 4.2B demonstrates no change in SLC19A2 expression over the time course of 1% O_2 exposure. Alternatively, SLC19A3 expression levels increased after 12 h of 1% O_2 treatment and reached ~11.5 fold after 48 h.

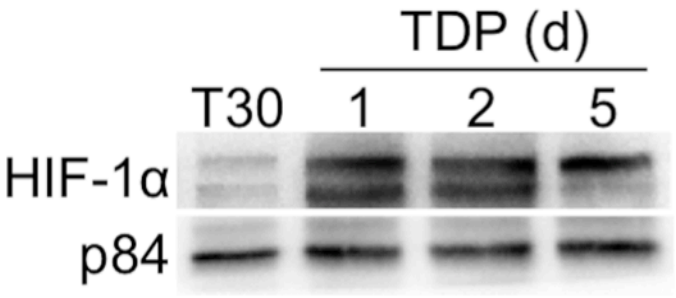

Figure 4.1. Stabilization and nuclear localization of HIF-1α in SK-N-BE cells during thiamine deficiency. Representative Western blot for HIF-1α in SK-N-BE cells exposed to PTD medium for 1, 2 and 5 days with p84 used as loading control.

4.4.3. Impact of thiamine deficiency on the expression of known HIF-1α target genes and thiamine transporters

To determine the effect of thiamine deficiency on the expression of GLUT1, VEGF and aldolase A, mRNA expression levels were quantified using qRT-PCR. GLUT1, VEGF and aldolase A all showed a time dependent increase in gene expression reaching ~3.4, 6.2 and 3.0 fold, respectively after 5 days of thiamine deficiency (Figure 4.2C). As shown in Figure 4.2D, SLC19A2 expression levels showed no change with 1.06, 1.12 and 1.04 fold change after 1, 2 and 5 days, respectively. SLC19A3 expression levels increased over time with a fold change of ~0.8, 2.0, and 3.4 after 1, 2, and 5 days, respectively.

4.4.4. Attenuation of HIF-1α response under 1% O_2 exposure and thiamine deficiency

Western blotting was used to establish whether the HIF-1α inhibitor YC1 decreased HIF-1α protein stabilization and nuclear localization after treatment to SK-N-BE cells. Figure 4.3A demonstrates HIF-1α stabilization in nuclear samples treated under normoxic and hypoxic conditions for 48 h with or without the addition of YC1. Compared to normoxic control cells, the presence of YC1 had no effect on HIF-1α. Alternatively, there was a decrease in HIF-1α stabilization in cells treated with 1% O_2 with the addition of YC1. Figure 4.3B shows HIF-1α stabilization in cells treated with PTD with a decrease in HIF-1α expression in cells treated with PTD in addition to YC1, indicating YC1 treatment causes a reduction in HIF-1α nuclear localization.

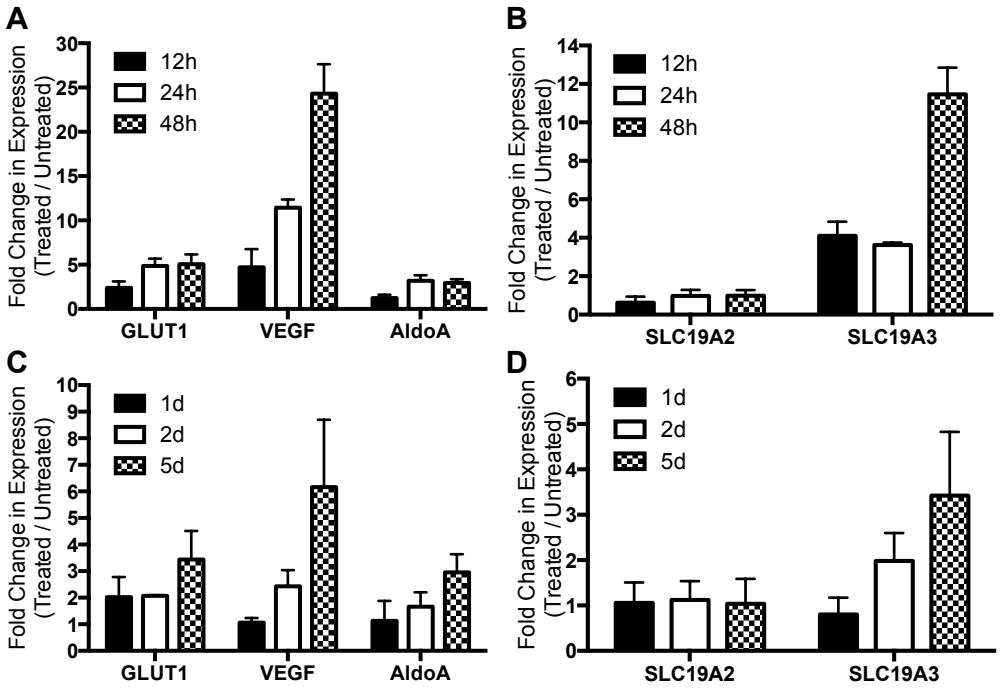

Figure 4.2. Effect of oxygen and thiamine deprivation on HIF-1α mediated gene expression. Fold change in gene expression for (A) GLUT1, VEGF and aldolase A and the (B) thiamine transporters SLC19A2 and SLC19A3 in SK-N-BE cells after 1% O_2 exposure for 12, 24, and 48h. Fold change in gene expression for (C) GLUT1, VEGF and aldolase A and the (D) thiamine transporters SLC19A2 and SLC19A3 in SK-N-BE cells after PTD treatment for 1, 2, and 5 days.

YC-1 significantly attenuated the HIF-1α mediated induction of GLUT1 and VEGF expression under hypoxic conditions but no significant change was found for aldolase A (Table 4.1). YC1 treatment also significantly attenuated the hypoxia-mediated induction of SLC19A3 (Table 4.1). HIF-1α-DN plasmid transfected into SK-N-BE cells followed by 1% O_2 for 48 h significantly attenuated the hypoxia mediated induction of GLUT1, VEGF, aldolase A and SLC19A3 (Table 4.1). As shown in Table 4.2, PTD treatment for 5 days with YC1 significantly attenuated the induction of gene expression for GLUT1, VEGF, aldolase A, and SLC19A3. SK-N-BE cells transfected with HIF-1α-DN and treated with PTD for 5 days also had a significantly attenuated induction of gene expression for GLUT1, VEGF and SLC19A3 (Table 4.2).

4.4.5. Impact of thiamine deficiency on pyruvate and lactate production

To establish whether limiting thiamine availability results in an accumulation of pyruvate and lactate, intracellular pyruvate and lactate levels were quantified in cell lysates after exposure to PTD for 1, 2 and 5 days. Figure 4.4A demonstrates a time dependent increase in cellular pyruvate levels during PTD treatment compared to T30 cells increasing to ~2.5 fold after 5 days. There was no change in intracellular lactate production after 1 and 2 days of PTD treatment as shown in Figure 4.4B. However, there was a slight increase of ~1.6 fold in intracellular lactate accumulation after day 5 of PTD exposure. Levels of extracellular lactate did not change over 1 and 2 days, but increased ~3 fold after 5 days of PTD treatment relative to T30 (Figure 4.4C).

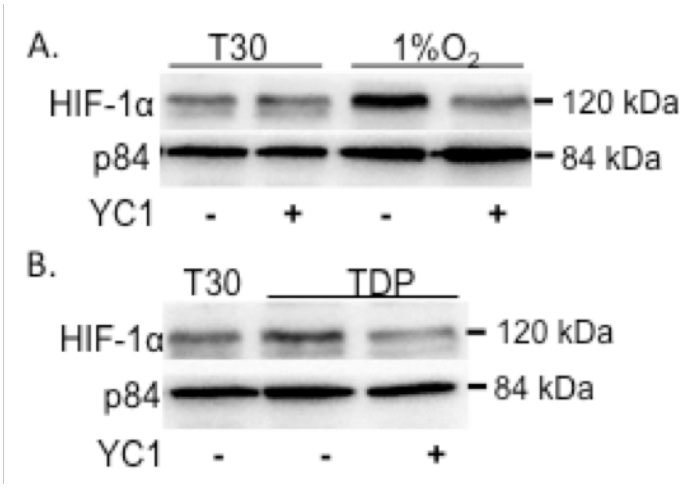

Figure 4.3. Effect of YC1 on HIF-1α stabilization after 1% O_2 and PTD exposure. Representative Western blots for the nuclear localization of HIF-1α in SK-N-BE cells grown in T30 media after (A) 1% O_2 exposure for 48 h and (B) PTD media for 5 days compared to untreated control cells in the presence and absence of YC1 with p84 as loading control.

Table 4.1. Attenuation in gene expression in 1% O_2. Cells were treated with or without YC1 or HIF-1α DN in 1% O_2 for 48 hours. Results are expressed as the average fold change ± SD compared to SK-N-BE cells cultured in normoxic conditions (untreated) with n = 3 independent experiments. Statistically significant ($p<0.05$) comparisons between untreated and YC1 (∗), and untreated and HIFDN (∗∗).

Gene	**Untreated**	**YC1**	**HIFDN**
GLUT1	1.86 ± 0.72	0.66 ± 0.16*	1.22 ± 0.63**
VEGF	23.1 ± 6.61	7.06 ± 1.94*	10.2 ± 4.57 **
Aldolase A	5.24 ± 1.72	2.67 ± 0.15	1.01 ± 0.44**
SLC19A2	0.31 ± 0.19	0.34 ± 0.14	0.46 ± 0.09
SLC19A3	6.54 ± 1.64	2.24 ± 0.81*	2.26 ± 0.37**

Table 4.2. Attenuation in gene expression in TD. Cells were treated with or without YC1 or HIF-1α DN in PTD for 5 days. Results are expressed as the average fold change ± SD compared to SK-N-BE cells cultured in T30 conditions (untreated) with n = 3 independent experiments. Statistically significant ($p<0.05$) comparisons between untreated and YC1 (∗), and untreated and HIFDN (∗∗).

Gene	**Untreated**	**YC1**	**HIFDN**
GLUT1	3.96 ± 1.23	1.01 ± 0.21*	0.99 ± 0.78**
VEGF	5.05 ± 2.25	2.36 ± 0.37*	1.89 ± 0.88 **
Aldolase A	2.55 ± 0.53	1.43 ± 0.52*	1.84 ± 1.06
SLC19A2	1.04 ± 0.54	0.79 ± 0.16	0.91 ± 0.51
SLC19A3	3.22 ± 1.73	0.91 ± 0.67*	1.35 ± 0.19**

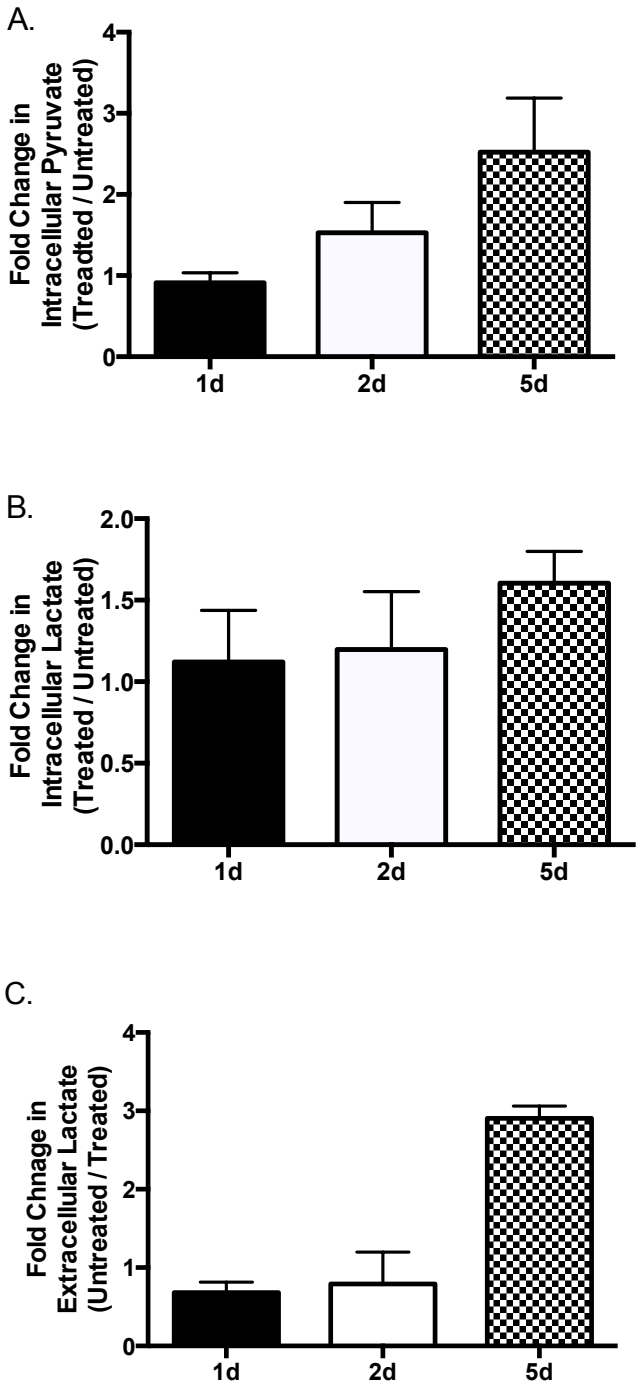

Figure 4.4. Impact of thiamine deficiency on pyruvate and lactate levels. The fold change in the intracellular levels of (A) pyruvate and (B) lactate and (C) extracellular lactate levels after exposure to PTD for 1, 2 and 5 days. Results are expressed as the average fold change ± standard deviation compared to SK-N-BE cells cultured under T30 conditions with n = 3 independent experiments.

4.5. DISCUSSION

Hypoxia/ischemia is associated with a number of pathologies including cancer, diabetes, Alzheimer's, and cardio/cerebro-vascular disease (Isenberg *et al.* 1986, Thornalley 2005, Karuppagounder *et al.* 2009). HIF-1α is a well characterized transcription factor that plays a central role in the cellular adaptation to low oxygen, mediating a reprogramming of glycolytic metabolism (Rodríguez-Enríquez *et al.* 2010). Both hypoxia and TD are associated with decreased ATP, increased cellular glycolysis, lactate secretion, and tissue acidosis (Gavrilescu and Peters 1931, Bonanno and Polse 1987, Swanson *et al.* 1995). This metabolic congruency may suggest a common adaptive response that shifts towards a metabolic phenotype with a reduced need for thiamine and oxygen.

Our findings that TD increases HIF-1α stabilization and gene expression suggest the metabolic consequences of TD may centralize with HIF-1α mediated metabolic reprogramming. HIF-1α induces the expression of the glucose transporter GLUT1, resulting in an increase in glucose consumption under hypoxic stress (Chen *et al.* 2001). An increase in glucose consumption was found in PDH deficient fibroblast cells compared to normal cells, demonstrating an increased reliance on glycolytic metabolism when devoid of this thiamine dependent enzyme (Borud and Strømme 1977). Our findings also demonstrated an increase in expression of GLUT1 during TD that may be a contributor for increasing glucose consumption. TD increased expression of the HIF-1α target gene aldolase A, a glycolytic enzyme that converts fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone. The increased expression of aldolase A may assist in increasing glycolysis to maximize ATP generation. HIF-1α also induces

angiogenesis through the increased expression of VEGF in an attempt to increase vascular density and supply of oxygen (Choi *et al.* 2003). Both hypoxia and TD induced VEGF expression in SK-N-BE cells. Although it is unknown if TD is associated with angiogenesis, increases in pyruvate have demonstrated angiogenic activity *in vitro* and *in vivo* (Lee *et al.* 2001). Thus, the resulting effects of TD on cellular metabolism and subsequent sequelae associated with TD disorders may be mediated through HIF-1α effects on metabolic reprogramming.

When thiamine availability is limited, an adaptive transcriptional upregulation of the thiamine transporter SLC19A3 has been observed in intestinal and renal tissues in an attempt to increase thiamine absorption and reduce clearance (Reidling and Said 2005, Ashokkumar *et al.* 2006). Consistent with these findings we observed that TD treatment of SK-N-BE cells resulted in an upregulation of SLC19A3 expression with no change in SLC19A2. We have previously demonstrated the upregulation of SLC19A3 and an increase in thiamine transport during hypoxic exposure to breast cancer cells that was reduced by HIF-1α shRNA (Sweet *et al.* 2010). Synonymous to these findings, hypoxia increased SLC19A3 in SK-N-BE cells and was attenuated by the HIF-1α inhibitor YC1 and the dominant negative form of HIF-1α in TD and hypoxia treatments. This further demonstrates a potential regulatory role of HIF-1α in mediating thiamine transporter expression during hypoxic and nutritional stress.

Although stabilization of HIF-1α is most commonly associated with low oxygen tensions, reduced degradation and induction of HIF-1α mediated gene expression is also associated with the accumulation of metabolic intermediates independent of oxygen. In particular, increases in cellular pyruvate and lactate have been shown to stabilize HIF-1α

and mediate gene expression (Lu *et al.* 2002, McFate *et al.* 2008). Pyruvate has been suggested to stabilize HIF-1α through inhibition of HIF-1α degradation, however the exact mechanism remains undetermined (Lu *et al.* 2002). Pyruvate is formed within the Embden-Meyerhof pathway and is converted to acetyl-CoA by PDH. Reduced expression of PDH has been reported under a state of TD indicating the potential role of thiamine in regulating the activity of this thiamine-dependent enzyme (Pekovich *et al.* 1998). It has been suggested that activity of α-KGDH is reduced prior to the onset of pathologic lesions while PDH activities is only reduced at later stages (Gibson *et al.* 2000). However, only changes in α-KGDH and not PDH activity has been typically reported in whole brain homogenate of thiamine-deficient animals (Butterworth *et al.* 1986, Bubber *et al.* 2004). Thiamine deficiency has been associated with inducing region specific lesions in the brain and as such changes in enzyme activity within whole brain homogenate may not represent regions affected by TD (Calingasan *et al.* 1999, Jankowska-Kulawy *et al.* 2010). While no reports to date have demonstrated direct increases in pyruvate levels within the whole brain of TD animals, a decrease in PDH activity and reduced pyruvate flux within nerve terminals of the forebrain in TD rats was recently reported (Jankowska-Kulawy *et al.* 2010).

When in excess, pyruvate can be interconverted to lactate by lactate dehydrogenase (Mole *et al.* 1973, Brooks 1985). An increase in lactate production is an indicator of HIF-1α mediated glycolytic shift and a common clinical sign of thiamine deficiency (Oriot *et al.* 1991). Our findings demonstrated that TD increased the extracellular secretion of lactate, consistent with the metabolic impact of TD and hypoxia. Although lactate has been reported to stimulate HIF-1α protein accumulation, it

is suggested that lactate requires the conversion to pyruvate to induce pseudo-hypoxia (Lu *et al.* 2002). However, it is unclear if the increase in pyruvate is due to inter-conversion from lactate or a combination of the reduction in the functionality of other thiamine-dependent enzymes.

In conclusion, our results indicate TD induces HIF-1α mediated gene expression similar to that observed during hypoxic stress. These results are the first to demonstrate a potential transcriptional congruency between hypoxia and TD that may explain the clinical manifestations occurring in pathologies associated with thiamine deficiency. In addition, the potential role of HIF-1α in thiamine transporter expression may provide new insights into the adaptive regulation during hypoxic and nutritional stress.

CHAPTER 5

INVOLVEMENT OF HIF-1 IN THE ADAPTIVE REGULATION OF THE THIAMINE TRANSPORTER SLC19A3 [3]

[3] Sweet, Rebecca L., Zera, Kristy A., Foskey, Adam M., and Zastre, Jason A. To be submitted to *Journal of Biological Chemistry*.

5.1. ABSTRACT

Thiamine is an essential vitamin acting as a cofactor for enzymes involved in multiple metabolic pathways. Under hypoxic conditions, cells undergo a shift in metabolism favoring a glycolytic phenotype. The upregulation of the thiamine transporter SLC19A3 under hypoxic conditions has been previously reported. However, the mechanism of SLC19A3 adaptive regulation in hypoxia is unknown. Protein expression and nuclear localization of HIF-1α and transcription factors required for basal expression of SLC19A3 was characterized after hypoxic exposure. HIF-1α was the only transcription factor to increase in expression in both whole cell lysate and nuclear fractions under hypoxic conditions compared to normoxic conditions. *In silico* sequence analysis identified four putative hypoxic response elements within the SLC19A3 promoter region. Deletion analysis demonstrated the region close in proximity to the transcription start site was involved in the response to hypoxia. Mutation of two hypoxic response elements upstream of the transcriptional start site (HRE-55 and HRE-47) attenuated the promoter activity during hypoxia. Therefore, HIF-1α may be involved in the trans-activation of SLC19A3 expression during hypoxia.

5.2. INTRODUCTION

Vitamin B1 (thiamine) is a water-soluble vitamin requiring dietary ingestion to meet the metabolic requirement of mammalian cells. The structure of thiamine consists of a quaternary amine with a pyrimidine core linked to a thiazole ring, existing as a cation at physiological pH (Komai *et al.* 1974). Due to thiamine's hydrophilic nature, cells utilize specific transport mechanisms for intracellular uptake. Thiamine transport is mediated

through Solute Carrier transporters belonging to the SLC19 family. The family is comprised of three proton-linked transporters, SLC19A1, SLC19A2 and SLC19A3. Of these, SCL19A2 (low affinity) and SLC19A3 (high affinity) have been characterized to transport thiamine while SLC19A1 transports reduced folate with a high degree of substrate specificity (Ganapathy *et al.* 2004).

The activated form of thiamine, thiamine pyrophosphate, acts as a cofactor for enzymes involved in glycolytic metabolism. Thiamine dependent enzymes include pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH), and transketolase (TKT) (Horecker and Smyrniotis 1953, Park and Gubler 1969). Due to thiamine's intimate involvement in cellular metabolism, adaptive processes are required to increase supply to meet metabolic demands during physiological stress. Regulation of SLC19A3 but not SLC19A2 transporter expression has been reported in response to stimuli such as chronic alcohol exposure, thiamine deficiency, and hypoxia (Subramanian *et al.* 2010, Sweet *et al.* 2010, Nabokina *et al.* 2013). A significant decrease in SLC19A3 mRNA expression was observed after exposure to chronic ethanol in renal epithelial cells, which correlated with a decrease in thiamine transport (Subramanian *et al.* 2010). Alternatively, SLC19A3 expression and transport capacity increased after exposure to thiamine deficiency (TD) (Nabokina *et al.* 2013). A role for the transcriptional factor SP1 has been shown in the adaptive regulation of SLC19A3 to TD conditions (Nabokina *et al.* 2013).

The involvement of hypoxia inducible factor 1-alpha (HIF-1α) in mediating SLC19A3 expression during thiamine deficiency and hypoxia has been recently suggested (Sweet *et al.* 2010, Schänzer *et al.* 2014, Sweet and Zastre Accepted 2013).

HIF-1α is well characterized to regulate genes involved in proliferation and survival, angiogenesis, erythropoeisis, pH regulation, and glycolytic metabolism (Eltzschig *et al.* 2005, Kim *et al.* 2006) (Mueller *et al.* 2000, Chen *et al.* 2001). An upregulation in SLC19A3 expression levels was found in both hypoxia and thiamine deficiency, which was attenuated in the presence of a dominant negative form of HIF-1α, YC1 (a HIF-1α inhibitor), and shRNA (Sweet *et al.* 2010, Sweet and Zastre Accepted 2013). Therefore the objective of this chapter was to investigate the role of HIF-1α in trans-activating SLC19A3 transporter expression.

5.3. MATERIALS AND METHODS

5.3.1. Cell Culture

See Chapter 2 for the culturing description of BT474 breast cancer cells.

5.3.2. Hypoxic Treatments

Hypoxia chamber settings and treatment details are previously described in Chapter 2.

5.3.3. Western Blotting

Whole cell lysates and nuclear lysates were prepared as described in Chapter 2. Immunoblot analysis and antibody specifications are also described in Chapter 2.

5.3.4. Promoter Constructs

A pGL2-TK-HRE plasmid containing three subcloned copies of a HRE (5'-GTGACTA**CGTG**CTGCCTAG-3') from the inducible nitric oxide synthase promoter was described previously and generously donated by Dr. Giovanni Melillo (Rapisarda *et al.* 2002). The region contained the three subcloned HREs was restriction digested with KpnI (Promega) and NheI (Promega) and inserted into a pGL3 vector (pGL3-TK-HRE). Promoter constructs for SLC19A2 and SLC19A3 were created by cloning the promoter region of the gene of interest from genomic DNA (Clontech) with Pfu polymerase (Agilient). Primers used for cloning were designed corresponding to known sequences (Table 5.1) (Reidling and Said 2003, Nabokina and Said 2004). The PCR products and pGL3 basic plasmid were restriction digested with NheI and KpnI at 37°C for 1 h followed by 65°C for 15 min. Digested fragments and plasmids were ligated using T4 DNA ligase (Promega). Sequences were verified through the Georgia Genomics sequencing facility (UGA).

5.3.5. Deletion Constructs

In order to determine which regions of the SLC19A3 promoter are involved in adaptive regulation, 5' truncations of the full SLC19A3 promoter were made from -1957/+59 to -32/+59. These deletion constructs were generated by PCR using Pfu polymerase, genomic DNA, and the oligonucleotide primers indicated in Table 5.1. For the full promoter, -970/+59, -473/+59 and -32/+59 the reverse primer was SLC19A3 +59 and an NheI site was added to the 5' end of the primer. For the -1957/+13 construct the reverse primer was SLC19A3 +13 with an Nhe site added to the 5'end of the primer. All

forward primers for the constructs had a KpnI site added to the 5' end. The PCR products and pGL3 basic plasmid were restriction digested with Nhe (Promega) and KpnI (Promega) at 37°C for 1 h followed by 65°C for 15 min. Digested fragments and plasmids were ligated using T4 DNA ligase (Promega). Sequences were verified through the Georgia Genomics sequencing facility.

5.3.6. Site-directed Mutagenesis

To further investigate the role of each putative HRE in SLC19A3 adaptive regulation, site-directed mutagenesis was employed to create mutation constructs. Mutation constructs were prepared following the manufacturer's protocol for the Quikchange site-directed mutagenesis II kit (Agilent Technologies). Briefly, primers were designed to include the desired mutation flanked by an unmodified nucleotide sequence (Table 5.2). After mutant strand synthesis, digestion of the parental methylated DNA was performed by adding 1 μl of the *Dpn* I restriction enzyme (10 U/μl) directly to the amplification reaction. The reaction mixture was mixed by gently pipetting the solution up and down several times. The reaction mixture was then centrifuged at max speed for 1 min and immediately incubated at 37°C for 1 hour to digest the parental (i.e., the non-mutated) supercoiled dsDNA.

XL1-Blue supercompetent cells were gently thawed on ice and 50 μL was aliquoted into a prechilled 14 mL BD Falcon polypropylene round-bottom tube. 1 μL of the Dpn I treated DNA was transferred to the aliquoted suprercompetent cells. The transformation reaction was gently swirled and incubated on ice for 30 min, heat pulsed at 42°C for 45 sec then placed back on ice for 2 min. 0.5 ml of NZY^{+} broth (preheated to

42°C) was added to the supercompetent cells and incubated at 37°C for 1 hour with shaking at 225–250 rpm. Plasmids were harvested using E.Z.N.A. ® Fastfilter Plasmid Midi Kit (Omega Biotek). Sequences were verified through the Georgia genomics sequencing facility.

Table 5.1. Oligonucleotide primers used to isolate the SLC19A2 full promoter, SLC19A3 full promoter, and SLC19A3 deletion constructs. KpnI sites are underlined and NheI are italicized.

Primer Name	Sequence
SLC19A2	Sense: 5'-TAT CGG TAC CTA TGT AGC CCC CTC CAA CA-3' Antisense: 5'-TAT *GCT AGC* CCC CTT CCT TCT CCT CCT C-3'
SLC19A3 -1957 (Full)	Sense: 5'-CGG GCT ACC AGA CTG TGG CTA TGA G-3'
SLC19A3 -970	Sense: 5'-CGG GGT ACC TAG GGA GGC TGA GGC AGA AG-3'
SLC19A3 -473	Sense: 5'-CGG GGT ACC CCA GGA AAC CAT CCC ACT CT-3'
SLC19A3 -32	Sense: 5'-CGG GGT ACC TCC GGG CCA GGC AGG CTC CG-3'
SLC19A3 +59	Antisense: 5'-CTA *GCT AGC* ATC GCT CAC TTG CCG CA-3'
SLC19A3 +13	Antisense: 5'-CGG *GCT AGC* ATC CAG GCG CTC TTG GTG-3'

Table 5.2. Primers for SLC19A3 promoter mutation constructs. Hypoxic response element locations are indicated in bold and mutations are indicated by the underline.

Primer Set	Primer Sequence
-1336	Sense: 5'-CAA AAA AAT TAA TGT GA**A A**TG GTG GCA CCC ACC TGC AAG CC-3' Antisense: 5'- GGC TTG CAG GTG GGT GCC ACC ATT TCA CAT TAA TTT TTT TG-3'
-908	Sense: 5'-GTT CGA GTG AGC CAA GAT **AAT G**CC ATT GCA CTC CAG CCC-3' Antisense: 5'- GGG CTG GAG TGC AAT GGC ATT ATC TTG GCT CAC TGC AAC-3'
-47a	Sense: 5'-CAT ATG CAA AG**C GTG** GGG G**AA TG**G CCC CGG GCT C-3' Antisense: 5'-AGC CCG GGG CCA TTC CCC CAC GCT TTG CAT ATG-3'
-47b	Sense: 5'-CAT ATG CAA AG**C GTG** GGG G**CG AA**G CCC CGG GCT C-3' Antisense: 5'-GAG CCC GGG GCT TCG CCC CCA CGC TTT GCA TAT G-3'
-55	Sense: 5'-CAT ATG CAA AG**A ATG** GGG G**CG TG**G CCC CGG GCT C-3' Antisense: 5'-GAG CCC GGG GCC ACG CCC CCA TTC TTT GCA TAT G-3'
-55/47	Sense: 5'-CAT ATG CAA AG**A ATG** GGG G**AA TG**G CCC CGG GCT C-3' Antisense: 5'-GAG CCC GGG GCC ATT CCC CCA TTC TTT GCA TAT G-3'

5.3.6. Transfection of BT474 cells for Luciferase assay

BT474 cells were reverse transfected with a pGL3-promoter constructs using Lipofectamine LTX (Life Technologies, Carlsbad, CA). Plasmid constructs were co-transfected with the pRL-SV40 vector encoding the *Renilla* luciferase gene used as a control for transfection efficiency. Transfection complexes were prepared as per manufacturers protocol with a reagent (μL):DNA(μg) ratio of 3.6:1. The firefly luciferase plasmid:renilla ratio was 25:1. Briefly, 9 μL of Lipofectamine LTX was added to 150 μL serum free media. In a separate tube, 2.5 μg plasmid DNA (pGL3-promtoer construct encoding a modified firefly luciferase gene of empty pGL3 vector), 0.1 μg Renilla, and 2.5 μL PLUS^{TM} reagent were added to 150 μL serum free media. The contents of the tubes were combined and incubated at room temperature for 5 min. 250 μL of transfection complex was added to 35 mm dishes followed by 100,000 cells/cm^2 in 2 mL medium. Cells were incubated with transfection complex for 6 h at which time media was changed. Treatments were started 24 h after transfection. The results are represented as a fold change of the ratio of firefly to *Renilla* luminescence in hypoxic to normoxic values.

5.3.7. Luciferase Assays

The Dual-Luciferase Reporter Assay System (Promega) was used to study promoter activity. A dual reporter assay allows for separate readings of firefly and renilla luminescence. Activity of the experimental reporter was normalized to the activity of the internal control in order to minimize experimental variability caused by differences in cell viability or transfection efficiency. After treatment with 1 % O_2 for 48 h, promoter activity of transfected plasmid constructs were assayed using Dual Luciferase Reporter®

Assay following manufacturer's protocol. Results were normalized to total protein measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL).

5.4. RESULTS

5.4.1. Expression of transcriptional factors after hypoxic exposure

Both SP1 and SP3 are involved in the minimal promoter activity of SLC19A3 and have been suggested to be hypoxia responsive (Figure 5.1A) (Discher *et al.* 1998, Nabokina and Said 2004). To investigate the effects of hypoxia on the protein expression of SP1 and SP3, Western Blots were performed on whole cell lysate and nuclear fractions of BT474 cells. HIF-1α protein expression increased after 1% O_2 exposure up to 72 h compared to normoxia in both whole cell lysate and nuclear fractions with fold changes of 3.6 and 9, respectively (Figure 5.1B, C, D and E). In whole cell lysate fractions, SP1 and SP3 protein expression increased 2 and 1.7 fold after 24 h 1% O_2 exposure, respectively. However, after 72 h 1% O_2 exposure compared to normoxia, the fold change in expression decreased to 1.17 for SP1 and 0.7 for SP3 (Figure 5.1B and D). In contrast, no change in the nuclear localization for both SP1 and SP3 was found up to 72 h of 1% O_2 exposure compared to normoxia (Figure 5.1C and E).

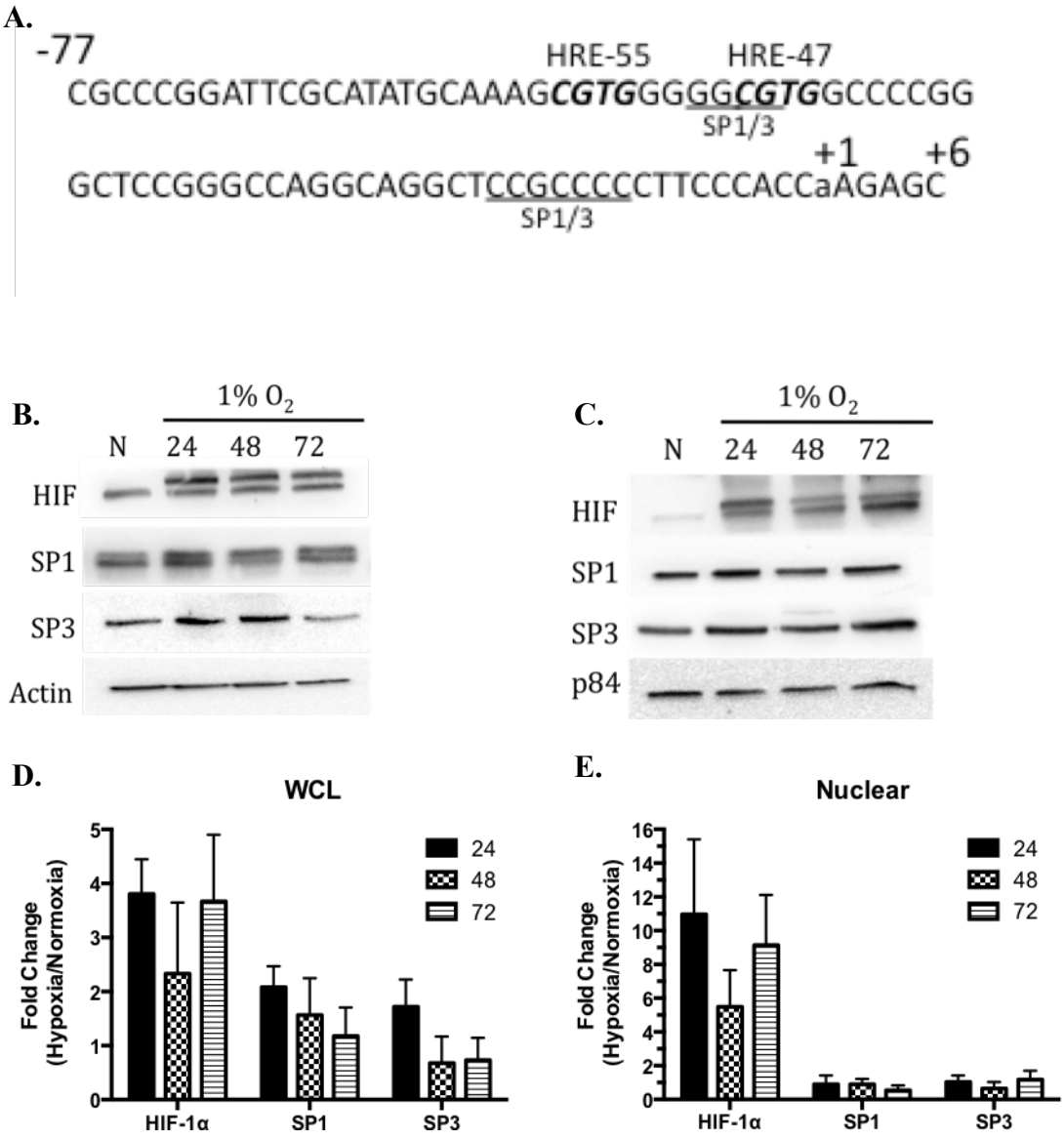

Figure 5.1. Effects of hypoxia on expression of transcription factors. (A) SLC19A3 minimal promoter region. Location of HREs are indicated by bold and italic and SP1/SP3 binding elements are underlined. +1 indicating the location of the transcriptional start site. Western blot of transcription factor expression in (B) WCL and (C) nuclear lysates after 1% O_2 exposure up to 72 h. Densitometry of transcription factor protein expression in (D) WCL and (E) nuclear lysates after 1% O_2 exposure up to 72 h. Data are presented with n=5 independent experiments.

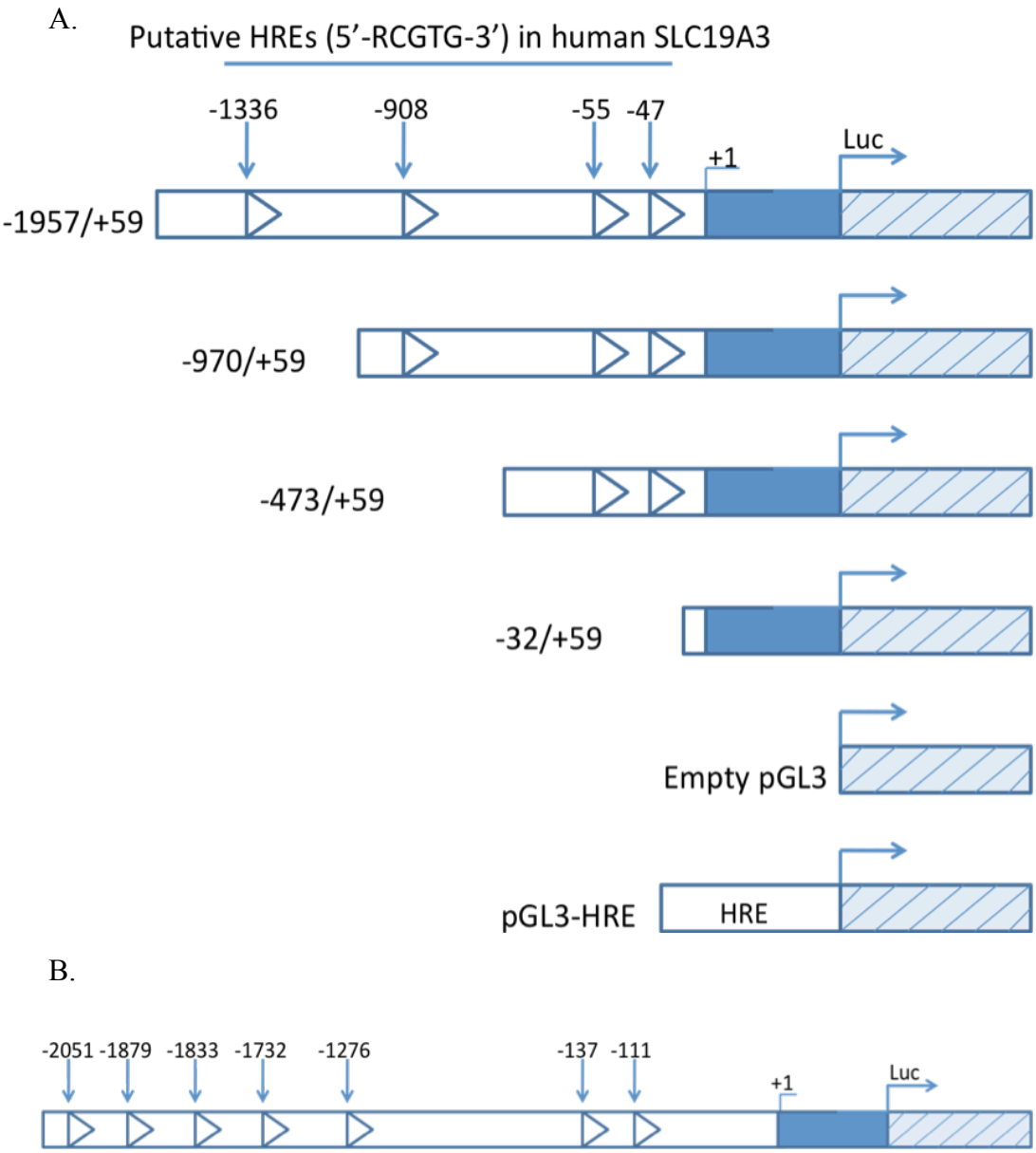

Figure 5.2. SLC19A3 and SLC19A2 promoter constructs. (A) 2016 bp promoter fragment of the SLC19A3 gene and was subcloned into the firefly luciferase reporter gene vector (pGL3), and the series of 5'-deletions of the full length -1957/+59. (B) 2653 bp promoter fragment of the SLC19A2 gene was subcloned into the firely luciferase reporter gene vector (pGL3). The location of the putative HRE sites (5'-RCGTG-3') are indicated by open arrowheads.

5.4.2. SLC19A2 and SLC19A3 promoter activity after 1% O_2 exposure

In silico sequence analysis revealed seven and four putative HREs within the SLC19A2 and SLC19A3 promoter regions, respectively, with the sequence 5'-RCGTG-3' upstream of the transcription start site (Figure 5.2). With +1 indicating the location of the transcriptional start site, the putative HREs in the SLC19A2 promoter region were located at -2051, -1879, -1732, -1276, -137 and -111 and -1336, -908, -55 and -47 for SLC19A3.

Luciferase reporter assays were utilized to investigate the hypoxia responsiveness of the SLC19A2 and SLC19A3 promoters. An empty pGL3 basic vector and pGL3-HRE plasmid were used as negative and positive controls, respectively. The empty pGL3 basic demonstrated no change in luciferase activity after hypoxic exposure compared to normoxia, while pGL3-HRE gave an ~45 fold induction after hypoxic exposure (Figure 5.3A). After 48 h exposure to hypoxia SLC19A3 showed an approximately 2.8 fold increase in promoter activity while there was no change in SLC19A2 (Figure 5.3B).

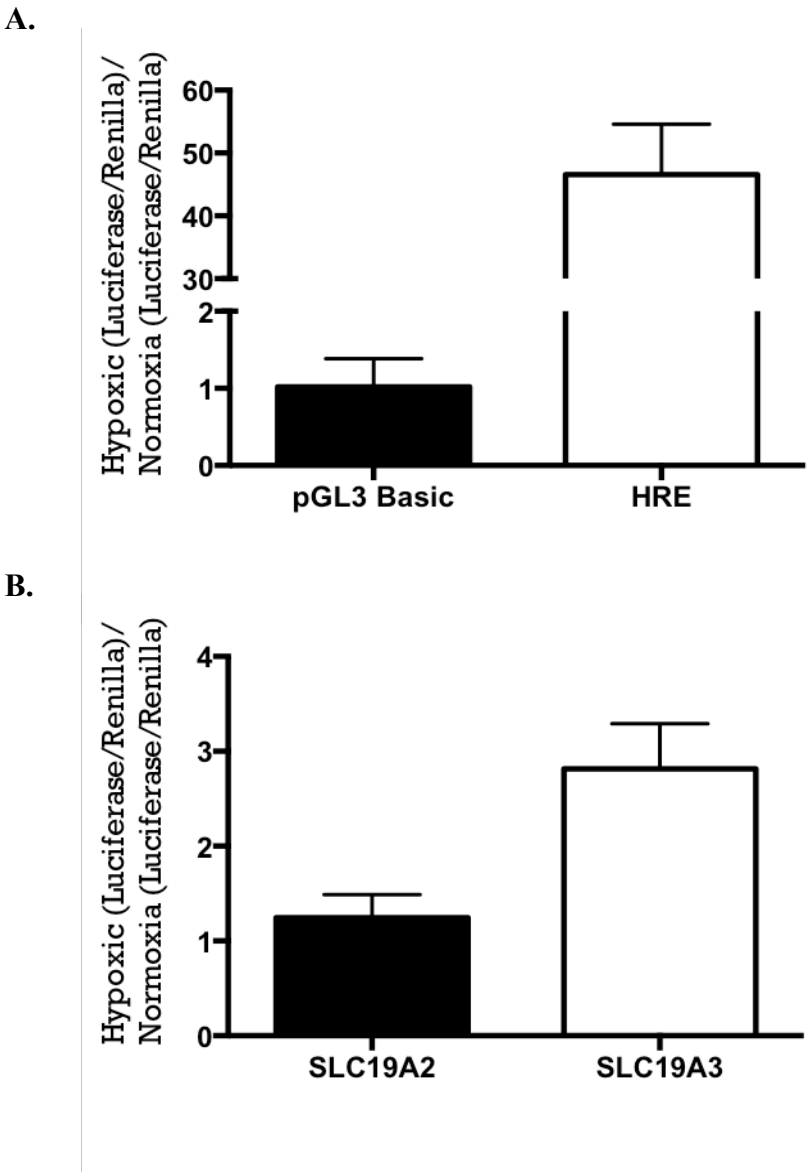

Figure 5.3. Promoter activity after hypoxia exposure. (A) Empty pGL3 Basic (Basic) and pGL3-TK-HRE (HRE) after 48 h 1% O_2 exposure. (B) Full SLC19A2 and full SLC19A3 promoter activity after 48 h 1% O_2 exposure. Promoter activity is expressed as a ratio of firely/*Renilla* luciferase activity. Data are presented as fold changes +/- standard deviation with n=5 independent experiments.

5.4.3. Identification of HREs in the SLC19A3 promoter critical for hypoxia-inducible transcription

The deletion constructs of the SLC19A3 promoter represented in Figure 5.2A were created to identify which of the putative HREs might be responsible for mediating a hypoxic transcriptional response. The promoter activity of the deletion constructs was examined in BT474 cells under normoxic and hypoxic conditions for 48 h (Figure 5.4A). Truncation of the promoter to lengths of 1,029 bp (-970/+59) and 532 bp (-473/+59) resulted in similar promoter activity after 48 h 1% O_2 exposure compared to the full SLC19A3 promoter (-1957/+59) with fold changes of 3.1, 2.4 and 2.8, respectively (Figures 5.3 B and 5.4 A). Truncation of the promoter to 91 bp (-32/+59 construct), which removes all of the putative HREs before the transcriptional start site resulted in a decrease of the promoter activity to approximately 1.5 fold after hypoxia exposure. In addition, a fourth deletion construct containing all four putative HREs but lacking excess bases after the transcription start site showed a decrease in SLC19A3 promoter activity from 2.8 fold (full SLC19A3) to 2 fold (-1957/+13) after hypoxic exposure.

To further investigate which putative HREs play a role in the hypoxia responsiveness of SLC19A3, we created site-directed mutants. Mutations at -1336 (-1336Mut), -908 (-908Mut), and -55 (-55Mut) demonstrated no change in the hypoxia responsiveness of the SLC19A3 promoter. Since HRE-47 overlaps with a reported SP1/SP3 binding site in the minimal promoter region, two mutations were made for this HRE (Figure 5.1A). The first mutation -47aMut was of the CGTG sequence of the HRE to AATG, which also mutated the SP1/SP3 binding site. The second mutation -47bMut was of the CGTG sequence of the HRE to CGAA, leaving the SP1/SP3 binding site

untouched. Both of the HRE-47 mutations led to a slight decrease (2 and 1.9 fold change) in the promoter activity of SLC19A3 after 1% O_2 exposure for 48 h compared to the full SLC19A3 promoter. Interestingly, when both sites -55 and -47A (-55/47Mut) were mutated, there was a loss in the hypoxia responsiveness with a fold change of ~1 after hypoxia exposure compared to normoxia (Figure 5.4 B).

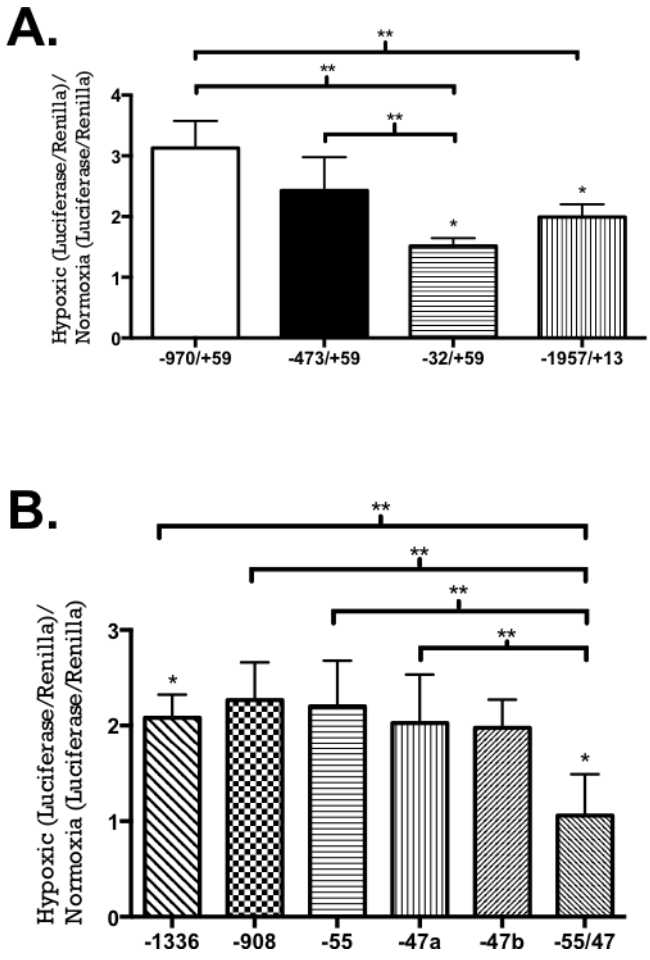

Figure 5.4. Identification of regions in the SLC19A3 promoter that are involved in hypoxia induced transcription. Promoter activity under hypoxic conditions compared to normoxic conditions in SLC19A3 (A) deletion constructs and (B) mutation constructs. Promoter activity is expressed as a ratio of firely/*Renilla* luciferase activity. Data are presented as fold changes +/- standard deviation with N=5 independent experiments. (*) Statistically significant differences compared to SLC19A3 full promoter ($p < 0.05$). (**) Statistically significant differences ($p < 0.05$).

5.5. DISCUSSION

We have previously reported an induction in the SLC19A3 mRNA expression after hypoxic exposure in breast cancer and neuroblastoma cell lines (Sweet *et al.* 2010, Sweet and Zastre Accepted 2013). Recently, an increase in the protein expression of THTR2 (SLC19A3) was observed after hypoxic exposure in human fibroblasts, which correlated with an increase in HIF-1α protein stabilization (Schänzer *et al.* 2014). Using the HIF-1α inhibitor YC1 and a dominant negative form of HIF-1α, results suggest there may be a role for HIF-1α in the adaptive regulation of SLC19A3 (Sweet *et al.* 2010) (Sweet and Zastre Accepted 2013). Recent studies focusing on the adaptive regulation of SLC19A3 have suggested a role for SP1. In an intestinal cell line model, extracellular thiamine levels were reported to increase transcription factor SP1 protein expression and binding pattern to the SLC19A3 promoter region (Nabokina *et al.* 2013). It was concluded the adaptive regulation of SLC19A3 in thiamine deficient conditions is mediated via transcriptional mechanism involving SP1 (Nabokina *et al.* 2013). In addition, SP1 expression is negatively affected by alcohol exposure. SP1 mRNA and protein expression was down regulated following chronic ethanol treatment in a mouse model (Rulten *et al.* 2006). The reduction in SP1 correlated with the down regulation of genes containing SP1 recognition sequences in their promoter regions (Rulten *et al.* 2006). SP1 and SP3 are ubiquitously expressed transcription factors that are involved in basal transcription of many genes including housekeeping genes (Suske 1999, Li *et al.* 2004). The sequence motif they bind to is identical, however, they have the ability to activate or repress transcription in a gene specific manner (Suske 1999) (Li *et al.* 2004).

Hypoxia has been shown to increase nuclear localization of SP1 in human umbilical vein endothelial cells and a role for SP1 in the involvement of facilitating promoter activation by hypoxia has been shown by a number of groups (Xu *et al.* 2000, Lee *et al.* 2003, Sánchez-Elsner *et al.* 2004). Several hypoxia-responsive genes such as EPO and VEGF contain SP1/3 binding sites within their promoter regions (Chin *et al.* 1995, Loeffler *et al.* 2005). However, there are conflicting reports regarding the role of SP1 during hypoxic conditions. Exposure of myocytes to hypoxia resulted in a decrease in nuclear localization and DNA binding of SP3, which positively correlated with an increase in β-enolase transcriptional activation with no change in SP1 (Discher *et al.* 1998). For the UDP-glucose dehydrogenase (UGDH) gene, hypoxic exposure resulted in decreased binding of SP1 to the promoter region (Bontemps *et al.* 2003). Our results indicated hypoxia had a slight increase in SP1/3 expression but no change in SP1/3 nuclear translocation. This suggests SP1/3 may not be involved in upregulating SLC19A3 during hypoxia. Alternatively, there was an increase in HIF-1α protein expression in both whole cell lysate and nuclear translocation, indicating a potential role for HIF-1α in the adaptive regulation of SLC19A3.

HIF-1α regulates gene expression by binding to hypoxia responsive elements in the promoter region containing the consensus sequence 5'-RCGTG-3'. Using mutation and deletion constructs we have identified two putative HREs (-55 and -47) upstream of the transcription start site that are involved in the hypoxic responsiveness of SLC19A3. Mutation of HRE-55 or HRE-47 alone had no effect on promoter activity during hypoxia while mutation of both simultaneously demonstrated no hypoxia responsiveness. This data suggests that two putative HREs closest to the transcription start site may be

primarily responsible for the stimulation of SLC19A3 promoter activity during hypoxia. Interestingly, expression of SLC19A2 was not upregulated in hypoxia even though the promoter contains 7 HREs. Most known functional HREs consist of two contiguous sites commonly in close proximity to one another and are separated by less than 9 bp (reviewed in (Kimura *et al.* 2001)). One sequence is termed the hypoxia binding sequence and the other is the ancillary sequence (Kimura *et al.* 2001). In the SLC19A3 promoter region, HRE-55 and HRE-47 are separated by four base pairs while the closest HREs in the SLC19A2 promoter have 22 bp between them. The positioning of the HREs within the promoter region may explain the hypoxia responsiveness observed in SLC19A3 but not SLC19A2.

In conclusion, we have shown hypoxic exposure induces activation of SLC19A3 promoter activity and not SLC19A2, consistent with previous findings on the adaptive regulation during hypoxia and thiamine deficiency. Two putative HREs upstream at -55 and -47 of the transcriptional start site are essential for mediating the hypoxia responsiveness. Further work is required to discern if HIF-1α is directly binding to this region or indirectly trans-activating SLC19A3 expression.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1. SUMMARY

Cancer cells have an increased demand for growth factors in order to supply an altered metabolism and a high proliferation rate. Nutrient uptake and metabolism are in part controlled by oncogene-mediated regulation to increase the cellular supply of carbon sources (Kim *et al.* 2004, Wise *et al.* 2008). Most nutrients require the presence of specific transporters since they do not readily diffuse across the plasma membrane due to their hydrophilicity. Therefore, tumor cells increase nutrient entry into cells through the upregulation of specific transporters in the plasma membrane. A coordinated enhancement in coenzyme supply such as thiamine may be required to support enzyme activity in rapidly dividing cells. The overall goal of this research was to investigate the adaptive regulation of thiamine transporters during exposure to physiological stressors such as limited oxygen availability and nutrient deprivation.

The expression of thiamine homeostasis genes in cancer has previously been studied with the most notable changes in SLC19A3 compared to normal tissues. A decrease in the thiamine transporter SLC19A3 expression has been shown in breast, gastric and colon cancer in comparison to corresponding normal tissues (Liu *et al.* 2003, Liu *et al.* 2009, Ikehata *et al.* 2012). Epigenetic repression through hypermethylation and histone deacetylation of the SLC19A3 promoter has been linked to the decrease in

expression observed in cancers (Liu *et al.* 2009, Ikehata *et al.* 2012). Methylation of the SLC19A3 promoter was reported in the plasma of early- and advanced-stage breast cancer patients as well as gastric cancer (Ng *et al.* 2011). Our group found a slight decrease, but not statistically significant for SLC19A3 gene expression in breast cancer tissue samples compared to normal breast tissue (Zastre *et al.* 2013). Alternatively, we also reported a significant increase in the gene expression of SLC19A2 (Zastre *et al.* 2013). Upregulation of SLC19A2 and downregulation of SLC19A3 was also verified in several breast cancer cell lines compared to human mammary epithelial cells (hMECs).

While SLC19A3 expression appears to be repressed in a number of cancers, these studies did not address potential cellular heterogeneity within tumors due to microenvironment effects such as hypoxia. Our lab has demonstrated that hypoxic exposure of breast cancer cells resulted in upregulation of SLC19A3 expression compared to cells grown under normoxic conditions (Sweet *et al.* 2010). A corresponding 2-fold increase in thiamine transport was also found (Sweet *et al.* 2010). Increased thiamine utilization in tumor cells has been shown to support the biosynthesis of nucleic acid precursors and neurotransmitters, demonstrating the importance of thiamine in cancer cell metabolism (Singleton and Martin 2001). We have reported an attenuated hypoxic response in SLC19A3 expression in cells transfected with HIF-1a shRNA after hypoxia exposure (Sweet *et al.* 2010). These results demonstrate the potential involvement of HIF-1a in regulating SLC19A3 expression.

When thiamine availability is limited, an adaptive transcriptional upregulation of the thiamine transporter SLC19A3 has been observed in intestinal and renal tissues in an attempt to increase thiamine absorption and reduce clearance (Reidling and Said 2005,

Ashokkumar *et al.* 2006). Consistent with these findings we observed that TD treatment of SK-N-BE cells resulted in an up-regulation of SLC19A3 expression with no change in SLC19A2. Synonymous to our previous findings, hypoxia increased SLC19A3 in SK-N-BE cells that was attenuated by the HIF-1α inhibitor YC1 and the dominant negative form of HIF-1α in TD and hypoxia treatments. This further demonstrates a potential regulatory role of HIF-1α in mediating thiamine transporter expression during hypoxic and nutritional stress. Our results indicate thiamine deficiency induces HIF-1α mediated gene expression similar to that observed in hypoxia/ischemia. The induction of HIF-1α may provide a nutritional connection to the activation of this important regulator of glycolytic metabolism and a metabolic switch required for cellular adaptation to TD. These results are the first to demonstrate a transcriptional relationship between hypoxia and thiamine deficiency that may explain the clinical manifestations occurring in pathologies associated with thiamine deficiency.

A potential role for HIF-1α in the regulation of SLC19A3 was demonstrated through attenuation in upregulation with HIF-1α shRNA, DN- HIF-1α, and YC1. Using *in silico* sequence analysis we have identified four putative HRE elements with the characteristic motif 5'-RCGTG-3' in the SLC19A3 promoter region. In corroboration with our previous findings, an induction in promoter activity of SLC19A3 after hypoxic exposure with no change in SLC19A2 compared to normoxia was found. The increase in promoter activity after hypoxic exposure was diminished when HREs located at -55 and -47 (-55/47Mut) were both mutated, indicating their involvement in the regulation of SLC19A3 promoter activity to hypoxic exposure. Interestingly, HRE55 and HRE47 in the SLC19A3 promoter region follow the separation pattern of most known functional

HREs, consisting of two contiguous sites separated by less than 9 bp (Kimura *et al.* 2001). Although these results do not elucidate the mechanism for hypoxia-mediated regulation of SLC19A3, it provides strong support for HIF-1α involvement in transactivating SLC19A3 expression.

In summary, adaptive regulation of the thiamine transporter SLC19A3 by HIF-1α is a novel finding. The data presented herein strongly suggest the involvement of the thiamine transporter SLC19A3 in the adaptive response of cells to physiological stresses such as hypoxia and nutrient deprivation. In addition, the role of HIF-1α in adaptive thiamine transport provides potential strategies that can modulate thiamine availability in TD associated syndromes. Further investigation of the molecular mechanisms involved in maintaining and regulating micronutrient homeostasis would not only establish a mechanistic comprehension into the adaptive regulation of micronutrients, but would provide insights to potential strategies to modulate nutrient availability in many etiologies.

6.2. EXPERIMENTAL LIMITATIONS

This section describes the experimental limitations and assumptions made with respect to the cell systems and methodologies used to investigate the adaptive regulation of vitamin B1 transporters in response to physiological stresses.

6.2.1. In vitro cell culture systems

While *in vitro* cell culture systems are widely used throughout research, it is well understood their use presents certain limitations when interpreting data. We utilized

breast and neuroblastoma cell line models to study the adaptive responses to physiological stresses measured through alterations in metabolite levels and changes in gene expression. In particular, we are interested in the effects of altered oxygen and thiamine levels. While *in vitro* culturing limits observations to a two-dimensional system, future work will aim to include the use of *in vivo* models to study diffusion and reperfusion limitations of oxygen.

6.2.1.1. Hypoxic conditions

In a physiological environment, many factors contribute to the oxygen availability to cells. Specifically in a tumor microenvironment, oxygen levels form a gradient with cells furthest from blood vessels receiving the least amount of oxygen exposure. Using an *in vitro* cell line model it is hard to recapitulate a similar microenvironment with a dynamic oxygen exposure. We are unable to account for the potential exposure of cells to a range of low oxygen conditions in a tumor microenvironment (0.1-2%) (Vaupel *et al.* 1989). Therefore, we have selected a treatment of 1% oxygen for hypoxic conditions. 1% oxygen falls in the middle of the range of low oxygen levels cells could potentially be exposed to within a tumor without creating an anoxic state.

6.2.1.2. Nutrient availability

Culturing of immortalized cells in an *in vitro* environment requires the use of specific media components in order to support cell proliferation and survival. Cell culture media for *in vitro* culturing can be cell type specific with different medias containing a range of nutrients, amino acids and buffering systems. For our studies we utilized

custom-made thiamine deficient RPMI media in order to control for thiamine exposure to cells (Sweet and Zastre Accepted 2013). The only component of the formulation changed for this media was the amount of thiamine. The physiological concentration of glucose has been reported to range from 1 to 8 mM (Silver and Erecinska 1994, Abi-Saab *et al.* 2002). It is common for culture media to contain up to 25 mM glucose, however, normal RPMI contains 11 mM glucose. While we have controlled for thiamine concentrations, excess glucose has been linked to HIF-1α stabilization (Lu *et al.* 2002). Therefore, future studies aim to further refine the *in vitro* model to closer mimic physiological concentrations of glucose.

6.3. FUTURE DIRECTIONS

6.3.1. Thiamine in hypoxic tumor microenvironments

Prolonged hypoxia/ischemia has been linked to cell death in cardiomyopathy, chronic heart failure and heart dysfunction (Ohata *et al.* 1994, Tanaka *et al.* 1994, Bishopric *et al.* 2001). Interestingly, thiamine deficiency has also been shown to result in heart failure (Ozawa *et al.* 2001, Singleton and Martin 2001). Patients presenting with congestive heart failure are commonly thiamine deficient and therefore previous studies have investigated the effect of thiamine supplementation on ischemic damage to cells (Brady *et al.* 1995). In an experimental myocardial infarction rat model, thiamine supplementation resulted in a cyto-protective effect against ischemic damage (Vinogradov *et al.* 1990). The number of ischemic lesions was also reduced after thiamine administration in an infarct model (Larrieu *et al.* 1987). The protection of cardiomyocytes from hypoxic stress by thiamine is reported to be through an increase in

the expression of heat shock protein-70 (Hsp70) and a decrease in apoptosis as measured by caspase activity (Shin *et al.* 2004).

Previous reports in the literature have focused on the effect of thiamine on hypoxia induced cell death. However, the function of thiamine homeostasis genes during the adaptive regulation under hypoxic conditions is unknown. The thiamine transporter SLC19A3 increases in expression after hypoxic exposure in breast cancer, neuroblastoma and wild type human non-neoplastic cells (Sweet *et al.* 2010, Schänzer *et al.* 2014, Sweet and Zastre Accepted 2013). The increase in SLC19A3 expression and the corresponding enhancement in thiamine transport under hypoxia may be a critical pro-survival response to supply thiamine in support of the PPP and maintenance of tumor cell metabolism and proliferation within hypoxic microenvironments. However, the functional role of an increase in expression of SLC19A3 under hypoxic conditions is unknown and fundamental knowledge regarding the role of thiamine in the hypoxic tumor microenvironment is lacking. Future work will aim to further our understanding of the effects of thiamine on the maintenance of tumor cell metabolism and proliferation within hypoxic microenvironments.

6.3.2. Adaptive regulation of SLC19A3 after ethanol exposure

TD can lead to mitochondrial dysfunction, metabolic and oxidative stress, and lactic acidosis, which have been linked to neurologic impairment in alcohol dependency (Butterworth 1995, Hazell and Butterworth 2009). TD in severe alcoholism involves poor nutrition, impaired enzyme utilization, and reduced intestinal and renal transport capacity (Hoyumpa 1980, Cook *et al.* 1998, Subramanian *et al.* 2010). Although, chronic alcohol

consumption is associated with a down regulation of thiamine transporters, TD has been recognized to stimulate an adaptive transcriptional upregulation of the thiamine transporter, SLC19A3 to increase intracellular supply (Ashokkumar *et al.* 2006). This antagonism between alcoholism and TD is not clearly understood due to a lack of fundamental knowledge regarding the regulatory elements mediating the adaptive expression of thiamine transporters. Future work for this project includes understanding the effect of ethanol on the transcriptional machinery controlling basal and adaptive expression of the thiamine transporters. Once the transcriptional pathways for the adaptive thiamine transporter expression during ethanol exposure are known, new insights and approaches into the underlying effects of alcohol consumption on thiamine transport capacity can be achieved.

6.3.3. Mechanistic understanding of TD induced lesions

Although cells adapt to hypoxia through the stabilization of HIF-1α, chronic hypoxia can ultimately lead to cell death. Aside from effects on metabolism, hypoxic signaling pathways may also induce apoptosis and necrosis (Shimizu *et al.* 1996). HIF-1α serves dual functions being involved in both pro-survival and pro-apoptotic responses. Consistent with findings that the functional HIF complex requires HIF-1α phosphorylation, HIF-1β preferentially binds phosphorylated HIF-1α when oxygen levels are reduced (Figure 6.1) (Semenza 2000, Suzuki *et al.* 2001). In addition, long term exposure to hypoxia results in the increase in expression of Bcl2/adenovirus EIB 19kD-interacting protein 3 (BNIP3), a member of the Bcl-2 pro-apoptotic protein family (Regula *et al.* 2002). *In situ* hybridization analysis of RNA expression in human breast

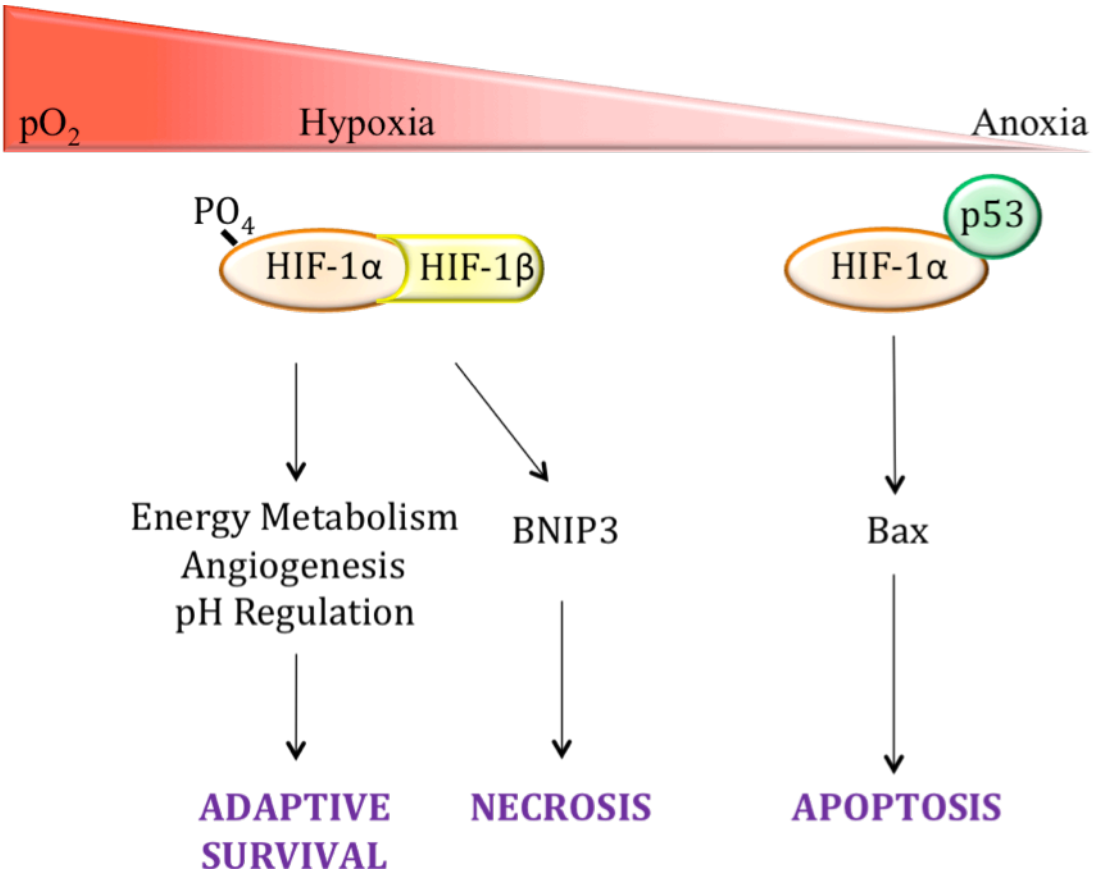

Figure 6.1. Phosphorylated and unphosphorylated forms of HIF-1α. The phosphorylated form of HIF-1α binds to HIF-1β and binds to the promoter region of HIF target genes. Genes that aid in adaptive survival can be involved in energy metabolism, angiogenesis and pH regulation. HIF can also activate BNIP3 which results in necrosis. The unphosphorylated form of HIF-1α can bind to p53 aiding in its stabilization and allowing the activation of proapoptotic genes.

tumors indicated BNIP3 to be in peri-necrotic areas of the tumor resulting from hypoxic stress (Sowter *et al.* 2001). BNIP3 can cause the mitochondrial permeability transition pore to open, activating caspase-independent necrotic-like cell death (Velde *et al.* 2000). Alternatively, when hypoxia is severe and prolonged, dephosphorylated HIF-1α binds to and stabilizes p53 (An *et al.* 1998, Suzuki *et al.* 2001). The stabilization of p53 in hypoxia has been shown to increase apoptotic cell death through transactivation of pro-apoptotic genes such as Bax (Graeber *et al.* 1994). Of interest, we have observed a change in the phosphorylation status of HIF-1α that may correlate with either adaptive survival or the induction of necrosis under thiamine deficient conditions (Figure 6.1) (Sweet and Zastre Accepted 2013).

Glutamate induced excitotoxic lesions have also been linked to ischemic injury. While glutamate is commonly released in synapses in the brain, neuronal and glial uptake systems rapidly transport glutamate from the extracellular space serving to reduce toxic effects (Schousboe 1981). During hypoxic conditions a reduction in glutamate uptake results in increased extracellular glutamate concentrations (Dallas *et al.* 2007) (Silverstein *et al.* 1986). During ischemia in cortical neurons, glutamate concentrations increase to levels that are extremely toxic (Choi *et al.* 1987).

Similarly, there is strong evidence for a role of glutamate in TD-induced cell death. There is an increase in the concentration of glutamate in regions vulnerable to TD (Hazell *et al.* 1993, Langlais and Zhang 1993, Hazell and Butterworth 2009). In addition, the increased extracellular glutamate in TD is suggested to result from decreased glutamate transporter expression and glutamate uptake (Hazell *et al.* 2003). While excitotoxicity from increased extracellular glutamate levels contribute to the selective

loss of neurons associated with TD, other mechanisms such as apoptosis and necrosis have been suggested to play a role in the pathogenesis of TD induced brain lesions (Matsushima *et al.* 1997). Overall, there are many proposals as to how TD results in cell death, however the exact mechanism of TD-induced lesions is unknown.

Comparable necrotic lesions were found in patients with hypoxia/ischemia and TD in the thalamus and mamillary bodies, suggesting congruency between the cellular response to hypoxic and TD stress (Vortmeyer and Colmant 1988, Vortmeyer *et al.* 1993). These observations may suggest a metabolic relationship between hypoxia and thiamine deficiency and may explain the similarities in neural lesions (Vortmeyer *et al.* 1993). Investigation of underlying cellular mechanisms of TD induced lesions may allow the realization of novel treatment options for patients with TD-related pathologies.

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APPENDIX

UP-REGULATION OF VITAMIN B1 HOMEOSTASIS GENES IN BREAST CANCER [4]

[4] Zastre, J. A., B. S. Hanberry, R. L. Sweet, A. C. McGinnis, K. R. Venuti, M. G. Bartlett and R. Govindarajan 2013. *The Journal of Nutritional Biochemistry*. 24: 1616-1624. Reprinted here with permission of publisher.

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 *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 *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 *et al.* 2011). During high glucose utilization, thiamine status is reduced demonstrating an integral connection between thiamine supply and metabolic flux (Elmadfa *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 *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 *et al.* 2000, Langbein *et al.* 2006). Transketolase activity has been shown to supply ~85% of the C5-ribose sugars for nucleic acid synthesis in cancer cells (Boros *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 *et al.* 2007{Langbein, 2006 #313, Schultz *et al.* 2008, Zhang *et al.* 2008, Xu *et al.* 2009). Additionally, glutaminolysis in cancer cells contributes to anaplerotic reactions that re-supply the TCA cycle with a-ketoglutarate (DeBerardinis *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 *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 *et al.* 2006, Schmidt *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 *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 *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.

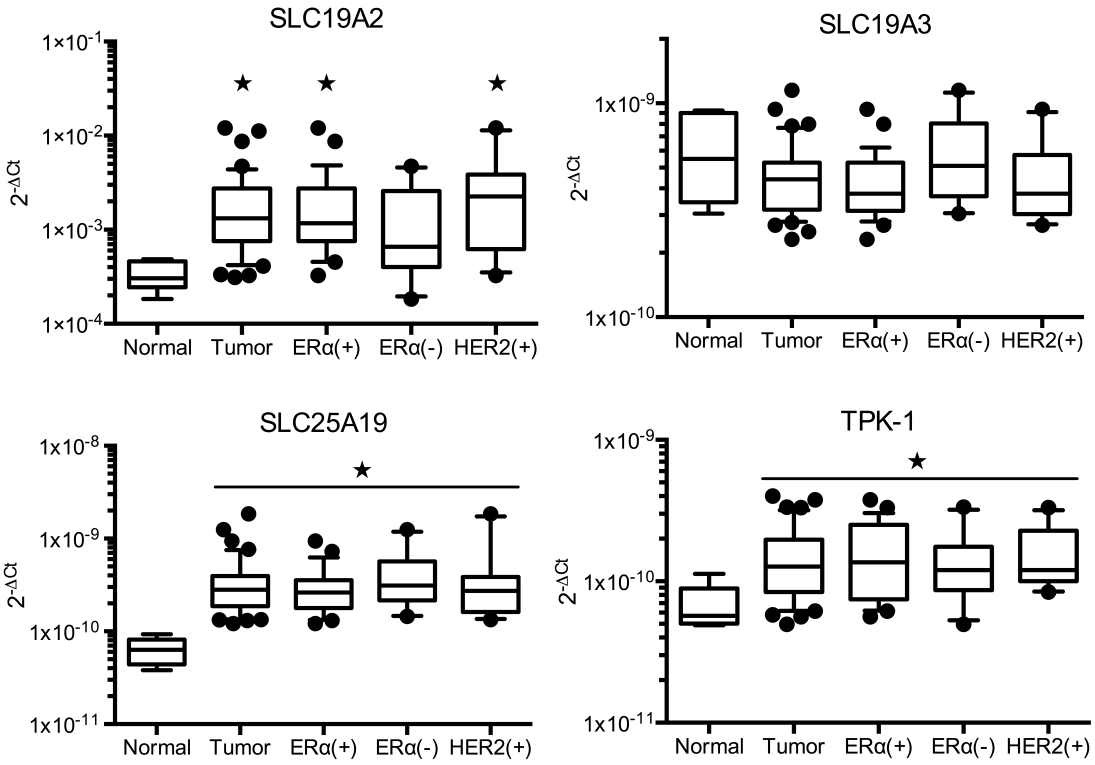

Figure A.1. Expression of thiamine homeostasis genes, SLC19A2, SLC19A3, SLC25A19 and TPK-1 in breast cancer and normal tissues from 48 clinical samples. Gene expression levels were determined by qRTPCR using TissueScan Breast Cancer cDNA Array II. Results are expressed as the Log2 gene expression normalized 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).

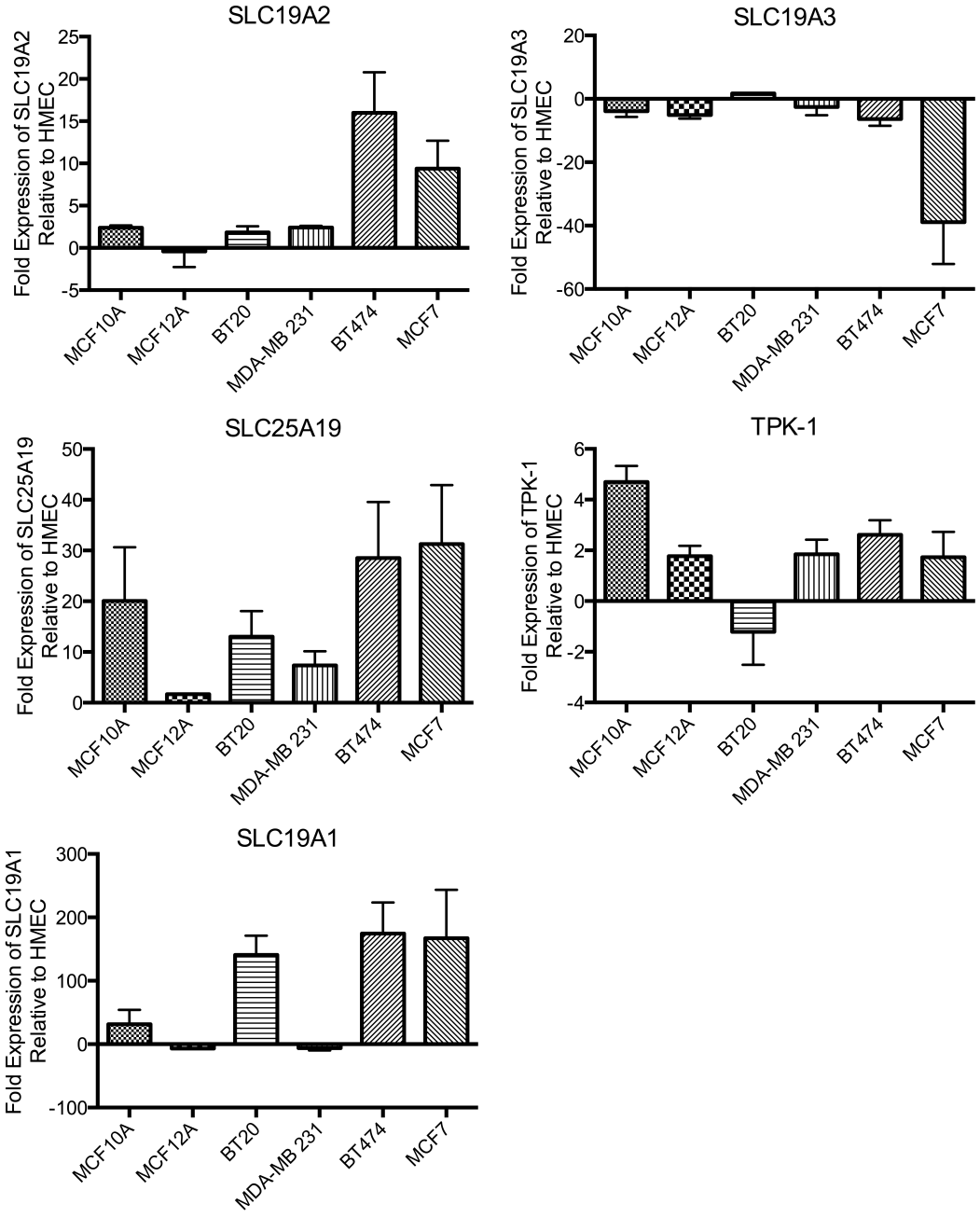

Figure A.2. Expression of thiamine homeostasis genes, SLC19A2, SLC19A3, SLC25A19, TPK-1, and SLC19A1 in immortalized non-tumorigenic breast epithelia and breast cancer cell lines compared to primary human mammary epithelial cells (HMECs). Gene expression levels were determined by qRTPCR using TBP as 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 *et al.* 2003, Ashokkumar *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).

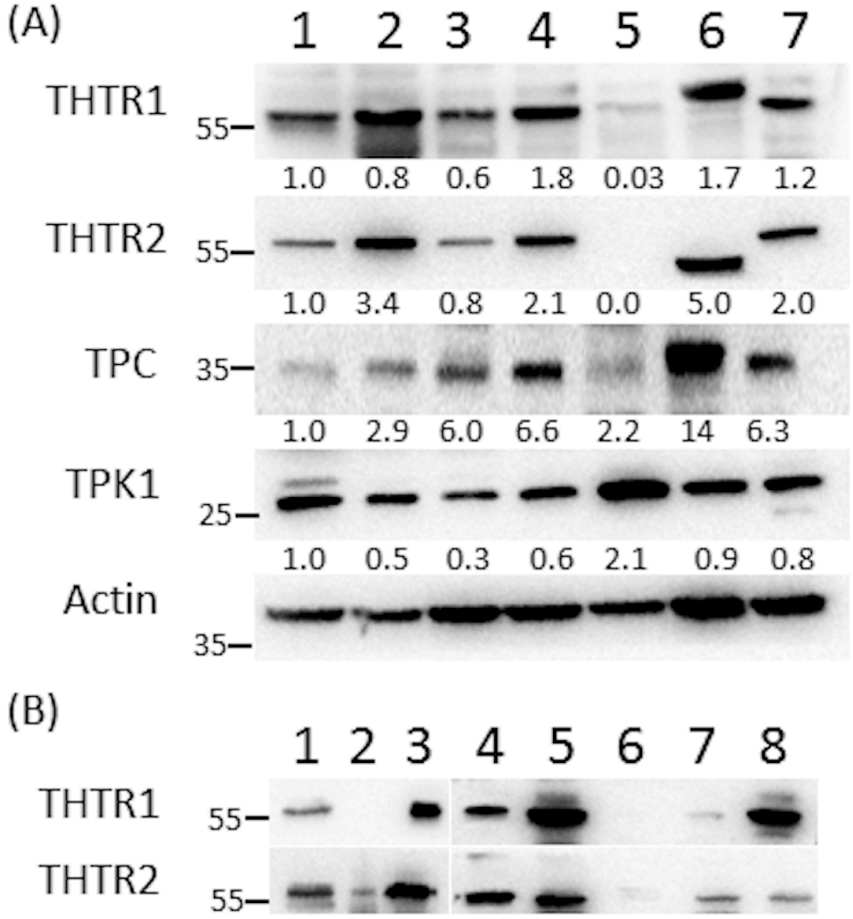

Figure A.3. Representative Western blots of THTR1, THTR2, TPC and TPK-1 in immortalized non-tumorigenic breast epithelia, breast cancer cell lines, and HMECs. (A) Whole cell lysate preparations of (1) HMEC, (2) MCF12A, (3) MCF10A, (4) BT474, (5) BT20, (6) MDA-MB-231, (7) MCF7 were immunofractionated 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.

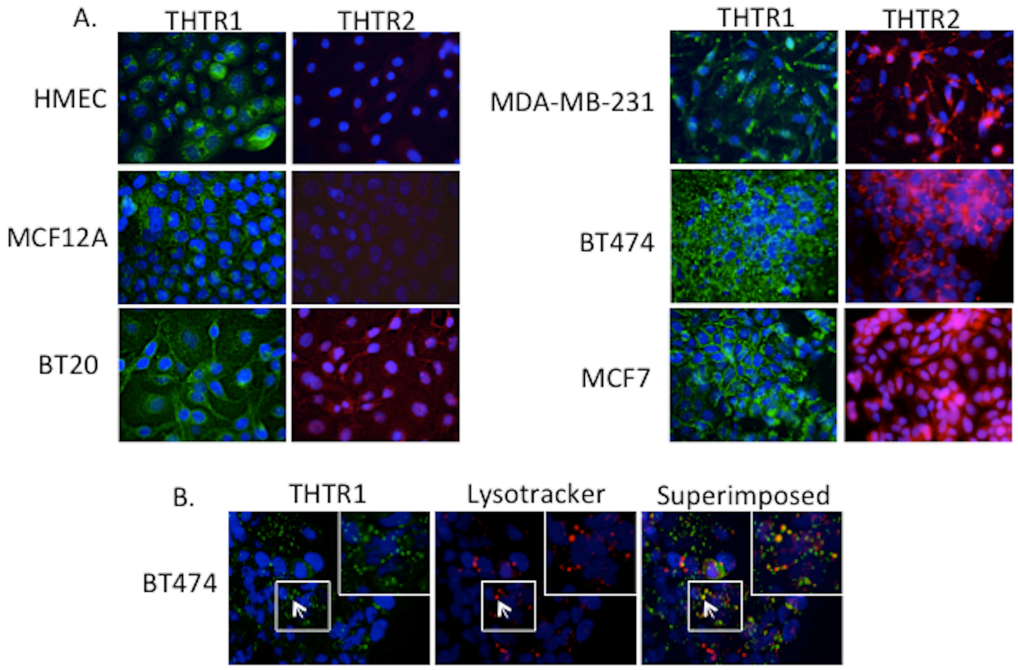

Figure A.4. Cellular localization of THTR1 and THTR2 by Immunofluorescence. (A) Localization of THTR1 and THTR2 in breast cancer cell lines compared with HMECs and MCF12A cells. (B) Co-localization of Lysotracker dye with THTR1 in BT474 cells.

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 *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 *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).

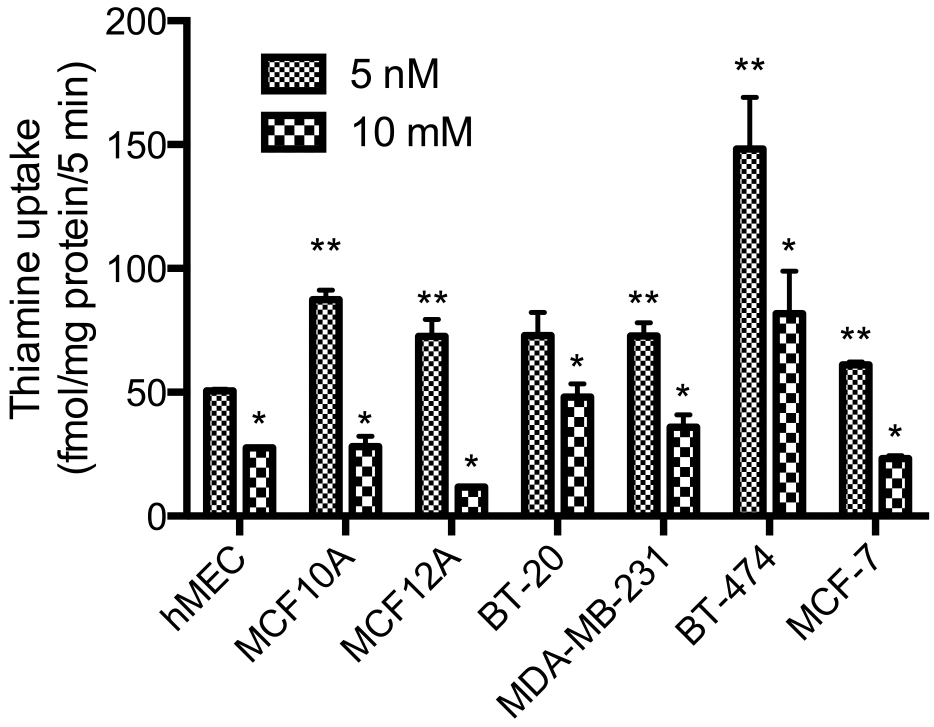

Figure A.5. Uptake of thiamine by breast cancer (BT20, MDA-MB-231, BT474, MCF7) and normal breast epithelial cell lines (HMEC, MCF10A, MCF12A). Thiamine uptake was performed for 5min at 37^0C in pH=7.4 transport buffer with or without excess (10mM) thiamine. Results reported as the mean +/- SEM normalized 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 (★★).

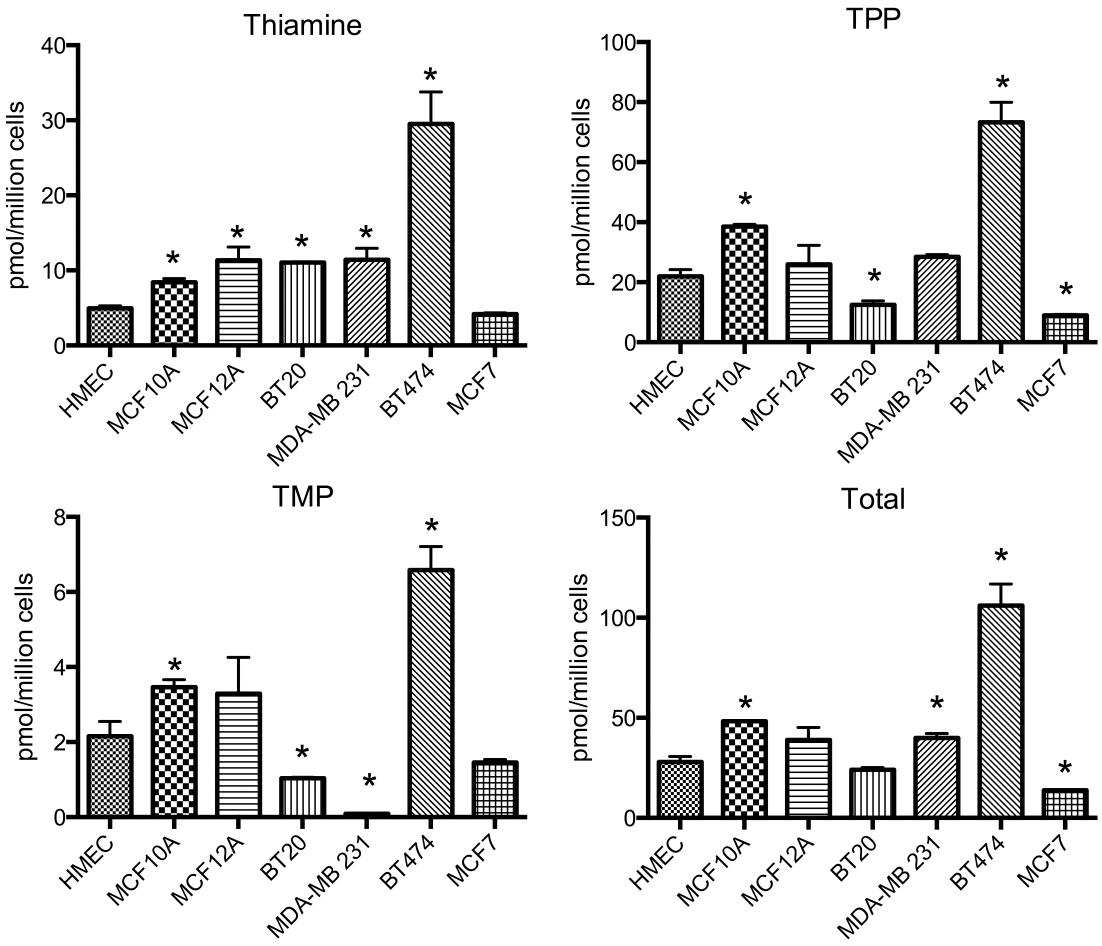

Figure A.6. Quantitation of intracellular thiamine and thiamine phosphate esters, TPP and TMP in breast cancer and normal breast epithelial cell lines. Thiamine and thiamine phosphates in cell lysates were derivatized to a thiochrome derivative for HPLC separation and detection by fluorescence. Quantitation 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 *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 *et al.* 2003, Liu *et al.* 2009, Ikehata *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 *et al.* 2003, Subramanian *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 *et al.* 2011). Tetraspanin proteins are important in cell motility and invasion and are associated with acting as a tumor metastasis suppressor (Richardson *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 *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 *et al.* 2003, Subramanian *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 *et al.* 1999, Ashokkumar *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 *et al.* 2008, Zhou *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 *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 *et al.* 2004, Ashokkumar *et al.* 2006). Recently, transport of thiamine across intestinal epithelial has also been found to be mediated by organic cation transporters (OCT1/3) (Lemos *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 *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 *et al.* 1997, Rindi and Laforenza 2000, Zhao *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 *et al.* 2001, Zhao *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 *et al.* 2000, Trachootham *et al.* 2009). Free thiamine has direct antioxidant properties as well as TPP being essential for glutathione production (Lukienko *et al.* 2000, Martin *et al.* 2003, Schmid *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.