Investigation of the Expression of Glucose Transporter Proteins in Human Cancer Cells

Carly Barron, BSc

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Supervisor: Evangelia Tsiani, PhD

Faculty of Applied Health Sciences
Brock University
St. Catharines, ON.

Abstract

Cancer cells are known to display increased glucose uptake and consumption. The glucose transporter (GLUT) proteins facilitate glucose uptake, however, their exact role in cancer metabolism remains unclear. The present study examined mRNA and protein expression of GLUT1, GLUT3, GLUT4 and GLUT12 in lung, breast and prostate cancer cells and corresponding noncancerous cells. Additionally, GLUT expression was determined in tumours from mice xenografted with human cancer cells. Differences in the mRNA and protein expression of GLUTs were found between cancerous and corresponding noncancerous cells. These findings demonstrate abundant expression of GLUT1 in cancer and highlight the importance of GLUT3 as it was expressed in several cancer cells and tumours. GLUT expression patterns *in vitro* were supported by the *in vivo* findings. The study of GLUT protein expression in cancer is important for understanding cancer metabolism and may lead to identification of biomarkers of cancer progression and development of target therapies.

Key words: epithelial cancer, GLUT1, GLUT3, GLUT4, GLUT12

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CHAPTER 1: LITERATURE REVIEW

1.1 Cancer

Unlike healthy cells, cancer cells share specific properties enabling malignant growth[1]. One of the most fundamental characteristics of cancer cells is their uncontrolled proliferative capacity, resulting from oncogenic mutations that deregulate signals controlling the cell growth-and-division cycle[1]. In order to sustain enhanced proliferation, cancer cells have increased requirements for sugars, fatty acids and amino acids. These nutrients provide energy and serve as the building blocks for macromolecules[2]. Although cancer cells display alterations in the metabolism of all classes of macromolecules, the role of carbohydrate metabolism in cancer has received attention in recent years because cancer cells are particularly dependent on glucose metabolism for energy production.

1.2 Glucose Metabolism in Cancer

Metabolic changes are a common feature of cancer cells. Although the glycolytic pathway is ubiquitous in the body, its role can differ between healthy and cancerous cells. The following sections describe how glucose is metabolized to provide energy and the modifications that may occur to this process in cancer.

1.2.1 Glycolysis

The ATP required for cellular processes is generated primarily through two pathways: glycolysis and the citric acid cycle. During glycolysis, one molecule of glucose is metabolized through a series of reactions and split into two pyruvate molecules. The reactions and enzymes of glycolysis can be seen in **Figure 1**. In the first step, glucose becomes phosphorylated to glucose-6-phosphate in an endergonic reaction requiring

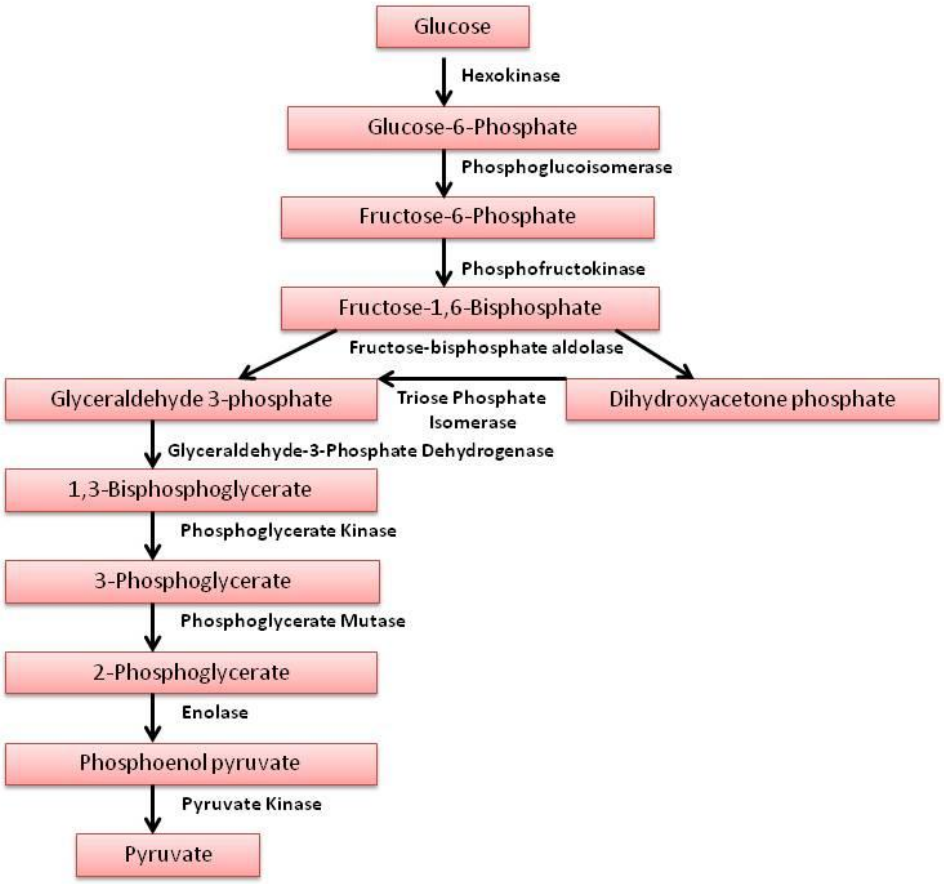

Figure 1. The metabolic pathway of glycolysis. Each glucose molecule that is metabolized through this process produces a net energy yield of 2 ATP. Although various cells of the body use this pathway, many types of cancers show a greater dependence on this pathway to provide energy[3].

energy from ATP. Next, glucose-6-phosphate is isomerized to fructose-6-phosphate. In the third step of glycolysis, phosphofructokinase catalyzes the transfer of ATP to fructose-6-phosphate to form fructose-1,6-bisphosphate. This reaction is thermodynamically favorable and it is known as a committed step because fructose-1,6-biphosphate can only react in glycolysis, unlike glucose-6-phosphate which can be shunted to other pathways. Fructose-1,6-biphosphate is then split into two 3-carbon units

that are oxidized to pyruvate, producing a net energy yield from each glucose molecule of two ATP and two NADH[3].

1.2.2 The Citric Acid Cycle

If adequate oxygen is present, pyruvate continues into the citric acid cycle. However, under conditions of limited oxygen, pyruvate becomes converted into lactate by the enzyme lactate dehydrogenase in a process known as anaerobic glycolysis. One NAD^+ is regenerated in the process of reducing pyruvate and is re-used in the glycolytic pathway

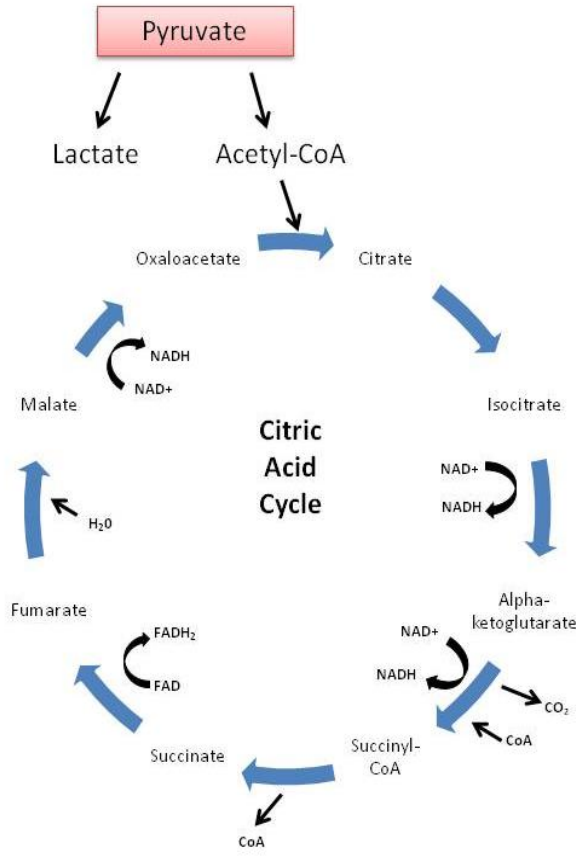

Figure 2. The fate of pyruvate. In healthy cells, pyruvate is converted to lactate under anaerobic conditions. If oxygen is present, pyruvate is converted to Acetyl-CoA and continues into the citric acid cycle. Cancer cells may display conversion of pyruvate to lactate even in the presence of oxygen[3].

so that ATP production can continue to occur in the absence of oxygen.

Under aerobic conditions, pyruvate is transported into the mitochondria and converted to acetyl Coenzyme A (CoA) by the pyruvate dehydrogenase complex. The two-carbon acyl unit of acetyl CoA enters the citric acid cycle by combining with oxaloacetate to form citrate. The series of reactions of the citric acid cycle can be seen in **Figure 2**. Citrate is further oxidized to release carbon dioxide and produce NADH from NAD^+ with each oxidative decarboxylation. The cycle is completed when an oxaloacetate molecule is regenerated. For each molecule of glucose oxidized, two full turns of the citric acid cycle are completed (one per pyruvate), yielding a total of 6 NADH, 2 $FADH_2$ and 2 GTP[3].

1.2.3 Oxidative Phosphorylation

Oxidative phosphorylation takes places across the inner mitochondrial membrane. It uses oxidation of the high energy electrons from NADH and FADH2, produced in glycolysis and the citric acid cycle, to pump protons out of the mitochondrial matrix and into the inter membrane space. The electron transport chain is performed by electron carrier protein complexes embedded in the inner mitochondrial membrane which receive electrons in sequence from NADH dehydrogenase → ubiquinone → cytochrome C reductase → cytochrome C → cytochrome C oxidase (**Figure 3**). Oxygen acts as the final electron acceptor combining with protons to form water. As the electrons are passed from protein to protein, energy is used to pump H+ out of the matrix across the inner mitochondrial membrane into the intermembrane space. This creates a proton gradient which drives protons to pass back into the matrix through the ATP synthase channel and

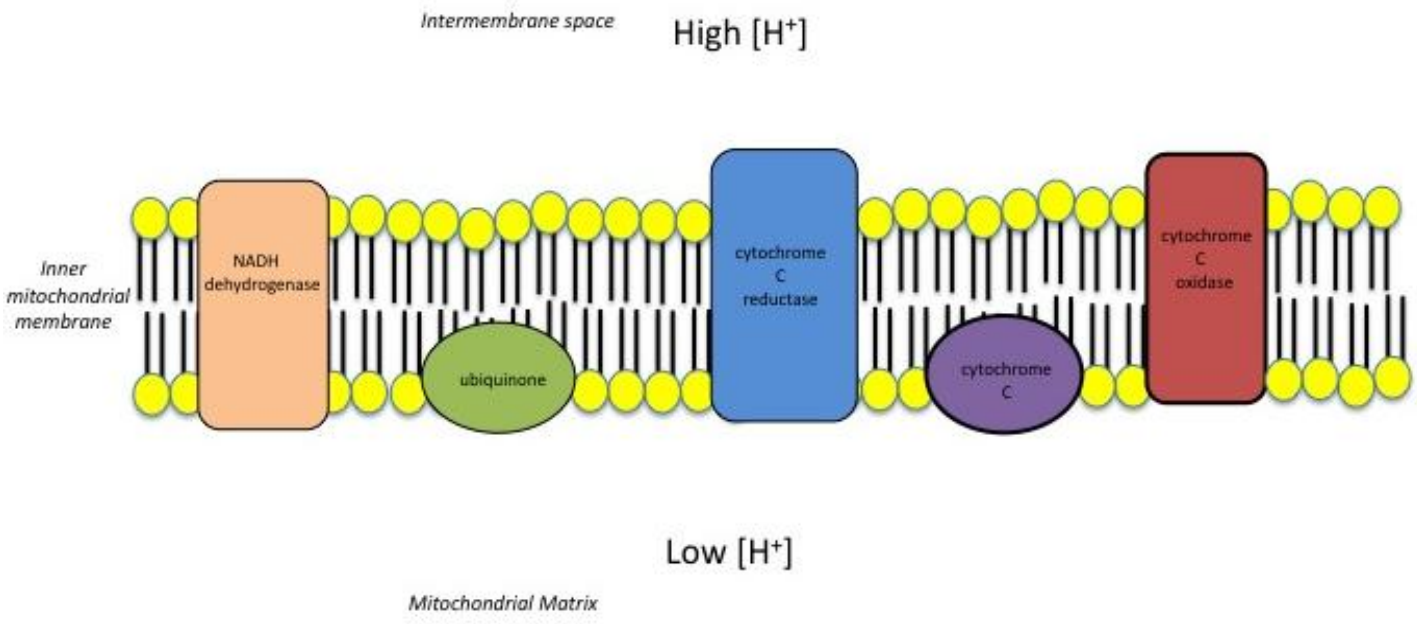

Figure 3. The electron transport chain. Electron carriers of the electron transport chain are found in the inner mitochondrial membrane. Healthy cells use these carriers to oxidize high energy electrons from NADH and $FADH_2$, pumping protons out of the mitochondrial matrix in the process and creating a proton gradient that is used to phosphorylate ADP to ATP[3].

produces ATP from ADP and PO_4^{2-} in the process. The net yield of ATP produced from one glucose molecule catabolized through the aforementioned aerobic pathways in eukaryotes is approximately 30, depending on the number of protons available for ATP synthesis. This represents a significant increase in energy yield compared to the 2 ATP produced in the anaerobic conversion of glucose to lactate.

1.2.4 The Warburg Effect

In 1920, the renowned biochemist Otto Warburg observed that human and animal tumours rely on the conversion of glucose into lactate for energy production instead of mitochondrial oxidation, even in the presence of oxygen[4]. His observation is supported

by the elevated levels of lactate dehydrogenase that have been found in various cancers[5]. The preferred conversion of glucose to lactate for energy production in the presence of oxygen (referred to in this text as aerobic glycolysis) in tumour cells became known as the "Warburg Effect".

There has been great interest in understanding why tumour cells, with increased metabolic needs, use such an inefficient pathway to derive energy from glucose. Initially, it was suggested that tumour cells use aerobic glycolysis because of mitochondrial defects preventing ATP production by oxidative phosphorylation[4]. However, this hypothesis was countered by studies showing that aerobic glycolysis is reversible and in most cases there is no permanent mitochondrial damage in cancer cells. This was demonstrated using short hairpin RNA-mediated knockdown of lactate dehydrogenase in mammary tumour cells, where inhibition of lactate production stimulated mitochondrial respiration in these cells[6]. In another study, dichloroacetate, a small molecule inhibitor of pyruvate dehydrogenase kinase, was used to activate pyruvate dehydrogenase and induce cancer cells to oxidize pyruvate in the citric acid cycle[7]. Although the breast cancer, non-small-cell lung cancer, and glioblastoma cells used were able to perform mitochondria-based glucose oxidation after this induction, apoptosis was increased. This indicates that aerobic glycolysis is also vital for cancer cell survival. Furthermore, there is some evidence to suggest that a degree of mitochondrial metabolism is needed for ROS production in tumourigenesis and growth[8,9].

The current theory behind the Warburg effect explains that tumour cells switch to aerobic glycolysis in order to produce intermediates needed for cell growth and division[10]. Healthy cells and cancer cells both use glucose to produce ATP and to

generate metabolites required for synthesis of amino acids, fatty acids and nucleotides. Although some ATP is needed in the reactions that produce macromolecules, in proliferating cancer cells there is a greater demand to covert glucose to macromolecular precursors than to produce ATP through oxidative phosphorylation by metabolizing glucose to CO_2 and H_20[10]. Glucose can provide six carbons for macromolecule synthesis or be shunted through the pentose phosphate pathway to generate NADPH needed for the synthesis of fatty-acids and nucleotides[10]. Although cancer cells do not use the most efficient pathway to produce ATP, with a constant supply of glucose and nutrients in the blood, aerobic glycolysis appears to represent a good survival strategy[10].

Furthermore, acidification of the local environment due to excess lactate secretion of tumour cells has been shown to promote invasion and enhance cell motility. Lactate was shown to stimulate fibroblast expression of hyaluronan, a polysaccharide found in the extracellular matrix, and its receptor CD44[11]. The effect of hyaluronan is to separate cells by diminishing contact between them, this creates an environment that allows the spread of cancer cells[11]. Lactate also has direct effects on cell motility, as shown by its ability to induce expression of Transforming growth factor-beta2 (TGF-β2), a key regulator of invasion, in glioma cells[12].

1.3 Glucose Transporter Proteins

Due to its hydrophilic nature, glucose requires specific carrier proteins to cross the plasma membrane of cells. Glucose uptake across the plasma membrane is the rate-limiting step for glucose consumption in healthy cells and tumours. There are two

families of membrane-associated carriers that mediate the transport of glucose into the cell: the facilitative glucose transporter (GLUT) proteins and the sodium-coupled glucose cotransporter (SGLT) proteins[13].

The SGLT family consists of 12 members from the human genome that function as cotransporters for sugars, anions, vitamins, and short-chain fatty acids[14]. These proteins are expressed in the epithelial cells of the small intestine and kidney, as well as the brain, muscle and thyroid gland[14]. Three members of the family have a role in sugar transport, these are the sodium-dependent glucose cotransporters (SGLT1 and SGLT2), and SGLT3 which is believed to act as a glucose sensor[14]. SGLT proteins transport glucose into the cell against its concentration gradient using energy provided by the sodium gradient across the cell membrane[14].

Fourteen members of the GLUT protein family have been identified in humans[15]. These transporters show a high degree of homology and share the following common features: (1) GLUTs are predicted to have a tertiary structure of twelve transmembrane domains with intracellular amino- and carboxyl-ends)[16] (**Figure 4**): The transmembrane domains are numbered from N-terminus to C-terminus with a large intracellular loop existing between transmembrane domains 6 and 7[16]. (2) The presence of seven conserved glycine residues within the transmembrane helices: These residues are regarded as being essential for facilitative transporter function, this has been confirmed using site-directed mutagenesis where glycine residues at position 75, 76 and 79 were found to be necessary for transport activity of GLUT1 from *Xenopus* oocytes[17]. (3) Several basic and acidic residues at the intracellular surface of the proteins: The significance of some of these

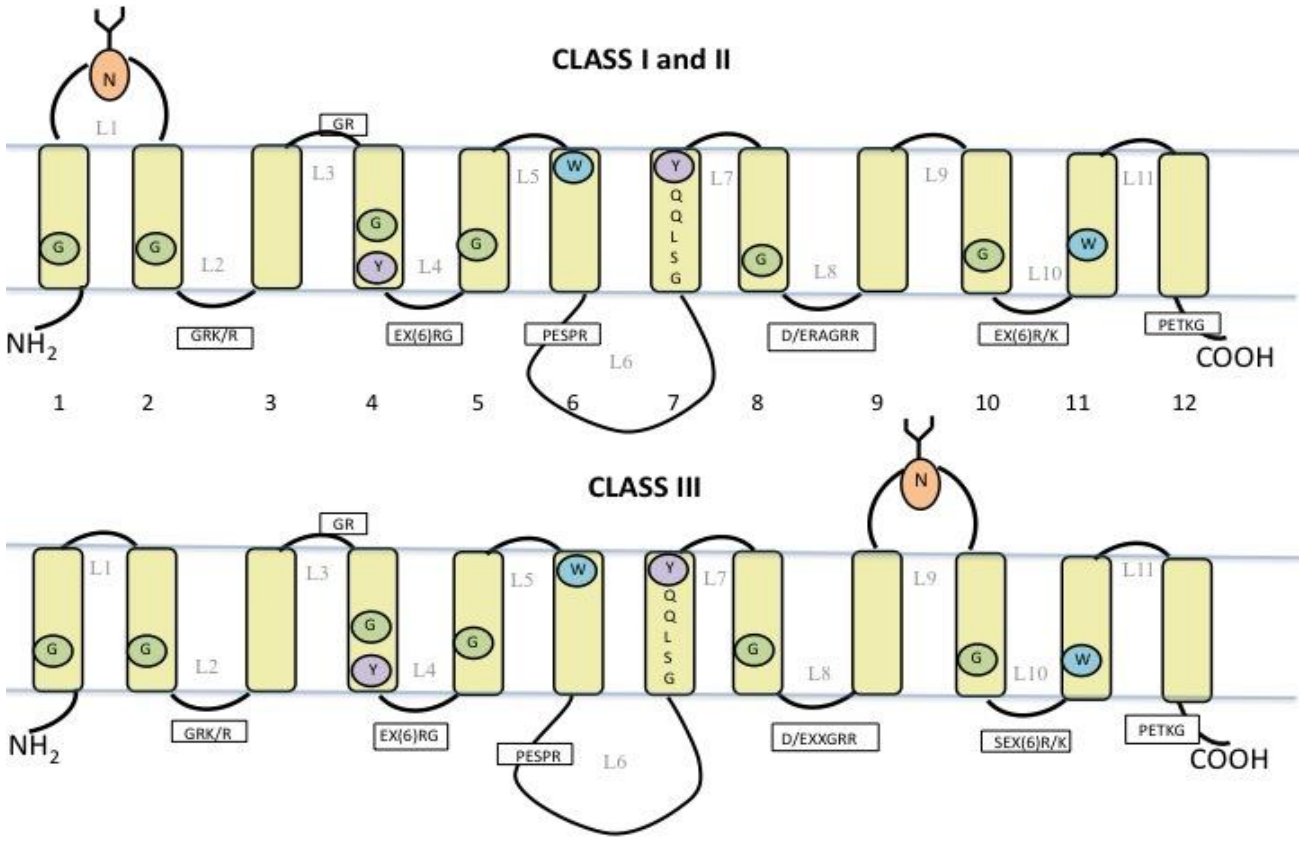

Figure 4. Schematic model of the structure of Class I and II and Class III members of the GLUT family. Twelve hydrophobic transmembrane domains are numbered and connected by hydrophilic loops. The characteristic N-glycosylation sites found between transmembrane domains 1 and 2 in Class I and II, and between transmembrane domains 9 and 10 in Class III can be seen. The seven conserved glycine residues and the conserved tryptophan and tyrosine residues are shown individually[16,24]. G, glycine; W, tryptophan; Y, tyrosine; L1-11, loops 1-11.

residues has been examined using mutational analysis; arginine and glutamate residues were found to be essential for the conformation of GLUT4 in COS-7 cells[18]. The amino acid motif R-X-G-R-R was also found to provide the proper membrane topology of GLUTs in *Xenopus* oocytes[19]. (4) Conserved tryptophan residues at helices 6 and 11: The tryptophan in helix 11 of GLUT1 has been shown to be essential for both transport activity and the binding of the glucose transport inhibitors cytochalasin B and

forskolin[20,21]. (5) Conserved tyrosine residues in helices 4 and 7: It has been demonstrated that these residues are important for the transport activity of GLUT1 and GLUT4[22,23].

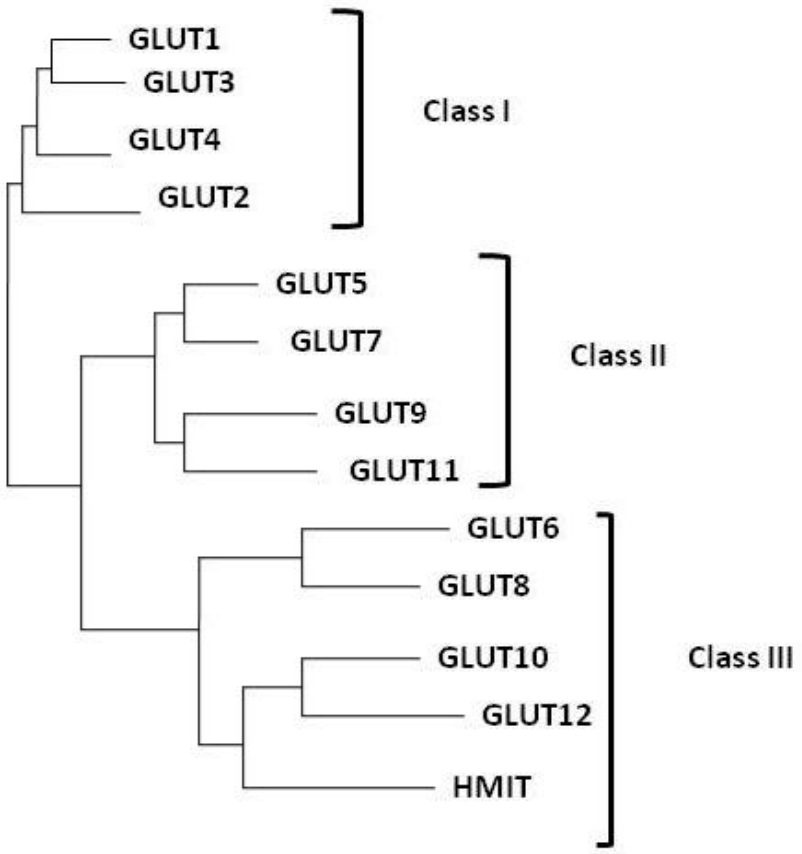

Figure 5. Dendogram of multiple alignment of the GLUT family. Branch lengths reflect the degree of difference between sequences. Adapted from Joost et. al (2001)[24]

The nomenclature of GLUT1-5 was based on the chronological order of cloning of their genes. Sequence information provided by various genome projects led to the identification of the novel sugar transporters and a new GLUT nomenclature was devised based on sequence homology[24]. The GLUTs can be grouped into three classes (**Figure 5**)

which differ in the position of a predicted long extracellular loop. In Class I (GLUT1, GLUT2, GLUT3, GLUT4) and II (GLUT5, GLUT7, GLUT9, GLUT11) proteins, this loop containing an N-linked glycosylation site is found between transmembrane domains 1 and 2. In Class III proteins, the extracellular loop with sites of N-linked glycosylation is located between transmembrane domains 9 and 10 (**Figure 4**)[24]. Glycosylation at this site has been shown to increase the efficiency of glucose transport[25]. An additional difference between classes is the presence of an internalization signal possessed by Class III transporters that localizes them intracellularly under steady state conditions[26]. This internalization signal has been identified as the motif YSRI for GLUT10 and dileucine for the remaining Class III transporters[26]. Although GLUT4 possesses internalization signals it is classified as a Class I transporter (See GLUT4 Structure/Affinity). Residues that are specific to the Class I transporters include a glutamine in helix 5 and the STSIF-motif in the extracellular loop 7[27,28]. Class II transporters lack a tryptophan following the conserved GPXXXP motif in helix 10 that is found in the other GLUT transporters. In GLUT1 this tryptophan was found to be important for binding of the ligands cytochalasin B and forskolin, but did not influence transport activity[20,21].

Unlike SGLT proteins which require energy to transport glucose, GLUT transporters move sugars via diffusion[29]. The transport of glucose by these proteins can be described as an alternating conformer model in which the transporter has mutually exclusive binding sites for glucose on its intracellular and extracellular side[15]. Binding of glucose to one of the sites triggers a change causing the transporter to switch to the other conformation and transport glucose across the plasma membrane during the process[15].

Table 1. Summary of the properties of the GLUT family

Protein	Major Isoform (aa)	Molecular Weight (kDa)	Substrates	Major Sites of Expression
GLUT1	492	54	Glucose (K_m 3 mM) Galactose (K_m 17 mM) Mannose (K_m 20 mM) Glucosamine (K_m 2.5 mM) DHA (K_m 1.1 mM)	Ubiquitous distribution
GLUT2	524	55	Glucose (K_m 17 mM) Galactose (K_m 92 mM) Mannose (K_m 125 mM) Glucosamine (K_m 0.8 mM) Fructose (K_m 76 mM)	Kidney, small intestine, liver, pancreas
GLUT3	496	45	2-deoxy-D-glucose (K_m 1.4 mM) Galactose (K_m 8.5 mM) Mannose Maltose Xylose DHA	Brain, nerves
GLUT4	509	55	Glucose (K_m 5 mM) DHA (K_m 0.98 mM) Glucosamine (K_m 0.8 mM)	Muscle, fat, heart
GLUT5	501	55	Fructose (K_m 6 mM)	Intestine, kidney, testis
GLUT6	507	46	Glucose	Spleen, brain, leukocytes
GLUT7	524	53	Glucose (K_m 0.3 mM) Fructose (K_m 0.2 mM)	Small intestine, colon, testis, prostate
GLUT8	477	51.5	Glucose (K_m 2 mM)	Testis, brain, adrenal gland, liver, spleen, fat, lung
GLUT9	511/540	66/46	Glucose (K_m 0.61 mM) Fructose (K_m 0.42 mM) Urate (K_m 0.9 mM)	Liver, kidney, placenta
GLUT10	541	57	2-deoxy-D-glucose (K_m 0.3 mM) Glucose Galactose	Heart, lung, brain, liver, muscle, pancreas, placenta, kidney
GLUT11	496	54	Glucose (K_m 0.16 mM) Fructose (K_m 0.16 mM)	Heart, muscle, kidney, placenta, fat, kidney, pancreas
GLUT12	617	67	Glucose	Muscle, heart, fat, small intestine, prostate, placenta
GLUT14	497/520	N/A	N/A	Testis
HMIT	618/629	69	Myoinositol (K_m 0.1 mM)	Brain

Glucose transport may be blocked by competitive inhibition by other sugars or metabolites at the extracellular or cytosolic sugar binding sites.

The GLUT proteins exhibit different substrate specificities, regulation and tissue expression[29]. The following summarizes the available data about the GLUT transporters with specific focus on Class I transporters GLUT1, GLUT3, GLUT4 and the Class III transporter GLUT12.

Class I

1.3.1 GLUT1

Structure

GLUT1 is the first discovered and best characterized facilitative glucose transporter. In humans, it consists of a protein sequence of 492 amino acids with a molecular weight of approximately 54 kDa[30]. The N-linked glycosylation site found in loop 1 is the most divergent region among species[30]. Overall, GLUT1 is a highly conserved isoform and exhibits 74-98% sequence identity among fish, chickens, humans, bovines, rats and mice[30].

Despite intensive study, a crystal structure of GLUT1 has not been determined[31]. GLUT1 is thought to exist as homodimers or homotetramers in human erythrocytes where its conversion between the two forms is dependent on the redox state[32]. In a reducing environment, tetrameric GLUTl was found to dissociate into GLUT1 dimers[32].

Affinity

In Xenopus oocytes, GLUT1 has been shown to transport glucose with a K_m of approximately 3 mM[33]. GLUT1 transports mainly glucose but can also transport galactose (K_m 17 mM), mannose (K_m 20 mM) and glucosamine (K_m 2.5 mM)[33,34]. In

addition, it has been shown to transport the oxidized form of vitamin C, dehydroascorbic acid with a K_m of 1.1 mM when using a *Xenopus* oocyte expression system[35].

Expression

GLUT1 is responsible for basal glucose uptake, therefore, is expressed in virtually all tissues under normal conditions[29]. It is referred to as the "Erythrocyte-Type Glucose Transporter" due to its high expression in erythrocyte cell membranes where it composes 3-5% of total membrane protein[30]. High levels of GLUT1 can also be found in endothelial and epithelial cells from blood-tissue barriers in the brain, eye, peripheral nerve, placenta and lactating mammary gland[36,37].

1.3.2 GLUT3

Structure/Affinity

GLUT3 consists of a protein sequence of 496 amino acids with a molecular weight of approximately 45 kDa[30]. GLUT3 transports glucose with a high affinity (K_m 1.4 mM for 2-deoxy-D-glucose)[46]. GLUT3 is also capable of transporting galactose (K_m 8.5), dehydroascorbic acid (K_m 8.5) and mannose and xylose, for which the K_m values have not been described[15,35]. In primary cultured cerebellar granule neurons it was demonstrated that GLUT3 has a higher catalytic center activity than GLUT1, 853 sec^{-1} versus 123 sec^{-1}, suggesting it is the more active transporter[38].

Expression

GLUT3 mRNA is ubiquitously expressed in human tissues, however, its protein is primarily found in the brain and testis[15]. The calculated turnover rate of GLUT3 compared to GLUT1 means that cells expressing GLUT3 have the capacity to transport seven times more glucose assuming an equal number of transporters on the plasma

membrane[38]. This high transport capacity and affinity of GLUT3 for glucose are especially important because GLUT3 introduces glucose into neurons from the interneuronal space where its concentration is low[29]. GLUT3 is highly expressed in the membrane of human testis as well as the spermatozoa[39].

1.3.3 GLUT4

Structure/Affinity

GLUT4 consists of a protein sequence of 509 amino acids with a molecular weight of approximately 55 kDa[30]. This protein is highly conserved in humans, bovines, rats and mice with 91-96% sequence similarity[30]. GLUT4 has a K_m for glucose of approximately 5 mM[30]. It can also transport dehydroascorbic acid (K_m 0.98 mM) and glucosamine (K_m 3.9 mM)[35,40].

Expression

GLUT4 is highly expressed in the insulin sensitive tissues including brown and white adipose tissue and skeletal and cardiac muscle[15]. It is the major glucose transporter in these tissues and thus plays a critical role in whole body glucose homeostasis.

Class III

The Class III facilitative glucose transporters are recently discovered and include GLUT6, GLUT8, GLUT10, GLUT12 and the H^+/myo-inositol co-transporter HMIT. They are not as well characterized as the other glucose transporters. These transporters are expressed in humans and were identified as a consequence of sequencing the human genome. In general, little is known about their functions and substrates.

1.3.4 GLUT12

Structure/Affinity

GLUT12 consists of a protein sequence of 617 amino acids with a molecular weight of approximately 67 kDa[30]. GLUT12 shows glucose transport activity but the affinity of the transporter for glucose remains unknown[26]. This glucose transport can be competitively inhibited by fructose, galactose and 2-DG[26].

Expression

GLUT12 expression is found in insulin-sensitive tissue including skeletal muscle, cardiac muscle and fat as well as the small intestine, prostate and placenta[41]. It was originally cloned in MCF-7 breast cancer cells. The expression of GLUT12 has been found to be stronger in ductal cell carcinoma *in situ* cells than in benign ducts of breast cancer tissues[42]. This suggests that although GLUT12 does not play a major role in glucose transport to healthy breast tissue, it becomes significant in providing sugars to malignant cells.

1.4 Molecular Mechanisms Driving the Warburg Effect

1.4.1 The PI3K-Akt Signaling Pathway

The phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases responsible for regulating growth and survival processes[43]. Three classes of PI3Ks exist based on structure and substrate specificity[44]. Class I enzymes consist of heterodimers that phosphorylate phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), Class II enzymes consist of a single catalytic subunit and preferentially use phosphatidylinositol or phosphatidylinositol-4-phosphate as substrates and Class III enzymes consist of a single catalytic subunit that only produces phosphatidylinositol-3-phosphate[44].

Class I enzymes are further divided into Class IA PI3Ks which can be activated by receptor tyrosine kinases, G protein coupled receptors (GPCRs) and oncogenes while Class IB PI3Ks are activated exclusively by GPCRs[44].

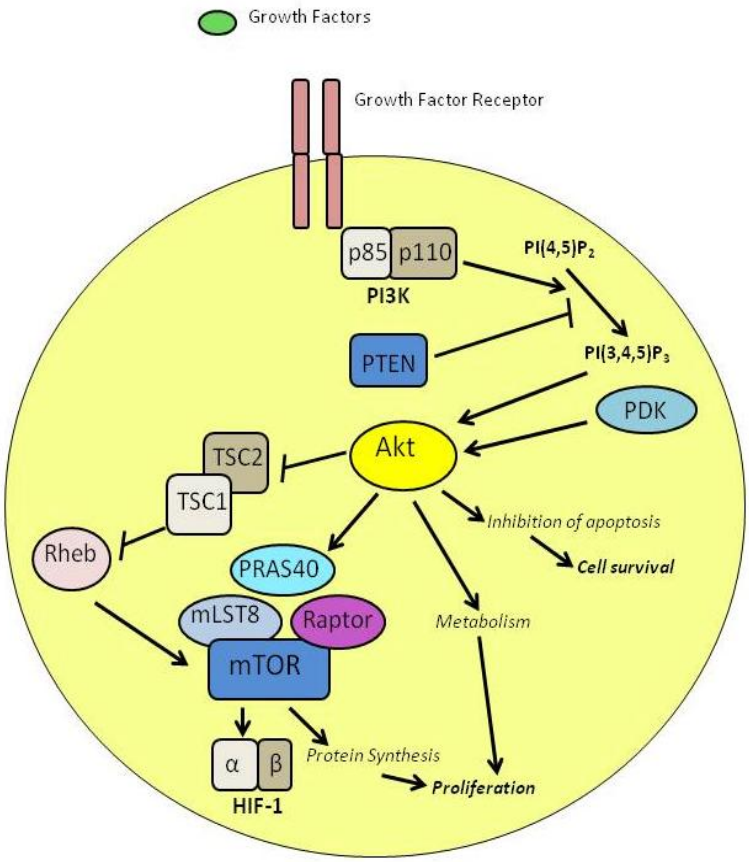

Figure 6. The PI3K signaling pathway. The family of lipid kinases termed PI3Ks has been found to have a key role in regulating cell survival and proliferation. Mutations that constitutively activate this pathway are common in cancer[43-45].

As seen in **Figure 6**, the Class IA PI3K pathway is initiated by a ligand binding to a cell surface receptor that recruits PI3K to the membrane by direct interaction of its Src homology domain 2 (SH2) domain, located within the p85 regulatory subunit, with tyrosine phosphate motifs on the activated receptor or adaptor proteins[44]. This removes inhibition of the p110 catalytic subunit which is then free to phosphorylate the membrane

phospholipids phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to generate phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$)[44]. PIP_3 serves as a plasma membrane docking site for proteins with pleckstrin-homology (PH) domains, including Akt, an oncoprotein that is frequently dysregulated in cancer[43]. Akt is a 57 kDa protein that exists in three isoforms (Akt1, Akt2, Akt3) all sharing a PH domain, a central kinase domain and a carboxy terminal regulatory domain[44]. In response to docking at the plasma membrane, Akt undergoes a conformational change exposing two amino-acid residues that must by phosphorylated for its full activation[44]. The first, Thr-308, is phosphorylated by the protein 3'- phosphoinositide- dependent kinase-1 (PDK-1) which is also recruited to the membrane by PIP_3. Ser-473 is the second amino-acid residue which is phosphorylated by the mammalian target of rapamycin complex 2 (mTORC2).

Mutations of the Class IA PI3Ks have been shown to occur frequently in cancer[45]. A common mutation occurs in the *PIK3CA* gene, encoding for the catalytic subunit p110α, which leads to the pathway being constitutively activated[45]. In addition, loss of the important tumour suppressor phosphatase and tensin homolog (PTEN) can result in uncontrolled PI3K signaling leading to cancer. This phosphatase is responsible for removing a phosphate group from PIP_3 converting it back to PIP_2 and thus preventing downstream signaling of the pathway.

Evidence indicates that Akt is involved in the Warburg Effect (**Figure 7).** Akt has been shown to promote glycolysis through a number of mechanisms. In both skeletal muscle and cancer cells, Akt has been shown to induce the expression of the glucose transporter (GLUT) proteins GLUT1 and GLUT3 and the translocation of GLUT4 to the plasma membrane, thereby allowing glucose to enter the cell[46-48]. Akt also

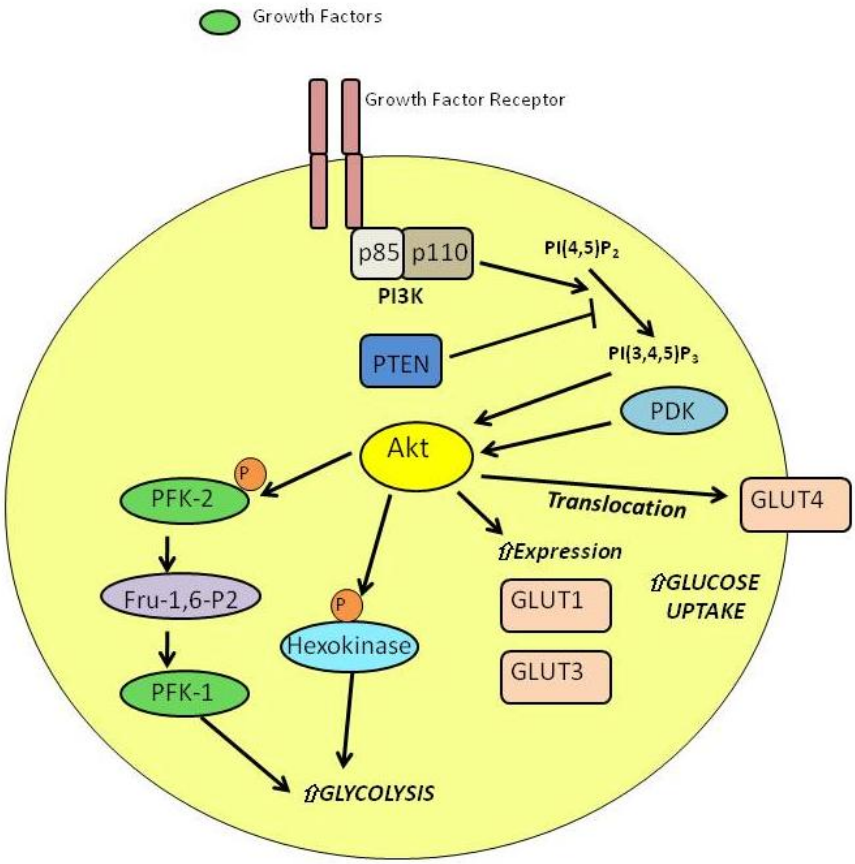

Figure 7. Akt in the Warburg Effect. Activation of the protein Akt through the PI3K pathway has been shown to promote glucose uptake and glycolysis in healthy and cancerous cells. This is achieved by inducing the expression and translocation of GLUT proteins[46-48], activating the enzyme hexokinase[49] and promoting the activation of PFK-1[50]. Because this pathway is frequently deregulated in cancer it is suggested these actions contribute to the Warburg Effect.

phosphorylates hexokinase, the enzyme of the first- and rate-limiting step in glycolysis[49]. Finally, in bovine heart tissue Akt has been shown to directly phosphorylate and activate phosphofructokinase-2 which catalyzes the formation of the product fructose-2,6-biphosphate (Fru-1,6-P_2)[50]. Fru-1,6-P2 is responsible for allosterically activating the most important regulatory enzyme of glycolysis, phosphofructokinase-1 (PFK-1). The ability of Akt to increase glycolysis without increasing oxidative phosphorylation suggests it may play an important role in contributing to the Warburg Effect.

1.4.2 HIF-1

The mammalian target of rapamycin (mTOR) is a 250 kDa serine/threonine kinase that controls growth and regulates protein synthesis[2]. It exists in two distinct complexes termed mTORC1 and mTORC2[51] (**Figure 8**). The mTORC1 complex consists of the mTOR catalytic subunit, regulatory associated protein of mTOR (raptor), proline-rich Akt substrate (PRAS40) and the protein mLST8. mTORC2 is composed of mTOR, rapamycin insensitive companion of mTOR (rictor), mammalian stress-activated protein kinase interacting protein 1 (MSIN1) and mLST8. Akt has been shown to activate the mTOR pathway by acting on two substrates[51]. Activated Akt phosphorylates the tuberous sclerosis complex (TSC) 2 and relieves the inhibitory effects of the tuberous sclerosis complex (TSC1/TSC2) upon the small G protein Rheb. When in the GTP-bound form, Rheb strongly activates mTORC1. Akt can also activate mTOR by phosphorylating PRAS40 to attenuate its inhibitory effects on mTORC1. This complex is responsible for regulating translation initiation and ribosome biogenesis through its downstream targets[51].

The hypoxia-inducible factor- 1 (HIF-1) complex is responsible for activating the transcription of genes in response to changes in tissue oxygenation[52]. HIF-1 is a heterodimer consisting of a HIF-1α and HIF-β subunit (**Figure 8**). Although HIF-1α is synthesized in healthy cells under conditions of normal oxygen concentration, it is hydroxylated by prolyl hydroxylases resulting in its recognition by von Hippel-Lindau tumour suppressor (VHL) and subsequent degradation[2]. Upon exposure to hypoxia, the HIF-1α and HIF-β subunits become rapidly stabilized[53]. Oncogenic mTOR activation or

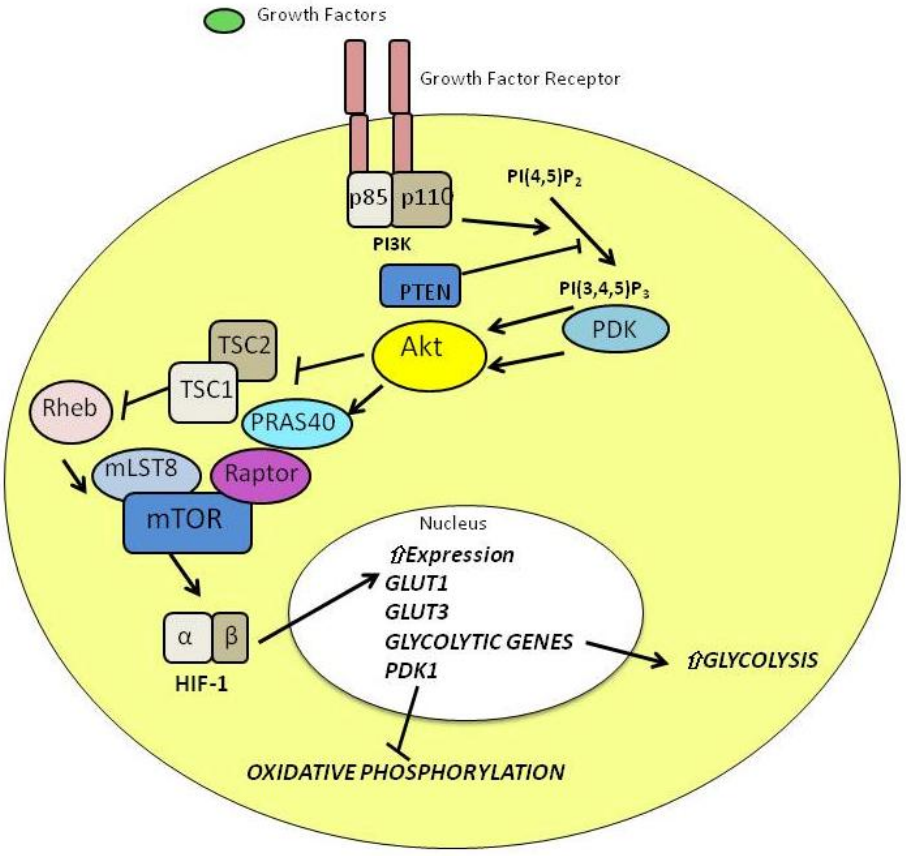

Figure 8. HIF-1 in the Warburg Effect. The overall effect of HIF-1 on metabolism is consistent with the Warburg phenotype in that it promotes glycolysis while preventing oxidative phosphorylation. Activation of HIF-1 has been shown to promote the transcription of glucose transporters[54] and glycolytic genes[55,56]. Additionally, it increases expression of PDK1 thereby inhibiting the flow of products into the citric acid cycle[57].

mutations in the tumour suppressor VHL can lead to HIF1 stabilization under normoxic conditions[53]. This occurs through increased translation of the hypoxia-inducible factor- 1 (HIF-1) α subunit of the HIF complex due to enhanced mTOR activity[52].

Evidence indicates that HIF-1 contributes to the Warburg effect by stimulating a number of genes that mediate glycolysis (**Figure 8**). In embryonic stem cells, lack of the HIF-1α subunit resulted in reduced GLUT1 and GLUT3 mRNA levels[54]. Although the GLUT1 and GLUT3 protein levels were not measured in this study, these findings

suggest increased GLUT expression by HIF-1. HIF-1 has been shown to stimulate the transcription of a number of glycolytic genes that possess hypoxia-response elements in their promoters, these include aldolase, enolase or lactate dehydrogenase, and the enzyme 6-phospho-2-kinase/fructose2, 6-biphosphatase[55,56]. In addition, HIF-1 stimulates mRNA and protein expression of pyruvate dehydrogenase kinase 1 (PDK1) which is responsible for phosphorylating and inhibiting the mitochondrial pyruvate dehydrogenase complex[57]. This prevents conversion of pyruvate into acetyl-CoA, leading to inhibition of the tricarboxylic acid cycle and enhancing production of lactate.

1.4.3 Ras

Ras GTPases function as molecular switches in processes governing cell proliferation, survival, and differentiation[58]. The three mammalian *ras* genes encode the four classical Ras proteins of 21 kDa: H-Ras, K-Ras4A, K-Ras4B, and N-Ras. These proteins show remarkable similarity, they are identical over the 85 N-terminal residues, and their identity within the following 80 residues is up to 90%[58].

A key function of Ras protein is to regulate the PI3K and the Mitogen Activated Protein Kinase (MAPK) signaling pathways. The Ras-MAPK signaling pathway can be seen in **Figure 9**. In the resting state, Ras binds to GDP. Ras proteins become active when bound to GTP, this occurs by tyrosine kinase receptor activation that inhibits GTPase activating proteins (GAPs) or activates guanine nucleotide exchange factors (GEFs)[58]. Most growth factors that signal through receptor tyrosine kinases (RTKs) or GPCRs activate Ras by recruiting the GEF Son of Sevenless (SOS) to the plasma

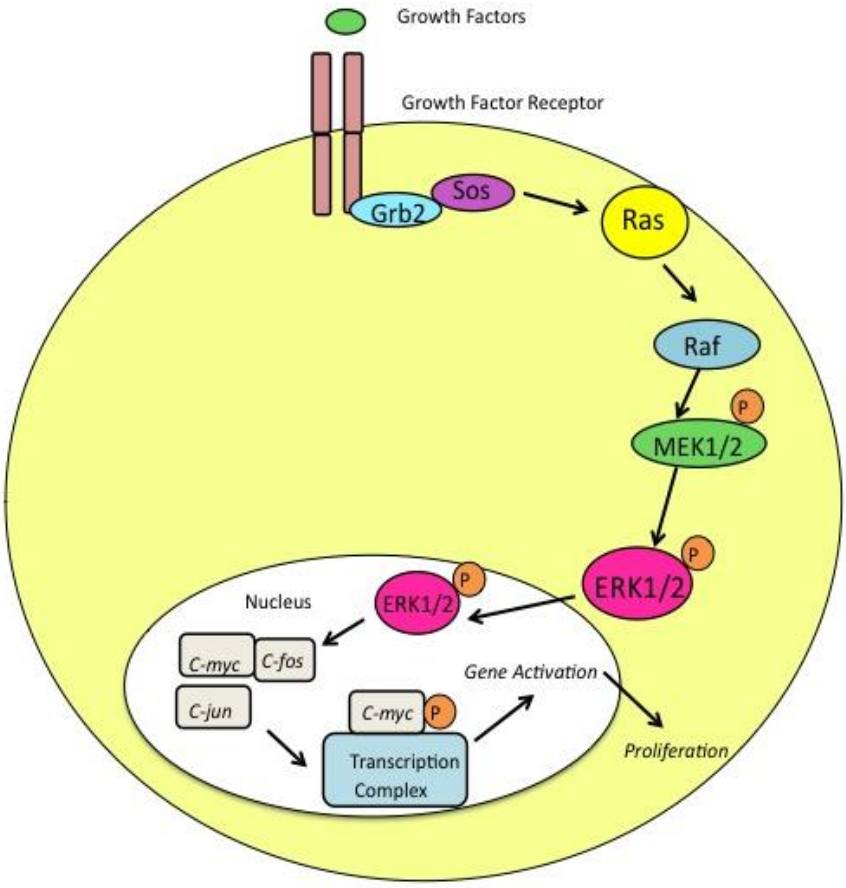

Figure 9. The Ras-MAPK signaling pathway. Activation of the Ras-MAPK pathway by growth factors and mitogen leads to growth and differentiation[58]. While this pathway is essential to healthy cells, oncogenic mutations of Ras can result in uncontrolled growth[58,60].

membrane[59]. SOS is found in a complex with the adaptor protein Growth Factor Receptor-Bound Protein-2 (GRB2). Similar to PI3K, the GRB2/SOS complex is translocated to the membrane by binding of GRB2 to phosphorylated tyrosine residues on RTKs or adaptor proteins[58]. GTP-bound Ras recruits and activates the serine/threonine kinase Raf. Activated Raf binds to and phosphorylates the dual-specificity mitogen activated kinases (MEK1/2), which in turn, activate the extracellular regulated kinases

1/2 (ERK1/2) by phosphorylation within a conserved Thr-Glu-Tyr motif. Upon activation, ERK1/2 translocates into the nucleus where it phosphorylates substrates including the transcription factors c-jun, c-fos and c-myc[58]. Alternatively, activated Ras may activate the p110 catalytic subunit of PI3K, independently of p85, and lead to the subsequent activation of Akt as earlier described[59].

In healthy cells Ras function is essential, however oncogenic mutations of Ras can lead to uncontrolled proliferation. Ras is found to be mutated in approximately 25% of human tumours[60]. Amino acid replacements at residue 12, and less commonly at residues 13 and 61, lock Ras proteins in a GTP-bound state in which they signal to downstream effectors even in the absence of extracellular stimuli[58].

In a study of NIH3T3 mouse fibroblasts transformed by an activated form of the K-ras oncogene, expression of lactate dehydrogenase and a number of glycolytic enzymes were found to be increased[60]. These cells consumed more glucose and produced more lactate than normal or reverted cells, providing evidence that Ras may play a role in the Warburg phenotype[60].

1.4.4 c-Myc

The MYC family of cellular oncogenes includes c-Myc, a transcription factor involved in the control of cell proliferation and death[61]. Myc also regulates genes involved in glucose metabolism. The targets of c-Myc are similar to HIF-1, in Rat-1 fibroblasts, c-Myc has been shown to directly transactivate genes encoding GLUT1, phosphofructokinase, and enolase[62]. Furthermore, lactate dehydrogenase-A has been

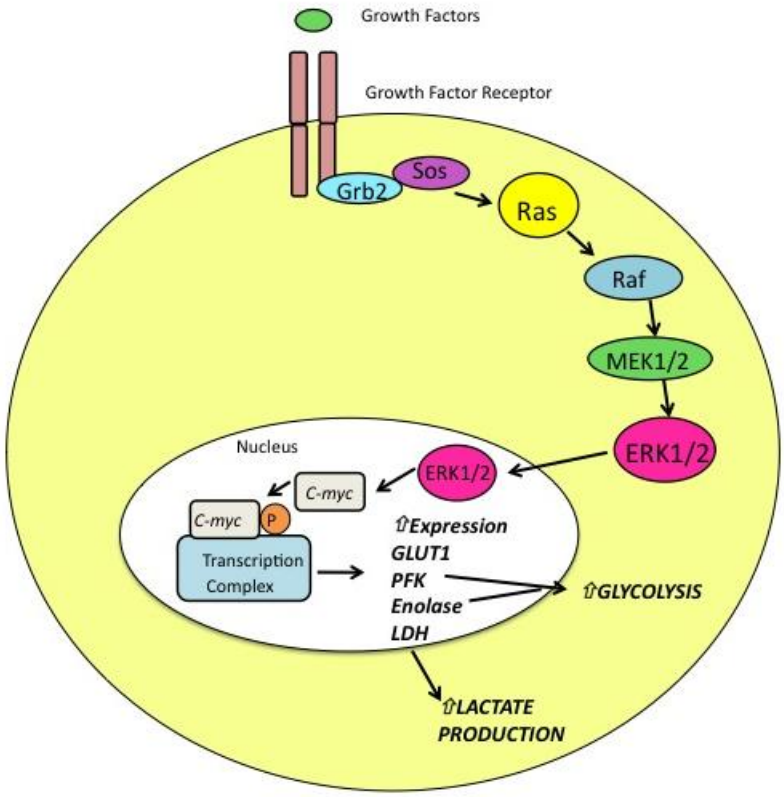

Figure 10. c-Myc in the Warburg Effect. The transcription factor c-Myc is suggested to contribute to the Warburg Effect by increasing the expression of genes that promote glycolysis[62] and lactate production[63] thereby enhancing aerobic glycolysis.

identified as a c-Myc-responsive gene[63]. This suggests c-Myc and HIF-1 may cooperate together to enhance aerobic glycolysis and contribute to the Warburg effect (**Figure 10**).

1.4.5 The tumour suppressor protein p53

Active p53 protein is composed of a tetramer made of four identical subunits, each consisting of an N-terminal transactivation domain, a proline-rich region, a DNA-binding core domain and, within the C-terminal regulatory domain, a tetramerization domain and an unstructured basic domain[64]. Wild type p53 is a sequence-specific DNA binding protein and transcription factor responsible for negatively regulating cell growth and division[65]. The p53 protein is activated by a variety of stress signals (**Figure 11**),

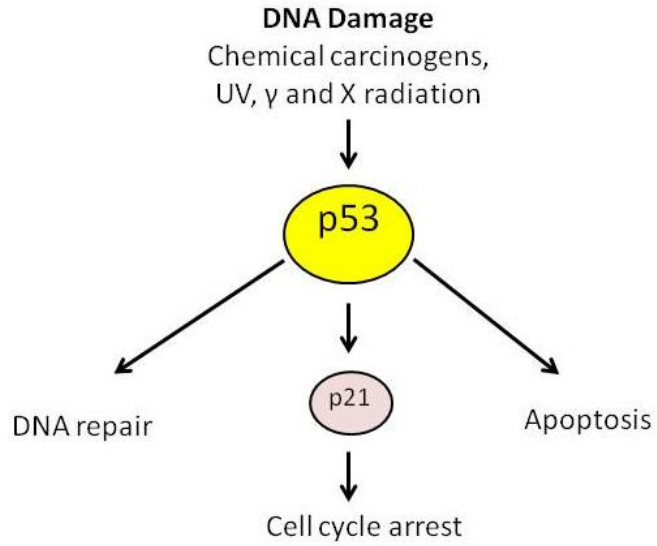

Figure 11. The tumour suppressor protein p53. In healthy cells, the p53 protein is activated by stress signals and initiates responses to prevent the formation of tumours such as DNA repair, cell cycle arrest and apoptosis[64]. Mutations in p53 are found in most cancers[64,66].

including DNA damage, and responds to the specific signal by initiation of cell cycle arrest, senescence or apoptosis among other outcomes[64]. The most common mutations of p53 are missense mutations that occur in the conserved DNA-binding domain in the central portion of the protein[64]. Most cancer-related mutations of p53 are clustered in the four 'hot spots' at codons 175, 248, 273 and 281/282[66]. In mutant p53 AS-30D hepatoma cells, overexpression of mutated p53 was found to significantly activate the promoter of hexokinase 2, the enzyme catalyzing the first step of glycolysis in which glucose is converted to glucose-6-phosphate[65]. In healthy cells, p53 blocks the glycolytic pathway by lowering levels of the glycolytic activator fructose-2,6-bisphosphate in cells through a gene identified as TP53-induced glycolysis and apoptosis regulator (TIGAR)[67] (**Figure 12**). This is also accomplished through inducing the expression of phosphatase and tensin homolog (PTEN), a tumour suppressor that inhibits activity of the PI3K pathway by

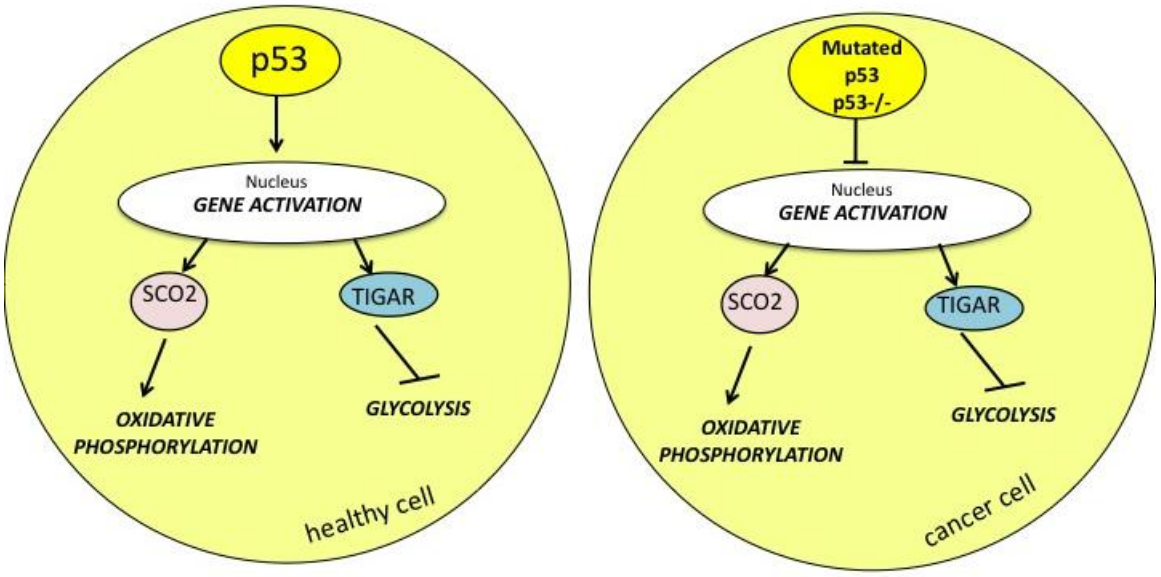

Figure 12. p53 in the Warburg Effect. In healthy cells, functional p53 induces the transcription of genes that promote oxidative phosphorylation and block glycolysis[64]. Cell with mutated or absent p53 lose these regulatory effects on metabolism[65,67-69].

removing a phosphate group from PIP_3[68]. In mice with a homozygous (-/-) disruption of the *TP53* gene, a significant decrease in oxygen consumption and mitochondrial respiration was observed[69]. These effects were found to be mediated by p53-induced transcription of synthesis of cytochrome c oxidase 2 (SCO2), which forms the cytochrome c oxidase complex of the electron transport chain[69]. Therefore, in addition to blocking glycolysis, p53 promotes oxidative phosphorylation. Loss of this gene may play an important role in contributing to the aerobic glycolysis observed in cancer cells.

1.4.6 ^{18}Fluoro-deoxy-glucose positron emission tomography (FDG-PET)

Malignant cells show increased glucose uptake both *in vitro* and *in vivo*[70]. In order to achieve a glycolytic rate that is approximately 30 fold higher than normal, tumours must

bring glucose into the cell at an elevated rate[70]. It is this characteristic that led to the development of a technique known as FDG-PET for tumour detection and monitoring[71]. ^{18}Fluoro-deoxy-glucose is a positron emitter that can be detected by PET scanners. ^{18}Fluoro-radiolabelled 2-deoxy-glucose analog is recognized as a substrate by glucose transporters. Tumour cells, with an increased rate of glycolysis, will have an increased uptake of FDG which serves as a substrate for hexokinase and is converted into its 6-phospho derivative. The resulting compound is stable and will not be metabolized further, allowing its accumulation to be visualized. Since tumours have an increased rate of glucose uptake compared to normal cells, FDG-PET is able to detect tumours and differentiate them from healthy tissue. In this fashion, glucose uptake serves as a functional biomarker in cancer that allows physicians to determine the extent of the disease and to guide treatment.

1.5 Regulation of Glucose Transporters

1.5.1 Regulation of GLUT1

The serine/threonine kinase Akt has been shown to induce GLUT1 gene transcription. This was demonstrated in mouse hepatoma cells where a hydroxytamoxifen-regulatable form of Akt responded to stimulation by hydroxytamoxifen by increasing GLUT1 transcription and protein accumulation[47]. Akt has also been implicated in the plasma membrane trafficking of GLUT1 in response to NF-κB transcription factors and cytokine stimulation[72-74].

Furthermore, expression of a kinase inactive Akt1 inhibited the ability of insulin to induce GLUT1 mRNA expression in the study by Barthel et al. (1999), suggesting that

Akt is an important mediator through which insulin regulates GLUT1 gene expression[47]. Chronic insulin stimulation has been shown to increase GLUT1 protein levels[75,76]; inhibition of the p70 S6 kinase with rapamycin almost completely abolished the increase in GLUT1 protein in response to insulin indicating activation of this downstream kinase from Akt is required in the process[75].

There is evidence that the ovarian hormone estrogen is capable of inducing GLUT1 expression. In MCF-7 breast cancer cells, treatment with estrogen was found to increase glycolysis via upregulation of GLUT1 protein[77]. A decreased rate of glycolysis and GLUT1 expression was observed due to the antiestrogenic effects of tamoxifen treatment.

Certain stressors have been shown to increase expression of GLUT1. Long-term treatment with hyperosmolarity (300 mM mannitol) was found to increase GLUT1 protein expression by 70%, and similar to insulin, this was mediated by the p70 S6 kinase[76]. Supporting the notion that GLUT1 is a stress inducible protein, exposure of L6 myotubes to prolonged low grade oxidative stress induced by glucose or xanthine oxidase was also shown to increase GLUT1 mRNA and protein levels[78]. Treatment with 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation that depletes ATP production, was shown to induce a 150% increase in GLUT1 protein expression[76]. This evidence suggests GLUT1 may be an important transporter for uptaking glucose to provide energy in low oxygen conditions.

Indeed, GLUT1 expression has been shown to be regulated by hypoxia through HIF-1[54]. Hypoxia response elements (HRE) located on the GLUT1 gene contain HIF-1 binding sites with the consensus sequence 5-RCGTG-3[79]. This was demonstrated using placental cell lines in which deletion of an 184bp hypoxia-HR of the rat GLUT1

promoter was found to reduce GLUT1 expression, indicating an important role of HIF-1 in modulating GLUT1 gene expression in response to low-oxygen conditions[79].

GLUT1 expression is up regulated by a number of oncogenes. c-Myc was found to induce GLUT1 gene expression in rat fibroblasts and livers[62]. A Rat1 fibroblast line expressing a protein that fuses Myc to the estrogen receptor ligand binding domain (MycER) was used to demonstrate GLUT1 behaves as a direct target gene of c-myc. This system uses exposure of the MycER protein to estrogenic compounds, such as 4-hydroxytamoxifen (4-HOTM), to translocate the ligand-bound MycER protein to the nucleus where it can activate target genes without new protein synthesis. After activation of MycER by 4-HOTM, expression of GLUT1 was found to be increased.

Colorectal cells with mutations in K-Ras or v-raf murine sarcoma viral oncogene homolog B1 (BRAF*)* were shown to upregulate the GLUT1 gene and display enhanced glucose uptake, glycolysis, and survival in low-glucose conditions[80]. Very few cells survived when wild-type KRAS alleles were subjected to a low-glucose environment. However, 4% of survivors acquired KRAS mutations that were not present in their parents. This finding suggests that glucose deprivation may select for mutant cells with KRAS mutations which can be a growth advantage because of increased GLUT1 expression.

Expression of the GLUT1 gene is down-regulated by the tumour suppressor gene p53[81]. Cotransfection of osteosarcoma-derived SaOS-2 cells, rhabdomyosarcoma-derived RD cells and C2C12 myotubes with GLUT1-P-Luc or GLUT4-P-Luc promoter-reporter constructs and wild-type p53 expression vectors was found to dose dependently decrease

GLUT1 and GLUT4 promoter activity. This highlights the link between GLUT1 overexpression observed in various cancers and mutations in p53.

In addition to increasing the expression of this transporter, insulin has been shown to promote GLUT1 translocation to the plasma membrane[82]. Evidence indicates that insulin-like growth factor I (IGF-I) is capable of regulating localization of GLUTs. In L6 myotubes, acute stimulation with IGF-I did not increase expression of GLUT1 protein, however an increase in GLUT1 protein in the plasma membrane enriched fraction of cells was seen[83]. The translocation of GLUT1 by IGF-1 was found to be similar to that of insulin with respect to the increase in cell surface GLUT1 over basal levels[84].

1.5.2 Regulation of GLUT3

The serine/threonine kinase Akt has been implicated in the regulation of GLUT3 expression. This was demonstrated in L6 skeletal muscle cells in which cells overexpressing wild-type Akt or a constitutively active membrane-targeted Akt displayed a significant increase in glucose uptake. The increased transport of glucose into the cell was found to be mediated by the cellular synthesis of GLUT3[46].

Like GLUT1, long-term insulin stimulation has been shown to increase mRNA and protein expression of GLUT3[75,76]. In L6 myotubes, this was suggested to occur in a MEK/ERK dependent manner as inhibitors of both proteins resulted in a large reduction in the insulin-stimulated increase in GLUT3 protein[76]. There is also evidence suggesting GLUT3 to be a stress induced protein which may be regulated by similar pathways as GLUT1. p70 S6 kinase has also been implicated in the increase in GLUT3 protein levels after long-term hyperosmolarity treatment[76]. Furthermore, like GLUT1, GLUT3 was

Table 2. Summary of known contributors/mediators of GLUT1 expression

Contributor/mediator	Tissue	Effect on regulation	Pathway Involved	Reference
Akt activation	Mouse hepatoma cells (Hepa1c1c7) FL5.12 hematopoietic myeloid cells	Increases transcription	mTOR/Raptor	47 72-74
Insulin	Mouse hepatoma cells (Hepa1c1c7) L6 muscle cells	Increases mRNA and protein expression	Akt/mTOR/4E-BP1	47 75,76,82
Estrogen	MCF-7 breast cancer cells	Increases protein expression	N/A	77
Stress *Hyperosmolarity (300 mM mannitol)* *2,4-dinitrophenol (0.5 mM)* *Oxidative stress (50 milliunits/ml glucose oxidase, 20 milliunits/ml xanthine oxidase)*	L6 muscle cell L6 muscle cell L6 muscle cell	Increases protein expression Increases mRNA and protein expression Increases mRNA and protein expression	p70 S6 kinase and p38 MAPK dependent N/A Increases GLUT1 transcription rate	76 76 78
Hypoxia/HIF-1	Trophoblast-derived human (BeWo) and rat (Rcho-1) cells	Induces mRNA expression	HIF-1 binding site on hypoxia response element 5-RCGTG-3	79
c-Myc	Rat1 fibroblasts	Induces mRNA expression	Directly transactivates *SLC2A1* gene	62
K-ras	Colorectal cancer cells	Induces mRNA expression	Directly transactivates *SLC2A1* gene	80
B-raf	Colorectal cancer cells	Induces mRNA expression	Directly transactivates *SLC2A1* gene	80
p53	SaOS-2 cells, rhabdomyosarcoma-derived RD cells, C2C12 myotubes	Downregulates mRNA expression	Represses promoter activity	81
IGF-I	L6 muscle cells	Regulates translocation to the plasma membrane	N/A	83, 84

shown to possess an HRE and expression of this gene was induced by formation of the HIF-1α complex in response to hypoxia[85]. This has been demonstrated in a number of models including neurons and carcinomas[86-88].

In murine brain and mouse neuroblastoma cells, it was confirmed that phosphorylated cyclic AMP-regulatory element-binding (pCREB)1 protein binds the GLUT3 promoter region and activates GLUT3 expression in neurons[89]. Cyclic-AMP (cAMP) signaling involves a conserved second messenger pathway which is regulated by heterotrimeric G proteins. These proteins transduce extracellular signals via G protein-coupled receptors and activate the enzyme adenylyl cyclase. This enzyme is responsible for the conversion of ATP to cAMP which then activates the cAMP-dependent protein kinase A. The transcription factor CREB is activated by protein kinase A by phosphorylation on Ser-133. In the study by Rajakumar et al. (2004) GLUT3 gene expression was transactivated by Ser-133 phosphorylation of CREB that led to enhanced binding to the site of the transcription factor activator protein 1 (AP-1) on the GLUT3 gene[89]. The Sp family of transcription factors was also implicated in the regulation of GLUT3 expression. These proteins bind to genes with a DNA control sequence T GGGCGGAAT, otherwise known as a GC box, to regulate expression of multiple target genes. In the case of GLUT3, Sp3 was found to activate gene expression by binding to a site in the 5'-flanking region of the gene, while Sp1 was found to functions as a repressor of GLUT3 expression when binding in this region.

In p53−/− mouse embryonic fibroblasts, the loss of p53 was found to activate the NF-κB transcription factor and increase the rate of aerobic glycolysis and upregulate GLUT3[90]. This expression was reduced by knockdown of the kinases IKKα and IKKβ

that allow for the activation of NF-κB, indicating that functional p53 may suppress the expression of GLUT3 by inhibiting this pathway. This is the first study demonstrating down-regulation of GLUT3 by the tumour suppressor GLUT3. Therefore, additional studies in other models are needed to clarify the regulation of this transporter by p53.

Acute insulin stimulation affects the localization of GLUT3 by inducing translocation to the plasma membrane[84]. In comparison, IGF-I stimulation results in 67% greater cell surface levels of GLUT3 than insulin[84]. It is possible that IGF-I-mediated GLUT3 translocation is responsible for the higher rate glucose uptake in the presence of IGF-I as compared to insulin.

The energy sensor AMP-activated protein kinase exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits[91]. It is activated by metabolic stressors which deplete ATP and increase AMP levels, allowing AMP to bind to the γ subunit of AMPK. This induces a conformational change promoting phosphorylation on the AMPK α subunit by upstream kinases. Once activated, AMPK stimulates catabolic pathways while switching off anabolic pathways[91]. In neuronal cells, AMPK has been shown to facilitate the translocation of GLUT3 to the cell surface after excitation[92]. Indirect activation of AMPK by glutamate excitation resulting in an increased AMP:ATP ratio, or pharmacological activation of AMPK with 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) resulted in increased GLUT3 surface expression. This was found to be prevented by inhibition of AMPK with compound C or knockdown with siRNA. However, in human embryonic kidney cells, AMPK was found to increase GLUT3-mediated glucose uptake in a manner that did not involve transporter translocation[93]. These findings suggest AMPK may have different regulatory effects on

GLUT3 depending on the cell type and stimulus. Further research to determine the mechanisms by which AMPK stimulates GLUT3-mediated glucose uptake is needed.

Table 3. Summary of known contributors/mediators of GLUT3 expression

Contributor/ mediator	**Tissue**	**Effect on regulation**	**Pathway Involved**	**Reference**
Akt activation	L6 muscle cells	Increases protein expression	N/A	46
Insulin	L6 muscle cells	Increases mRNA and protein expression	MEK/ERK dependent	75, 76
Stress *Hyperosmolarity (300 mM mannitol)*	L6 muscle cells	Increases protein expression	p70 S6 Kinase dependent	76
Hypoxia/HIF-1	Hep-G2 human hepatocellular carcinoma cells (ATCC HB 8095) L8 rat muscle cells, Hepa-1c1c7 rat hepatoma cells, rat cerebrum and retina, BeWo choriocarcinoma cells	Induces gene expression	HIF-1 binding site on hypoxia response element	85-88
cAMP-pCREB	Mammalian neurons	Induces gene expression	Binding to transcription factor activator protein 1 (AP-1) on the *SLC2A3* gene	89
sp3	Mammalian neurons	Induces gene expression	Binding to GC box on *SLC2A3* gene	89
sp1	Mammalian neurons	Represses gene expression	Binding to GC box on *SLC2A3* gene	89
p53	Mouse embryonic fibroblasts	Downregulates mRNA expression	Inhibition of NF-κB pathway	90
IGF-I	L6 muscle cells	Regulates translocation to the plasma membrane	N/A	84
AMPK activation	Cerebellar granule neurons Human embryonic kidney cells (293T)	Regulates translocation to the plasma membrane Increases activity of transporter	N/A	92 93

1.5.3 Regulation of GLUT4

A 2.4 kb DNA segment at the 5′ region of the GLUT4 gene is responsible for tissue-specific expression of GLUT4 in adipose tissue, skeletal muscle, and cardiac muscle[95]. The myocyte enhancer factor 2 (MEF2) proteins are a family of transcription factors that play a role in muscle differentiation and development[94]. A MEF2 binding site has been identified in the GLUT4 promoter that is necessary for expression in skeletal muscle[94]. A region −742 to −712 relative to the initiation site for transcription, termed Domain I, has also been has been implicated in tissue-specific expression of GLUT4[95]. A factor termed GEF appears to operate in this region in association with MEF2A[96].

Downregulation of GLUT4 expression occurs in adipose tissue in obesity. This has been shown in obese and obese patients with type 2 diabetes in which adipocyte GLUT4 protein and mRNA levels were significantly decreased compared to lean body mass controls[97]. This study is supported by Sinha et al. (1991) where decreased levels of the muscle/fat GLUT4 isoform were found in adipose tissue of patients with type 2 diabetes[98].

Upregulation of GLUT4 in skeletal muscle has been observed in response to exercise. This can be achieved by a single session of exercise, as seen in rats that experienced a 2-fold increase in GLUT4 mRNA and a 50% increase in GLUT4 protein expression 16 hours after a prolonged exercise session[99]. This has also been demonstrated in humans, exercise training was shown to increase GLUT-4 gene expression immediately after exercise and it remained elevated three hours after the end of exercise[100]. These effects have been suggested to occur through activation of the energy sensor AMP-activated

protein kinase (AMPK), as increasing AMPK activity with the AICAR was shown to lead to transcriptional activation of the GLUT4 promoter in rats[101].

Like GLUT1 and GLUT3, expression of the GLUT4 gene may be down-regulated by the tumour suppressor gene p53[81]. The inhibitory effect of p53 on transcriptional activity of GLUT4 was found to be significantly greater than its effect on GLUT1. The reason for this difference is not understood but may reflects differences in the expression of the transporters since GLUT1 is ubiquitous and GLUT4 is tissue-specific.

In conditions of low plasma insulin, GLUT4 is stored in intracellular tubulo-vesicular compartments. Two motifs are responsible for the association of the protein with this compartment: a dileucine motif in the C-terminus and a FQQI motif in the N-terminus[30]. Upon insulin binding to its receptor, the PI3K and MAPK signaling cascades are activated, as previously described. In response to insulin, GLUT4 is rapidly translocated from endosomal compartments to the plasma membrane resulting in an increase in glucose uptake[29]. The precise pathways by which insulin signaling pathways control GLUT4 trafficking are still not fully understood. However, this is suggested to occur through Akt phosphorylation of the GTPase activating protein AS160. Knockdown of AS160 in adipocytes using short-hairpin RNA was found to increase basal GLUT4 levels on the adipocyte surface, suggesting that the role of this protein is to retain GLUT4 intracellularly and this hold is relieved upon insulin stimulation[102]. Disruption in the regulation of GLUT4 translocation by insulin is one of the key defects in insulin resistance and type 2 diabetes mellitus[31].

Acute IGF-I stimulation in L6 myotubes was found to induce GLUT4 translocation to the plasma membrane without affecting the total cellular content of this protein[83].

Prolonged (8 hour) stimulation, however, was found to increase total GLUT4 protein levels and thus the level found in the cell surface. These findings suggest that similar to insulin, IGF-I has acute and chronic effects on glucose uptake that are mediated through glucose transporters.

In addition to increasing GLUT4 expression, AMPK has been implicated in GLUT4 translocation that occurs after acute exercise. AICAR activation of AMPK is capable of increasing glucose uptake and GLUT4 translocation in skeletal muscle[103,104].

Table 4. Summary of known contributors/mediators of GLUT4 expression

Contributor/ mediator	**Tissue**	**Effect on regulation**	**Pathway Involved**	**Reference**
Obesity	Adipocytes	Decreases mRNA and protein expression	N/A	97, 98
Exercise	Muscle	Increases mRNA and protein expression	N/A	99, 100
AMPK activation	Muscle	Increases mRNA expression Regulates translocation to the plasma membrane	Transcriptional activation of GLUT-4 promoter N/A	101 103, 104
p53	SaOS-2 cells, rhabdomyosarcoma-derived RD cells, C2C12 myotubes	Downregulates mRNA expression	Represses promoter activity	81
Insulin	Adiopocytes Muscle cells	Regulates translocation to the plasma membrane	Akt/AS 160 dependent	102, 48
IGF-I *(3-10 nM)*	L6 muscle cells	Acute- Increases translocation to the plasma membrane Long-term- Increases protein expression	N/A	83

1.5.4 Regulation of GLUT12

There is limited evidence regarding the regulation of GLUT12. Given that other GLUTs have been shown to be regulated by estrogen, the effects of the hormones 17β-estradiol and dihydrotestosterone on GLUT12 expression were examined in MCF-7 cells[16]. Treatment with 17β-estradiol for up to 24 hours was found to have no effect on GLUT12 mRNA expression, however a significant increase in GLUT12 protein was seen was seen after treatment with 17β-estradiol or dihydrotestosterone for 24 hours. These findings suggest the GLUT12 mRNA translation rate or protein half-life may be increased by hormone treatment.

Additionally, this study looked at the effect of epidermal growth factor (EGF), a known activator of proliferation in breast cancer cells, on GLUT12 expression. While GLUT12 mRNA expression was found to significantly decrease after eight hours of treatment with 60 ng/ml EFG, an increase in GLUT12 protein was observed after two hours of treatment which began to decrease after 24 hours. Therefore, GLUT12 expression appears to be regulated at the mRNA and protein level by EGF, the mechanism by which this is achieved and possible negative feedback loops after long-term EGF treatment remain to be examined.

Similar to the previously mentioned GLUTs, there is evidence to suggest that repression of the *SLC2A12* gene occurs by the tumour suppressor p53. Using a small compound that re-activates p53, termed reactivation of p53 and induction of tumour cell apoptosis (RITA), p53 was pharmacologically reconstituted in cancer cell lines and HCT116 and HCT116 *TP53-/-* xenografts grown in immunodeficient mice[105]. Upon p53 reactivation, significant repression of the *SLCA12* gene in HCT116 xenografts occurred.

Using chromatin immunoprecipitation (ChIP) sequencing it was shown that upon RITA treatment, p53 binds to a consensus site in the *SLC2A12* gene. Taken together, these findings suggest that *SLC2A12* is a direct target of p53 and the expression of GLUT12 that occurs in cancer may be due to the mutation or loss of this tumour suppressor.

It has been shown in various cell lines that endogenous and overexpressed GLUT12 localizes to intracellular compartments and the plasma membrane[42,106,107]. GLUT12 contains dileucine motifs at the N-terminus and C-terminus similar to GLUT4[41]. There does not appear to be a continuous cycling mechanism for GLUT12, as plasma membrane associated GLUT12 is not endocytosed. In human skeletal muscle, insulin was shown to be capable of inducing translocation of GLUT12 to the plasma membrane[108]. This was confirmed in L6 myoblasts in which insulin stimulation caused a shift of GLUT12 from an intracellular location to the plasma membrane fraction. Inhibition of PI3K was found to block GLUT12 translocation in these cells, suggesting that activation of this pathway may be responsible for the translocation of this transporter in muscle.

In renal epithelial cells, it was demonstrated that treatment with high glucose (25 mM) and 10% serum causes translocation of endogenously expressed GLUT12 to the cytoplasm and plasma membrane[109]. GLUT12 was implicated in the increased glucose uptake observed after mitogen treatment and it was found that incubation with the mTOR inhibitor rapamycin decreased glucose uptake levels and restricted GLUT12 to a perinuclear location in these cells. The potential regulated trafficking of this transporter by mitogens and the mTOR pathway should be explored in additional tissues.

Table 5. Contributors/mediators of GLUT12 expression				
Contributor/mediator	**Tissue**	**Effect on regulation**	**Pathway Involved**	**Reference**
Estrogen	MCF-7 breast cancer cells	Increases protein expression	N/A	16
Epidermal Growth Factor (EGF) *(60 ng/ml)*	MCF-7 breast cancer cells	Transient increase in protein levels Long term decrease in mRNA and protein levels	N/A	16
p53	Various cancer cell lines	Downregulates mRNA expression	Binds to promoter of *SCL2A12* gene	105
Insulin	Muscle	Regulates translocation to the plasma membrane	PI3K dependent	108
mTOR activation	Renal epithelial cells	Regulates translocation to the plasma membrane	N/A	109

1.6 Cell Lines

Culture models are frequently used to investigate the physiology and biochemistry of cells. A primary culture can be established from a tissue that is fragmented, or dispersed by enzymatic or mechanical disruption. Once maintained in the appropriate growth medium, the first subculture will proliferate and these cells can be reseeded to give rise to a secondary culture, and so on. Finite cell lines have no gross genetic changes and a diploid chromosomal number[110]. These cells show anchorage dependence and grow as a monolayer, ceasing growth once confluence has been reached[110]. Human cells grown in

culture generally divide 50-100 times before entering into a senescent phase in which there is an observed decline in cell number[111].

Unlike finite cell lines, continuous cell lines have the ability to proliferate for an unlimited number of cell generations. Highly aggressive cancers that have accumulated genetic changes necessary for unlimited growth may spontaneously become continuous cell lines when cultured *in vitro*[110]. In order to expand the lifespan of cells, treatment with viral genes or carcinogenic chemicals may be used to sequester proteins inhibiting cell division and cause acquisition of mutations making the cells immortal[110]. Continuous cell lines may be aneuploid and have altered growth characteristics including anchorage independence and a loss of contact inhibition and density limitation on growth[110].

The amount of tissue available for a number of cancers is limited because acquiring tumour tissue is invasive, and in some cases not possible due to the location of the tumour. Furthermore, tumour tissue may contain varying amounts of non-malignant cells and supporting tissues. Immortalized cancer cell lines are frequently used as *in vitro* models in cancer research. There are a number of advantages to using a cell culture model to understand cancer biology. These cells resemble the primary tumour population but offer a homogenous population of cells with an unlimited replicative ability. Furthermore, cells that are cultured *in vitro* are grown in a controlled environment that is free from external factors that may affect the expression of certain proteins. The properties of the cell lines studied can be seen in **Table 6**.

MRC-5

The MRC-5 cell line was established in 1966 from the normal lung tissue of a 14-week old male fetus[112]. Previous research has indicated these cells are capable of 42 to 46

population doublings before the onset of senescence[112]. A number of studies have used MRC-5 cells as an established model of non-cancerous human lung fibroblasts.

SK-MES-1

The SK-MES1 cell line was derived from the pleural effusion of a 65 year old Caucasian male with squamous cell carcinoma[113].

H460

The H460 cell line was initiated in 1982 from the pleural fluid of a large cell lung cancer patient[114] and is an established large cell lung cancer epithelial cell line.

A549

The A549 cell line was initiated in 1972 through *in vitro* cultivation of lung carcinoma tissue from a 58-year-old Caucasian male[115]. This is a human lung adenocarcinoma epithelial cell line.

H1299

The H1299 cell line was derived from the lymph node of a 43 year old Caucasian male with non-small cell lung cancer[116].

184B5

The 184B5 cell line was established from normal mammary tissue from a 21 year old female obtained from a normal reduction mammoplasty[117]. This is a chemically transformed cell line that was established when cells from this tissue were exposed to benzo(a)pyrene, a carcinogenic polycyclic aromatic hydrocarbon. The 184B5 cell line appears to be immortal, but is not malignant. It is a human mammary epithelial cell line.

MDA-MB-231

The MDA-MB-231 cell line was isolated from pleural effusions of a 51 year old female Caucasian with breast cancer[118]. This cell line does not express estrogen receptors and therefore has been widely used to examine non-hormone sensitive breast cancer.

MCF-7

The MCF-7 cell line was initiated from the pleural effusion in a 69 year old female Caucasian with breast adenocarcinoma[119]. This cell line possess cytoplasmic estrogen receptors that are able to process estradiol and these cells are capable of forming domes as seen in differentiated mammary epithelium. MCF-7 is an established and widely used a human breast adenocarcinoma epithelial cell model.

PNT1A

The PNT1A cell line was initiated from the prostate of a 35 year old male at post-mortem[120]. This is a noncancerous prostate epithelium cell line that has been immortalized with the Simian vacuolating virus 40 (SV40).

22Rv1

The 22Rv1 cell line was derived from a serially propagated xenograft in mice after castration-induced regression and relapse of the parental CWR22 xenograft[121]. This cell line has been shown to express the androgen receptor. 22Rv1 is a human prostate adenocarcinoma epithelial cell line.

LNCaP

The LNCaP cell line was established in 1977 from a needle aspiration biopsy of the left supraclavicular lymph node of a 50 year old Caucasian male with metastatic prostate

carcinoma[122]. This cell line is both androgen receptor- and estrogen receptor-positive and has been used extensively as a model of hormone sensitive prostate cancer.

PC-3

The PC-3 cell line was derived from a 62 year old Caucasian male from the bone metastasis of a grade IV prostatic adenocarcinoma[123]. These cells do not express the androgen receptor and therefore are a widely used in vitro model of hormone insensitive prostate adenocarcinoma.

Table 6. Cell line properties

Cell Line	Histology	Mutation Status/ Hormone Responsiveness
SK-MES-1	Lung squamous cell carcinoma	p53 null
H460	Lung large cell carcinoma	KRAS mutant LKB1 null PIK3CA mutation
A549	Lung adenocarcinoma	KRAS mutant LKB1 null
H1299	Lung adenocarcinoma	p53 null
MRC-5	Lung fibroblast	No known mutations
MDA-MB-231	Breast adenocarcinoma	p53 null KRAS mutant Estrogen receptor negative
MCF-7	Breast adenocarcinoma	PIK3CA mutation Estrogen receptor positive
184B5	Mammary epithelium	No known mutations
22Rv1	Prostate adenocarcinoma	p53 null Androgen responsive
LNCaP	Prostate adenocarcinoma	PTEN null Androgen responsive
PC-3	Prostate adenocarcinoma	p53 null PTEN null
PNT1A	Prostate epithelium	No known mutations

1.6.1 Glucose Transporter Expression in Tumours and Cancer Cell Lines

Previous studies have suggested that increased glucose uptake in malignant cells is associated with an increased expression of glucose transporter proteins. The expression of select members of the GLUT family has been studied in various cancers. The following will review findings of GLUT expression in lung, breast and prostate tumours and cancer cell lines.

1.6.2 Glucose Transporter Expression in Lung Tumours and Cancer Cell Lines

Compared to healthy cells, tumour cells have been shown to increase the expression of GLUTs with a low K_m for glucose in order to meet demands[42]. GLUT1 and GLUT3 have high affinities for glucose and have been detected in various histological types of lung cancer at both the mRNA and protein level[124]. Ogawa et al. found GLUT1 to be amplified more than GLUT3 in lung tumours, however both proteins were correlated with proliferating cell nuclear antigen (PCNA), a nuclear protein that increases when DNA is being synthesized, indicating that GLUT-mediated glucose uptake may aid in the proliferation of cancer cells[124].

GLUT1 and GLUT3 have been detected by immunohistochemistry in poorly and undifferentiated non-small-cell lung cancer (NSCLC) tumours[71]. These findings are supported by a study by Kurata et al. in which expression of GLUT1 was found to be significantly higher in primary lung tumours than in normal lung tissue[125]. Analysis of autopsy samples from primary lung and liver tumours, normal tissues and metastatic liver tumours using reverse transcriptase PCR revealed no differences in GLUT1 expression between primary lung tumours and metastatic liver tumours. However, the expression of

GLUT3 and GLUT5 were found to be significantly higher in metastatic liver tumours, suggesting different GLUT expression patterns may exist between primary and metastatic tumours. Studies comparing other types of primary cancers and their metastatic tumours are needed.

Little data exists regarding the expression of GLUTs in lung cancer cell lines. In agreement with the findings from lung cancer tissue samples, A549 and H226 cells were found to have low basal expression of GLUT1[126]. In comparison, this study found that H1299 and H1650 cells expressed higher levels of GLUT1[126]. This study is supported by detection of GLUT1 in A549 cells by western blotting by Ito et al. (2002)[127].

The expression of glucose transporters in the lung cancer cell line H460 was determined using oligonucleotide DNA microarray[128]. Compared to a control pool of RNA, levels of GLUT1 in H460 cells were found to be increased and GLUT3 expression was 1.5-1.7 fold higher than the control. Further *in vitro* studies are required to establish the basal expression of GLUTs in lung cancer cell lines.

1.6.3 Glucose Transporter Expression in Breast Tumours and Cancer Cell Lines

Mixed evidence exists in the literature in regards to GLUT1 expression and breast cancer. Younes et al. found GLUT1 expression in only 42% of tumours and their findings are supported by subsequent studies in which GLUT1 was detected in only a portion of breast cancer tumours[129-131]. Contrary to these reports, Godoy et al. reported positive staining in 91% of the invasive ductal carcinomas analyzed with immunohistochemistry and high expression has been reported in other studies as well[132-134]. It is possible these

conflicting results are due to the method used to detect GLUT1 (immunohistochemistry vs. PCR) or differences in the histological types of breast cancer examined.

Expression of glucose transporter proteins that are not present in corresponding healthy cells has been documented in breast cancer[107]. This was observed in a study by Rogers et al. (2003) where GLUT12 expression at the mRNA and protein level was found in primary human breast cancers but not normal breast epithelium[42]. Furthermore, GLUT5 is highly expressed in human breast cancer but is absent in normal human breast tissue[135]. The presence of a high affinity fructose transporter (GLUT5) in neoplastic breast tissue indicates human breast cancer cells may have a specialized capacity to transport fructose. GLUT3 was detected at the mRNA and protein level in breast cancer tumours and found to be expressed at significantly higher levels in poorly differentiated tumours[136], suggesting this transporter may a useful marker in breast cancer staging.

Breast cancer cell lines are the most extensively studied with respect to GLUT expression. Hexose uptake assays and immunoblotting experiments have revealed that the breast carcinoma cell lines MCF-7 and MDA-468 express the glucose transporters GLUT1, GLUT2 and GLUT5[135]. GLUT1 expression is found more consistently in breast cancer cell lines than in tumours. When the invasiveness of the human breast cancer lines MCF-7, MDA-MB-435 and MDA-MB-231 was measured using an *in vitro* assay and compared with GLUT expression, it was found that GLUT1 protein levels increased with invasive potential[137]. GLUT2 and GLUT5, on the other hand, were found to be inversely associated with invasiveness[126]. GLUT3 was also detected in all three cell lines but did correlate with invasiveness. Therefore, data regarding expression of specific GLUTs in cancer cell lines may be indicative of prognosis in breast cancers.

Estrogen-receptor positive MCF-7 cells and estrogen-receptor negative MDA-MB-231 cells are used to represent models of early- and late-stage breast cancer, respectively. Since both cell lines display high rates of fructose transport and increased levels of GLUT5 mRNA and protein, Chan et al. performed GLUT5 knockdown by antisense oligonucleotide and found a decrease in cell proliferation[138]. This suggests gene silencing/down regulation of GLUT5 as an attractive therapy for breast cancer because it was effective in both an early and late stage model of breast cancer.

The GLUT12 protein has been demonstrated in the breast cancer cell lines MCF-7 and T-47D[16,41] while no detection was observed in the MDA-MB-231 and MDA-MB-435 cell lines. Expression of this transporter in other breast cancer cell lines remains to be investigated.

1.6.4 Glucose Transporter Expression in Prostate Tumours and Cancer Cell Lines

Limited and conflicting evidence exists regarding GLUT expression in prostate cancer. A study using immunohistochemistry found that while healthy prostate tissue expressed GLUT3 and GLUT5, prostate carcinoma did not[132]. In this study GLUT1 was detected in prostate carcinoma at lower expression levels than in the normal tissue[132], however other studies have failed to detect any GLUT1 protein expression in prostate tumours[139-141]. The protein responsible for glucose transport in prostate cancer may be GLUT12 as it was detected both at the mRNA and protein level in hyperplastic tissue sections[107]. Additionally, GLUT11 mRNA has been found to be up-regulated in prostate cancer as compared to benign prostate tissue[141].

The evidence regarding GLUT expression in prostate cancer cell lines is also extremely limited. GLUT1 mRNA has been detected in the DU145, PC-3 and LNCaP prostate cancer cell lines[142]. Additionally, a study using LNCaP cells and its derivatives C4, C4-2 and C4-2B indicated the presence of GLUT1 and GLUT12 mRNA in all cell lines examined[107]. Western blot analysis and immunofluorescence revealed the presence of the GLUT1 and GLUT12 proteins in these cell lines as well. These results are in contrast to the expression pattern observed in malignant prostate tissue which was negative for the GLUT1 protein[107]. Thus there is a need to address the relationship between GLUT expression in cancer cell lines and human tissues of the same cancer type.

1.7 Rationale

It is well established that tumour cells display an increased rate of glucose uptake and utilization compared to non-cancerous cells[4,143]. Therefore, it is important to understand the mechanisms by which these processes are achieved. Tumour cells may compensate by upregulating GLUT proteins. This hypothesis is supported by evidence that elevated expression of glucose transporters has been observed in most cancers[144]. Moreover, some cancers also show an abnormal tissue expression pattern compared to healthy tissues[42,132,135]. Deregulated expression of GLUTs, with different hexose affinities, may allow cancer cells to optimize their energy supply providing a fundamental advantage for growth.

Elevated uptake of glucose allows tumours to be visualized using FDG-PET, currently used for the diagnosis and staging of cancers. Higher glucose uptake on FDG-PET scans

is often correlated with more aggressive and advanced-staged tumours[145.]. A positive relationship between tumour retention of FDG and GLUT1 expression has been observed in a number of cancer types[146-151] suggesting that GLUT1 may serve as a prognostic biomarker in tumours. However, some studies have failed to find an association between FDG uptake and GLUT1 expression[152,153]. This indicates that other GLUTs may be responsible for the increased glucose uptake in these tumours and warrants the investigation of the expression of multiple GLUTs in cancer.

Another protein potentially responsible for the high rates of glucose uptake observed in cancer cells is the high affinity transporter GLUT3. Previous studies have detected this protein in various tumours[71,132,154], however its expression in cancer cell lines is less clear. Given the stimulatory effect of mitogens and growth factors on cancer cell growth, it is also important to understand cancer expression patterns of GLUTs that are regulated by these substances. These include GLUT4, normally restricted to insulin-sensitive tissues, and GLUT12 which has been described in breast and prostate tumours[42,107]. It is possible stimulation by mitogens and/or growth factors increase the expression of these GLUTs and GLUT4- and GLUT12-mediated glucose uptake by cancer cells. Furthermore, GLUT4 and GLUT12 have been suggested to be transcriptionally repressed by the tumor suppressor p53, indicating these proteins may facilitate tumor growth in cancers with mutated p53[81,105].

Limited data exists regarding the expression of GLUT4 in cancer. Previous studies show it has been sporadically detected in lung cancer tissue[140,152], however its expression has not been investigated in cancer cell lines. Although GLUT12 was originally identified in a breast cancer cell line, little work has been done subsequently to

characterize its expression in other cancer cells. Although there is some evidence to indicate that GLUTs 5, 8, 10 and 11 are expressed in cancer[140], the focus of this study was restricted to GLUT1, GLUT3, GLUT4 and GLUT12 due to the limited amount of commercially available products detecting GLUTs. Examining expression of the selected transporters in cancer cell lines will provide insight regarding which transporters may be facilitating the high rates of glucose uptake in tumours.

Support for GLUT expression as a prognostic indicator comes from studies that have found the expression of GLUTs to be associated with poor survival. This has been best studied with regards to GLUT1 and GLUT3. High expression levels of GLUT1 has been shown to be an indicator of poor survival in lung[71], pancreatic[155], colorectal[156], ovarian[157], breast[158], bladder[159] esophageal[160] and oral cancer[161] . A high level of GLUT3 expression has been shown to be associated with poor survival in lung[71], laryngeal[162] and oral cancer[161]. Furthermore, GLUT1 is an established marker of hypoxia which is a poor prognostic factor due its involvement in tumour resistance to radiotherapy and chemotherapy[163]. With increased knowledge about GLUT expression in aggressive cancers, these proteins can be used to help evaluate anticancer therapies by serving as biomarkers of tumour response to treatments. Therefore, understanding GLUT expression in cancer will aid in both monitoring tumours and planning a course of therapy.

Given that glucose uptake across the plasma membrane is considered the rate-limiting step for glucose consumption in tumour cells, this represents an ideal point to target for cancer therapy. Recent studies have focused on inhibiting the glucose transporter GLUT1 in order to prevent cancer cell growth. Incubation with an anti-GLUT1 antibody was shown to decrease cell proliferation and induce apoptosis in NSCLC and breast cancer

cell lines[164]. In addition, the anti-GLUT1 antibody was found to have a synergistic effect with the anti-cancer drugs cisplatin, paclitaxel and gefitinib in enhancing apoptosis. The glucose transporter inhibitor fasentin has been shown to increase apoptosis in prostate cancer and leukemia cells by sensitizing these cells to the death ligand FAS[165]. Furthermore, the flavonoid apigenin has been found to inhibit proliferation of prostate cancer cells by decreasing GLUT1 expression at both the mRNA and protein level[166]. Taken together, the results of these studies suggest that preventing glucose uptake by blocking glucose transporters is an attractive therapeutic strategy for the treatment of various cancers. Identification of novel inhibitors of glucose transport is important and the small-molecule compounds WZB27 and WZB115 have recently been shown to inhibit glucose transport in lung, breast, colon and cervical cancer cells more potently than fasentin or apigenin[167].

The increased dependence of tumour cells on sugars and the glycolytic pathway to generate ATP, along with the potential use of GLUTs as biomarkers and targets for therapeutics, provide important reasons to study GLUT expression in cancers. Although studies have indicated that cancer cells have increased and deregulated expression of GLUTs, a comprehensive understanding of the basal levels of glucose transporter expression in cancer is lacking. Expression and localization of the GLUTs in cancer cell lines has been studied less extensively than in tumour models. It is important to study GLUT expression in cell culture models as a first step in understanding the mechanism of glucose uptake by cancer cells and the function of these proteins. By using cancer cell lines, a uniform population of cells is available that will provide information about baseline expression patterns of GLUTs, not affected by hypoxia or treatment a patient

may be receiving. Established cancer cell lines are readily attainable and can be easily manipulated in future studies determining the function and regulation of GLUT proteins in cancer. Therefore, there are many advantages to studying GLUT expression in cancer cell lines rather than tumours.

As certain mutations have been shown to affect glucose transporter expression and distribution (See Regulation of Glucose Transporters), it is important to include cancer cells with different mutation statuses in the analysis of GLUT expression in cancer. The investigation of glucose transporter expression in a number of cancer cell lines, with different mutations and histologies, is important to provide a clear understanding of the role of GLUT proteins in cancer cell metabolism.

1.8 Hypotheses

In the present study it is hypothesized that:

1) Lung, breast and prostate cancer cells display an increased and abnormal pattern of GLUT expression compared to non-cancerous cells of the corresponding tissue type
2) Mutation status of oncogenes and tumour suppressor genes mediates specific patterns of GLUT expression
3) Epithelial tumour cells may express multiple GLUTs
4) Expression patterns of GLUTs in human tumours will be similar to that detected in tissue culture models of cancer

1.9 Objectives

The primary objectives of the study are to:

1) Examine the expression of the glucose transporters GLUT1, GLUT3, GLUT4 and GLUT12 at the mRNA level in lung, breast and prostate cancer cell lines as well as the corresponding non-cancerous cell lines of that tissue
2) Examine the expression of the glucose transporters GLUT1, GLUT3, GLUT4 and GLUT12 at the protein level in lung, breast and prostate cancer cell lines as well as the corresponding non-cancerous cell lines of that tissue
3) Determine the expression of GLUT1 and GLUT3 in tumours from nude mice xenografted with cancer cell lines

CHAPTER 2: METHODOLOGY

2.1 Materials

All tissue culture materials including Dulbecco's Modified Eagle Medium (D-MEM), Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), trypsin and antibiotic were purchased from GIBCO Life Technologies (Burlington, ON). Antibodies against GLUT3, GLUT4 and GLUT12 were purchased from Abcam (Cambridge, MA) and antibodies against GLUT1 and GLUT4 were purchased from Serotec (Toronto, ON). HRP-conjugated anti-rabbit secondary antibody and actin antibody were purchased from New England Biolabs (Mississauga, ON). Alexa Fluor 568 antibody was obtained from Molecular Probes (Hamilton, ON). The ECL Western Blotting Analysis System and SYBR Premix Ex TAQ DNA were obtained through Thermo-Fischer (Ottawa, ON). Polyvinylidene difluoride (PVDF) membranes, molecular weight protein standards and electrophoresis reagents were purchased from BioRad (Mississauga, ON). The RNeasy Mini Kit was purchased from Qiagen (Mississauga, ON) and SuperScript III First-Strand Synthesis SuperMix from Invitrogen (Burlington, ON). DNA-free was obtained from Ambion (Austin, TX). Vector biotinylated secondary, ABC reagent and Novared were obtained from Vector Laboratories (Burlington, ON). All other chemicals, including NaOH, HCL, NaCl and albumin bovine serum (BSA) were purchased from Sigma (St. Louis, MO). The MRC-5, SK-MES, H460, A549, H1299, 184B5, MDA-MB-231, MCF-7, PNT1A, 22Rv1, LNCaP and PC-3 cells were purchased from American Type Culture Collection (ATCC). The L6 cells were a kind gift of Dr. A. Klip (Hospital for Sick Children, Toronto, ON). The C2C12 cell lysate was a kind gift from Dr. Jeff Stuart (Brock University, St. Catharines, ON). The anti-GLUT1, -GLUT3

and -GLUT4 small interfering RNA (siRNA) transfection kit was obtained from Qiagen (Mississauga, Ontario, Canada).

2.2 Buffers and Solutions

The composition of each buffer and solution that will be used to perform the experiments is presented below:

Cell Lysis:

PBS Washing Buffer: 137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM NA_2HPO_4, 0.68mM $CaCl_2$, 0.49mM $MgCl_2$, add water to 1L and adjust pH to 7.4.

SDS Sample Buffer: 62.5mM Tris-HCl (pH 6.8), 10% glycerol 2% w/v SDS, 0.01% bromophenol blue, add 0.05% β-mercaptoethanol before use.

Cell Lysis Buffer: 20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM Na_3VO_4, 1µg/ml leupeptin, and add 1mM PMSF before use and chill on ice.

Western Blotting:

1.5M Tris-HCl Buffer (pH 8.8): 27.23g Tris base (18.15g/100ml), 80ml deionized water, adjust to pH 8.8 with 6N HCl. Bring to total volume 150ml with deionized water.

0.5M Tris-HCl Buffer (pH 6.8): 6g Tris base, 60ml deionized water, adjust to pH 6.8 with 6N HCl and bring total volume to 100ml with deionized water.

Resolving Gel Buffer: 33% v/v of 30% Acrylamide/ Bis Solution, .38M Tris-HCl, pH 8.8, .l% w/v SDS, bring to total volume 30ml with deionized water. Right before pouring the gel, add 150µl 10% APS made fresh daily and 15µl TEMED and swirl.

Stacking Gel Buffer: 13.3% v/v of 30% Acrylamide/ Bis Solution, .13M Tris-HCl, pH 6.8, .1% w/v SDS, bring to total volume 30ml with deionized water. Right before pouring the gel, add 150µl 10% APS made fresh daily and 30µl TEMED and swirl.

10x TBS (Tris-buffered saline): .20M Tris base, 1.65M NaCl, adjust pH to 7.6 with HCl. Use at 1x TBS. Dissolve in 1L of water.

Blocking Buffer:1x TBS, 0.1% Tween- 20 (100%), 5% w/v nonfat dry milk. Dissolved in water.

Wash Buffer TBS/T: 1x TBS and 0.1% Tween- 20

Primary Antibody Dilution Buffer: 5% w/v nonfat dry milk in TBS/T

10x Electrode Running Buffer: .13M Tris base, .96M Glycine, .017M SDS. Dissolve and bring volume to 500ml with DD water. Do not adjust pH with acid or base. Dilute 50ml of 10x stock with 450ml water before use.

Transfer Buffer: 25mM Tris base, 0.2M glycine, 20% methanol, dissolved in 800ml of water.

Polymerase Chain Reaction (PCR):

cDNA Synthesis Mix: 2μl 10X Reverse Transcriptase (RT) Buffer, 4 μl 25mM $MgCl_2$, 2 μl 0.1 M Dithiothreitol (DTT), 1 μl RNaseOUT (40 U/μl) and 1 μl Superscript III RT (200 U/μl) per reaction.

Primer Pair Master Mix: 0.5 μl of 10 pmol/μl primer FORWARD, 0.5 μl of 10 pmol/μl primer REVERSE, 2.75 μl H_20 and 6.25 μl SYBR Green Premix per reaction.

2.4 Cell Culture Techniques

A549, H1299, H460, PC-3, PNT1A and 22Rv1 cell lines were grown in RPMI media supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B). LNCaP cells were grown in RPMI media supplemented with 10% (v/v) FBS 1% (v/v) antibiotic-antimycotic solution, 10 mM HEPES buffer and 1 mM sodium pyruvate. SK-MES, MRC-5, MCF-7 and MDA-MB-231 cell lines were grown in D-MEM media supplemented with 10% (v/v) FBS, and 1% (v/v) antibiotic-antimycotic solution. The cells were maintained until they reached 80-90% confluency with the media replaced every 2-3 days.

2.5 Cell Pellet Preparation

All cell lines were grown in 75cm^2 flasks and two flasks per passage per cell line were collected as cell pellets. Once 80-90% cell confluency was reached, cells were washed twice with PBS. Two milliliters of trypsin was added to each flask to detach cells which were collected in PBS and centrifuged at 10,000 rpm for 5 minutes. The PBS was aspirated from each tube and the cell pellet was washed by adding 1 mL of PBS to each tube and breaking the pellet. The cells in PBS were centrifuged again at 4,000 rpm for 5 minutes. The PBS was aspirated from the tube and the samples were stored immediately at -80°C for further use in Real-Time PCR.

2.6 Real-Time PCR

Total RNA was purified from cell pellets using the Qiagen RNeasy Mini Kit following the manufacturer's protocol. After RNA was eluted it was treated with the DNA-free kit by Ambion to eliminate any DNA contamination within the RNA samples. Next, a spectrometer was used to determine the concentration of the RNA samples. Equal amounts of RNA (up to 5μg) were used to synthesize cDNA using the SuperScript III First-Strand Synthesis Supermix protocol by Invitrogen. Previously designed forward (FOR) and reverse (REV) primers were used to amplify human GLUT3, GLUT4, GLUT12, RPII-1 and RPII-2. These primers were designed based on modifications to existing PCR primer pairs for gene expression detection and quantification listed in PrimerBank, with annealing temperatures of 60°C (http://pga.mgh.harvard.edu/primer-bank/index.html). The GLUT 1 FOR and REV primers were designed with assistance from a post-doctorate fellow using Primer Bank. These primers have been efficiency

tested and the integrity of the product has been verified by gel electrophoresis (**Figure s.1**).

The sequence of the primers used are as follows:

GLUT1: FOR 5'-CCTGCAGGAGATGAAGGAAG-3'

REV 5'-TCGAAGATGCTCGTGGAGTA-3'

GLUT3: FOR 5'-GCTGGGCATCGTTGTTGGA -3'

REV 5'-GCACTTTGTAGGATAGCAGGAAG -3'

GLUT4: FOR 5'-TCG GGC TTC CAA CAG ATA GG -3'

REV 5'-AGC CAC GTC TCA TTG TAG CTC -3'

GLUT12: FOR 5'-CGG TTT CTG GTG ATG AAA GG -3'

REV 5'-TCC GCA TGT TGT CTT TTG AA -3'

RPII-1: FOR 5'-GGGTG CTGAGTGAGAAGGAC-3'

REV 5'-AGCCAT CAAAGGAGATGACG-3'

RPII-2: FOR 5'- GAAACGGTGGACGTGCTTAT-3'

REV 5'-TCTC CATGCCATACTTGCAC-3'

The cycling conditions used are as follows: 95°C for 1 min, 40 total cycles of 95°C for 10 sec, 60°C for 25 sec, and melt peak determination (95°C for 15 sec, increasing from 65°C to 95°C with 0.5°C increments for 5 sec each). Parallel reactions were carried out for the RPII housekeeping gene to calculate relative mRNA levels by real time PCR using the $2^{-[\Delta][\Delta]Ct}$ method. RPII-1 was used for GLUT3, GLUT4 and GLUT12, and RPII-2 was used for GLUT1.

2.7 Cell Lysis

All cell lines were grown in 75cm^2 flasks and two flasks per passage per cell line were collected for cell lysates. Once 80-90% cell confluency was reached, cells were washed twice with PBS and placed on ice. PBS was removed and 1.5 mL of lysis buffer was added to each flask. Cells were lysed and collected into 1.5 mL eppendorf tubes and stored at -20°C.

2.8 Protein Assay

Protein assay dye (BioRad) was prepared and filtered for protein concentration determination. A standard protein curve was created using BSA protein standards at 0, 0.1, 0.2, 0.4, 0.6, 0.8, 0.9 and 1.0mg/ml. 10µl of each protein standard and the same of each of cell lysate was transferred in triplicate into separate wells of a 96-well plate. 200µl of protein assay dye was then added to each well. A microplate was used to measure absorbance at 595nm and the final concentration of the protein samples was determined.

2.9 Western Blotting

An equal amount of SDS sample buffer was added to each cell lysate and the samples were then boiled for 5 minutes followed by centrifugation for 5 minutes. A total of 30-50 µg of each protein was subjected to SDS-PAGE electrophoresis on 10% polyacrylamide gels. Samples were then transferred from the gel to a PVDF membrane and incubated in blocking buffer for 1 h at room temperature. The membrane was then incubated in primary antibody solution overnight at 4°C. After washing in TBS/T, the membrane was

incubated with the appropriate HRP-conjugated secondary antibody for 1h at room temperature. Signals were detected using the ECL Plus Western Blotting Detection System and exposure to film. Densitometric analysis was performed using ImageJ software.

2.10 Immunohistochemistry

Tumour tissues fixed in formalin from Balb/c nude mice xenografted with A549, H1299 and PC-3 cells were obtained from the Translational Biology lab at the Juravinski Cancer Centre, Hamilton, ON. Four µm thick tumour sections were mounted onto slides and then deparaffinized by washes in xylene, ethanol and distilled water, respectively. Antigen unmasking was performed by boiling slides in 10 mM sodium citrate buffer for 30 minutes. Slides were then blocked with 5% normal goat serum in TBS/T at room temperature for one hour followed by incubation with primary antibody against GLUT-1 (1:250 dilution) or GLUT-3 (1:100 dilution) overnight at 4°C. Slides were incubated in Vector biotinylated secondary for 30 minutes, washed in TBS/T and then incubated in Vector ABC reagent for an additional 30 minutes. Vector Novared was used to stain slides that were then washed in distilled water and counterstained with hematoxylin. Slides were dehydrated with washes in ethanol followed by xylene and then mounted. Signals of antibody localization in the plasma membrane and cytoplasm were evaluated by a trained pathologist.

2.11 Immunofluorescence Microscopy

Cells were grown on coverslips in 6-well dishes then washed with cold PBS and fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Cells were washed with PBS then permeabilized and blocked with 0.5% triton-X and 5% FBS in PBS for one hour at room temperature. The cells were then incubated with primary antibody against GLUT-4 (1:100 dilution) overnight at 4°C. The slides were washed with PBS and incubated with anti-rabbit secondary antibody conjugated to Alexa Fluor 568 for one hour in the dark. After staining with DAPI for 5 minutes, the slides were mounted and examined immediately using appropriate excitation wavelength.

2.12 Small Interfering RNA (siRNA)

Cells were incubated with Hyperfect transfection reagent without or with siRNA against GLUTs for 24 hours. The transfection reagent was removed and cells were incubated for an additional 72 hours before collection or fixation.

2.13 Statistical Analysis

Real time PCR Ct values were normalized first to the housekeeping gene RPII-1 (GLUT3, GLUT4, GLUT12) or RPII-II (GLUT1). Values were then normalized to the appropriate noncancerous cell line (Figures 13-21) or to MRC-5 (Figures s.4-7) or the lowest GLUT expressed within that cell line (Figures s.8-10). Arbitrary densitometric values obtained from western blots were normalized first to the corresponding actin bands, then normalized to the appropriate noncancerous cell line. Statistical analysis was performed using SPSS v19.0 software. The results are presented as a mean +/- SEM of at

least three separate experiments. The assumptions of analysis of variance (ANOVA) were checked and data was analyzed by one-way ANOVA followed by a Tukey post-test to determine statistical differences between mean expression in cancer cells and the respective control cell line. Statistical significance was assumed at $P<0.05$.

CHAPTER 3: RESULTS

3.1 GLUT1 Expression in Cancer Cells

3.1.1 Lung Cancer Cells

The expression of GLUT1 was evaluated at the basal mRNA level through quantitative Real-Time PCR in SK-MES-1, H460, A549 and H1299 epithelial lung cancer cell lines and the noncancerous lung fibroblast line MRC-5 (**Figure 13 A**). Relative quantification revealed that GLUT1 mRNA levels were elevated in SK-MES-1 cells compared to MRC-5 cells (4.30 ± 0.61, $p<0.01$). GLUT1 mRNA levels were also higher in H460 cells (1.93 ± 0.63, NS), while A549 (0.7 ± 0.059, NS) and H1299 ($.12 \pm 0.004$, NS) cells showed lower levels of GLUT1 mRNA in comparison to the MRC-5 cell line.

GLUT1 protein expression has been documented in a number of cancers[16, 29, 132]. Western blotting was performed to examine whether the observed mRNA expression of GLUT1 in the lung cancer cell lines corresponded with expression of the GLUT1 protein (**Figure 13 B**). Similar to the mRNA data, SK-MES-1 cells had higher levels of GLUT1 protein compared to the MRC-5 cell line ($193.75\% \pm 9.87\%$, $p<0.01$). Similar patterns were observed between the mRNA and protein expression of GLUT1 in the H460 ($138.84 \pm 27.53\%$, NS) and A549 ($55.17 \pm 13.78\%$, NS) cell lines. In contrast to the low mRNA expression of GLUT1 in the H1299 cell line, expression of GLUT1 protein was 88% higher compared to MRC-5 cells ($188.67 \pm 6.79\%$, $p<0.05$).

3.1.2 Breast Cancer Cells

GLUT1 mRNA expression in the breast cancer cell lines MDA-MB-231 and MCF-7 was normalized to the mammary epithelial cell line 184B5 (**Figure 14 A**). The estrogen-

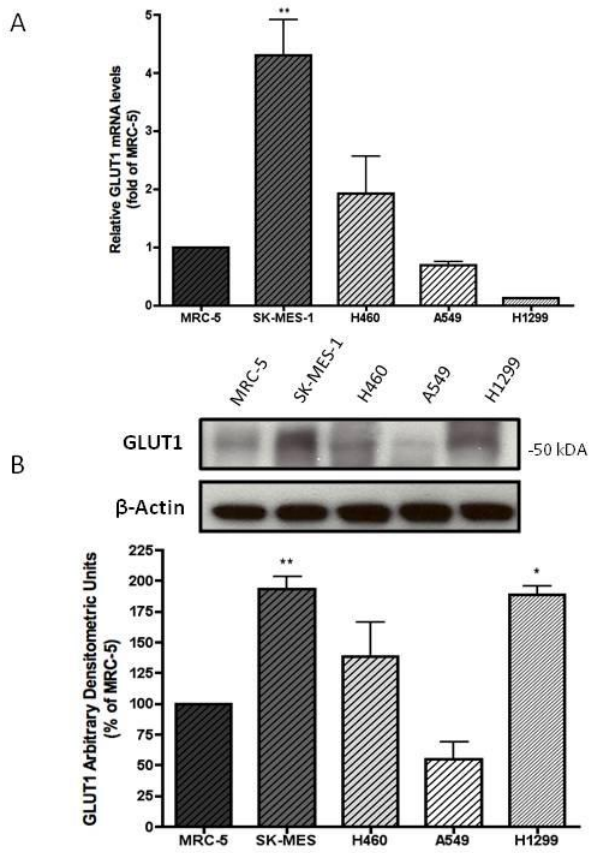

Figure 13. GLUT1 expression in lung cancer cells. A) Basal GLUT1 mRNA levels were determined by Real Time PCR and are expressed as a fold change of the lung fibroblast cell line MRC-5. B) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT1. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was calculated using Image J software. Protein levels are expressed as a percentage of the MRC-5 cell line. Results represent the mean ± SEM of three to four independent experiments. (*$p<0.05$, **$p<0.01$).

independent cell line MDA-MB-231 showed 1.57 fold higher GLUT1 mRNA expression compared to 184B5 cells (1.57 ± 0.32, NS), while estrogen-responsive MCF-7 cells had lower expression than the noncancerous mammary cell line (0.33 ± 0.037, NS).

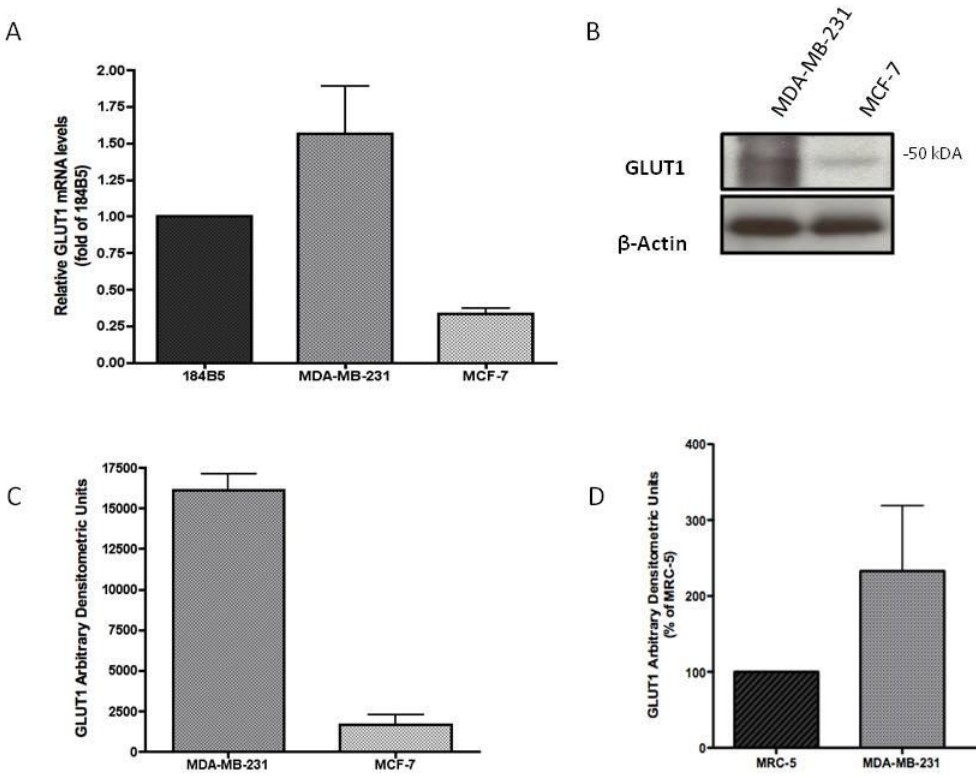

Figure 14. GLUT1 expression in breast cancer cells. A) Basal GLUT1 mRNA levels were determined by Real Time PCR and are expressed as a fold change of the mammary epithelial cell line 184B5. B) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT1. A representative immunoblot is shown. C) The densitometry of the bands expressed in arbitrary units was calculated using Image J software. Protein levels are expressed as a comparison of arbitrary units between the MDA-MB-231 and MCF-7 cell line. D) Protein levels are expressed as a percentage of the lung fibroblast MRC-5 cell line. Results represent the mean ± SEM of three to four independent experiments.

GLUT1 protein expression in the breast cancer cell lines was analyzed as arbitrary values (**Figure 14 C**) and normalized to the noncancerous cell line MRC-5 (**Figure 14 D**) as it was not possible to obtain 184B5 cells for cell lysate preparation during the course of the study. Densitometric analysis of western blotting revealed that like the mRNA data, the MDA-MB-231 cell line showed a stronger expression of the GLUT1 protein than MCF-7 cells . When compared to the MRC-5 cell line, the MDA-MB-231 (232.9 ±

86.27, NS) cell line showed a 2.3-fold higher level of GLUT1 protein while the MCF-7 cell line (25.57 ± 12.39, NS) expressed GLUT1 at a lower level than the lung fibroblast line.

3.1.3 Prostate Cancer Cells

GLUT1 mRNA expression was examined in the prostate cancer cell lines 22Rv1, LNCaP and PC-3 and expressed as a fold of the noncancerous prostate epithelial cell line PNT1A (**Figure 15 A**). GLUT1 expression was determined to be higher in all prostate cancer cell lines than in PNT1A cells. The androgen responsive cell lines 22Rv1 (1.83 ± 0.42, NS) and LNCaP (2.34 ± 0.68, NS) showed higher GLUT1 mRNA levels while the androgen insensitive cell line PC-3 showed an 8.88 fold increase in GLUT1 expression (8.88 ± 1.21, $p<0.001$).

Different patterns were observed with respect to protein expression, the 22Rv1 (8.82 ± 1.07%, $p<0.05$) cell line was found to express the GLUT1 protein at a lower level than the PNT1A cell line and the LNCaP (37.22 ± 16.0%, $p=0.077$) cell line showed low GLUT1 expression compared to PNT1A as well (**Figure 15 B**). Consistent with the mRNA data, PC-3 cells showed higher GLUT1 expression compared to PNT1A cells (154.49 ± 26.08%, NS).

In order to determine the specificity of the GLUT1 antibody used in western blotting, siRNA was used to silence GLUT1 in H1299 cells. Transfection with GLUT1 siRNA resulted in down-regulation of a band at 50 kDa suggesting this to be the specific band for the protein (**Figure s.2 A**).

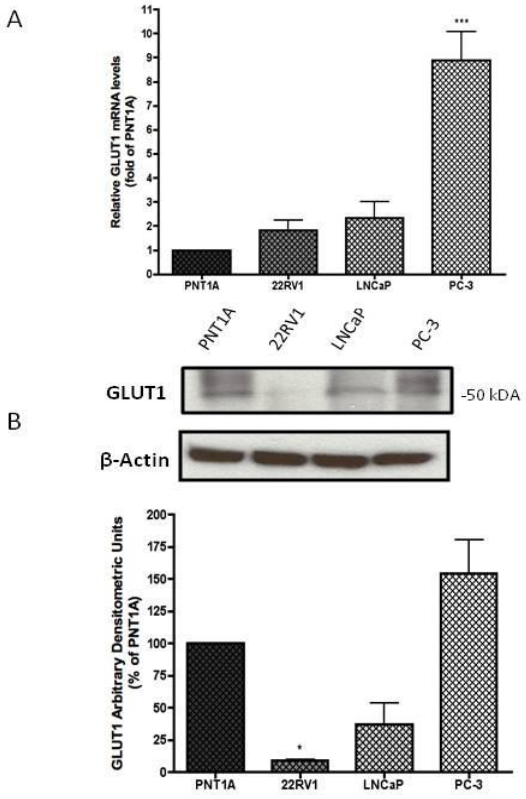

Figure 15. GLUT1 expression in prostate cancer cells. A) Basal GLUT1 mRNA levels were determined by Real Time PCR and are expressed as a fold change of the prostate epithelial cell line PNT1A. B) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT1. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was calculated using Image J software. Protein levels are expressed as a percentage of the PNT1A cell line. Results represent the mean ± SEM of three to four independent experiments. (*$p<0.05$, ***$p<0.001$).

3.2 GLUT3 Expression in Cancer Cells

3.2.1 Lung Cancer Cells

GLUT3 mRNA has been reported to be ubiquitously expressed in cells[15]. The H460 (5.05 ± 0.44, $p<0.01$) and A549 (2.62 ± 0.18, $p<0.05$) lung cancer cell lines showed higher levels of GLUT3 mRNA compared to the MRC-5 cell line (**Figure 16 A**).

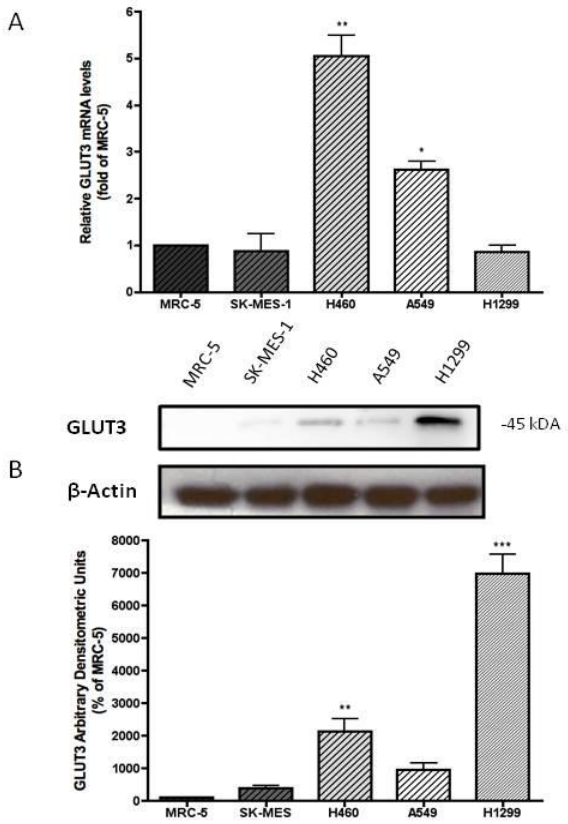

Figure 16. GLUT3 expression in lung cancer cells. A) Basal GLUT3 mRNA levels were determined by Real Time PCR and are expressed as a fold change of the lung fibroblast cell line MRC-5. B) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT3. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was calculated using Image J software. Protein levels are expressed as a percentage of the MRC-5 cell line. Results represent the mean ± SEM of three to four independent experiments. (*$p<0.05$, **$p<0.01$,***$p<0.001$).

Both SK-MES-1 (0.87 ± 0.38, NS) and H1299 (0.85 ± 0.15, NS) cells expressed GLUT3 mRNA at a level lower than that found in the MRC-5 cell line.

GLUT3 protein was not detected in the MRC-5 cell line, this is consistent with reports of GLUT3 expression primarily in the brain and testis[15] (**Figure 16 B**). In contrast, GLUT3 protein was detected in all of the lung cancer cell lines (**Figure 16 B**). The SK-MES-1 (398.67 ± 58.78%, NS) and A549 (943.47 ± 212.38%, NS) cancer cell lines were

found to have the lowest expression of the GLUT3 protein. H460 (2143.71 ± 367.34%, $p>0.01$) cells showed approximately 21-fold higher levels of GLUT3 protein compared to MRC-5, and the H1299 (6976.23 ± 600.49%, $p>0.001$) cell line showed the highest expression of GLUT3 at 70-fold higher than the noncancerous lung fibroblast cells.

3.2.2 Breast Cancer Cells

GLUT3 mRNA expression in the MDA-MB-231 breast cancer cell line was similar to that found in the noncancerous 184B5 mammary cells (1.05 ±0.11, NS) (**Figure 17 A**). MCF-7 cells showed minimal GLUT3 mRNA expression that was lower than the levels found in the 184B5 cell line (0.001 ± 0.0005, $p<0.001$).

Both breast cancer cell lines displayed GLUT3 expression at the protein level (**Figure 17 B**). In contrast to the low GLUT3 mRNA levels in MCF-7 cells, this cell line expressed the protein at a similar level to the MDA-MB-231 cell line. When expressed as a percentage of the MRC-5 cell line, both the MDA-MB-231 (1690.93 ± 213.31, $p<0.001$) and MCF-7 (1189.86 ± 170.22, $p<0.01$) breast cancer cell lines showed higher expression of the GLUT3 protein.

3.2.3 Prostate Cancer Cells

Expression of GLUT3 mRNA was higher in the 22Rv1 prostate cell line compared to the noncancerous PNT1A prostate epithelial cells (9.11 ± 3.01, $p<0.05$) (**Figure 18 A**). Both the LNCaP (0.029 ± 0.018, NS and PC-3 cell lines showed little expression of GLUT3 mRNA (0.0006 ± 0.0002, NS).

GLUT3 protein was detected in the PNT1A cell line (**Figure 18 B**), although this has not been reported in previous studies, healthy prostate tissue was shown to express

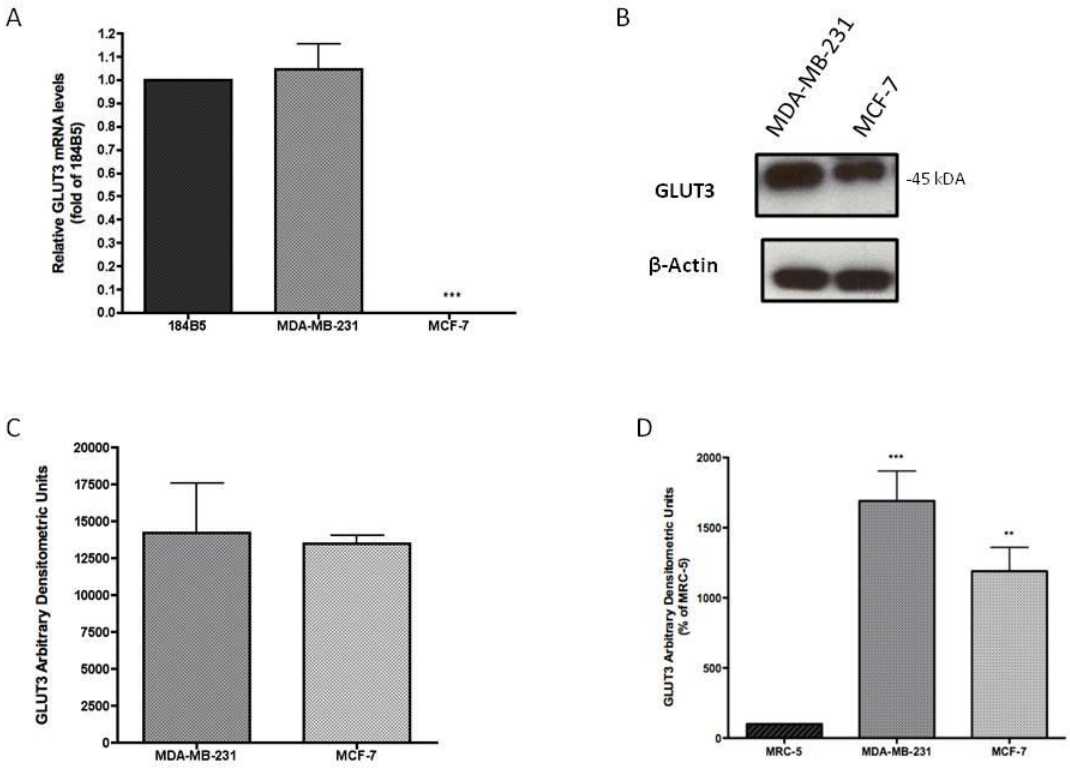

Figure 17. GLUT3 expression in breast cancer cells. A) Basal GLUT3 mRNA levels were determined by Real Time PCR and are expressed as a fold change of the mammary epithelial cell line 184B5. B) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT3. A representative immunoblot is shown. C) The densitometry of the bands expressed in arbitrary units was calculated using Image J software. Protein levels are expressed as a comparison of arbitrary units between the MDA-MB-231 and MCF-7 cell line. D) Protein levels are expressed as a percentage of the lung fibroblast MRC-5 cell line. Results represent the mean ± SEM of three to four independent experiments. (**p<0.01,***p<0.001).

GLUT3 through immunohistochemistry[132]. Unlike the high GLUT3 expression that was seen at the mRNA level, there was very low detection of the GLUT3 protein in the 22Rv1 cell line (19.39 ± 9.51%, NS) (**Figure 18 B**). LNCaP cells were also found to express GLUT3 at a lower level than the PNT1A cell line (48.12 ± 20.3%, NS). The PC-3

cell line, with low GLUT3 mRNA expression, had the greatest GLUT3 protein expression at 1.82-fold higher than that found in PNT1A cells (182.140 ± 45.75%, NS).

The specificity of the GLUT3 antibody used was also tested. When siRNA was used to silence GLUT3 in H1299 cells this resulted in down-regulation of a band at 45 kDa suggesting this to be the specific band for the protein (**Figure s.2 B**).

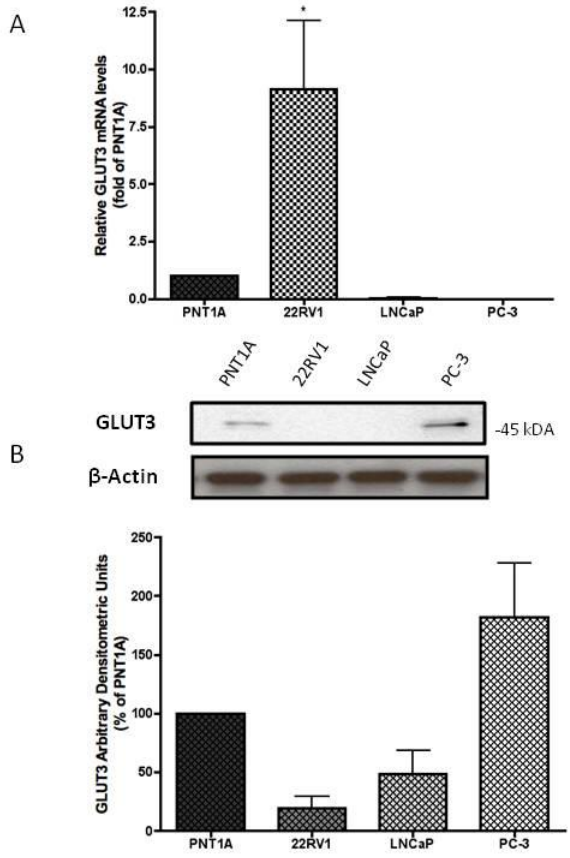

Figure 18. GLUT3 expression in prostate cancer cells. A) Basal GLUT3 mRNA levels were determined by Real Time PCR and are expressed as a fold change of the prostate epithelial cell line PNT1A. B) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT3. A representative immunoblot is shown. The densitometry of the bands expressed in arbitrary units was calculated using Image J software. Protein levels are expressed as a percentage of the PNT1A cell line. Results represent the mean ± SEM of three to four independent experiments. (*$p<0.05$).

3.3 GLUT4 Expression in Cancer Cells

3.3.1 mRNA Expression

GLUT4 mRNA expression is largely restricted to brown and white adipose tissue, skeletal and cardiac muscle[15]. GLUT4 mRNA was detected in the MRC-5 lung fibroblast cell line and to a lower extent in the H460 lung cancer cell line (0.53 ± 0.038, NS) (**Figure 19 A**). A 3-fold increase in GLUT4 mRNA was seen in the SK-MES-1 (3.25 ± 0.44, $p<0.01$), A549 (3.37 ± 0.63, $p<0.05$) and H1299 (3.51 ± 0.45, $p<0.01$) lung cancer cell lines compared to MRC-5 cells.

GLUT4 mRNA was detected in the 184B5 mammary cell line and at similar levels in the MDA-MB-231 breast cancer cell line (0.9 ± 0.24, NS) (**Figure 19 B**). MCF-7 cells showed 1.5-fold higher GLUT4 mRNA levels compared to the 184B5 cell line (1.5 ± 0.56, NS).

The 22Rv1 (7.39 ± 2.46, NS) and LNCaP (31.67 ± 4.83, $p<0.001$) prostate cancer cell lines showed markedly higher GLUT4 mRNA expression compared to the noncancerous PNT1A prostate epithelial cell line (**Figure 19 C**). PC-3 cells displayed low GLUT4 expression at the mRNA level (0.59 ± 0.082, NS).

3.3.2 Protein Expression

There was no detection of the GLUT4 protein using western blotting in both whole cell lysates (**Figure 20 A**) and total membrane fractionations (data not shown) of the lung, breast and prostate cancer cell lines and the corresponding noncancerous epithelial cell lines. GLUT4 protein was detected in a mouse C2C12 myotube sample (**Figure 20A**).

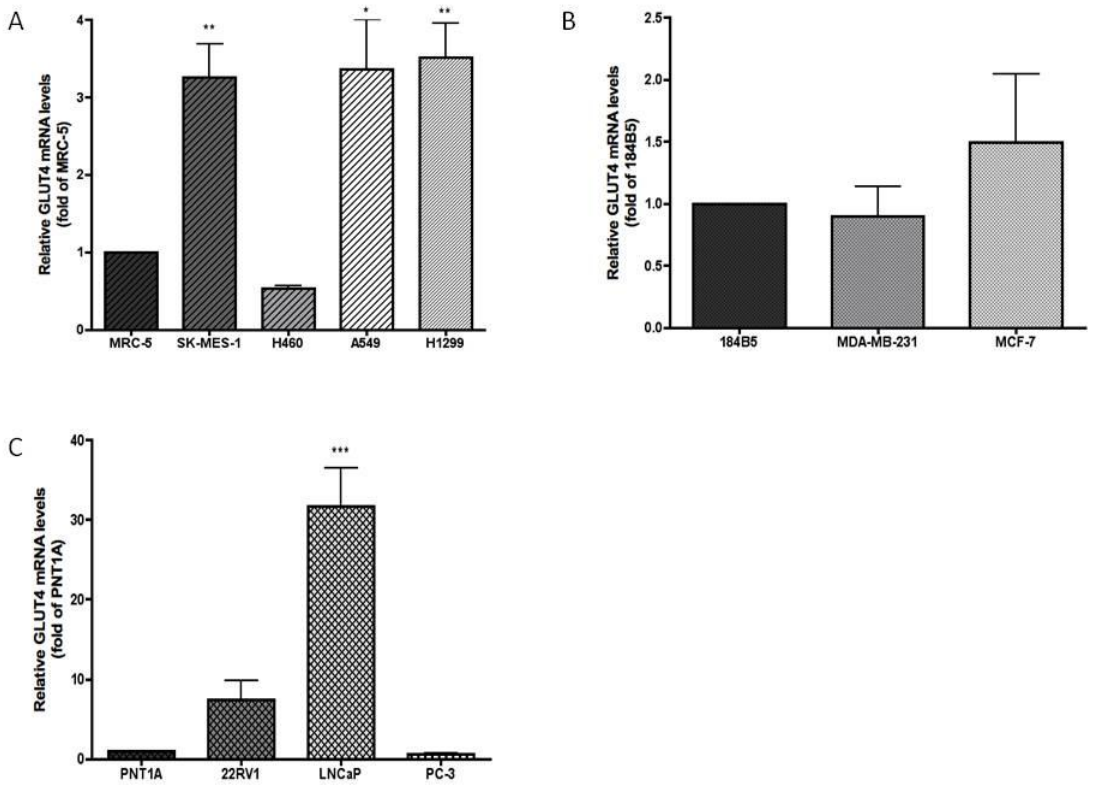

Figure 19. GLUT4 mRNA expression in cancer cells. A) Basal GLUT4 mRNA levels were determined in lung cancer cell lines by Real Time PCR and are expressed as a fold change of the lung fibroblast cell line MRC-5. B) Basal GLUT4 mRNA levels were determined in breast cancer cell lines by Real Time PCR and are expressed as a fold change of the mammary epithelial cell line 184B5. C) Basal GLUT4 mRNA levels were determined in prostate cancer cell lines by Real Time PCR and are expressed as a fold change of the prostate epithelial cell line PNT1A. Results represent the mean ± SEM of three to four independent experiments. (*p<0.05, **p<0.01,***p<0.001).

In order to determine if cancer cell lines expressed GLUT4 at the protein level, immunofluorescence microscopy was employed as another method of protein detection. A549 lung cancer cells and MCF-7 breast cancer cells were found to display cytoplasmic GLUT4 staining (**Figure 20 B-C**). L6 rat skeletal muscle cells were examined as a

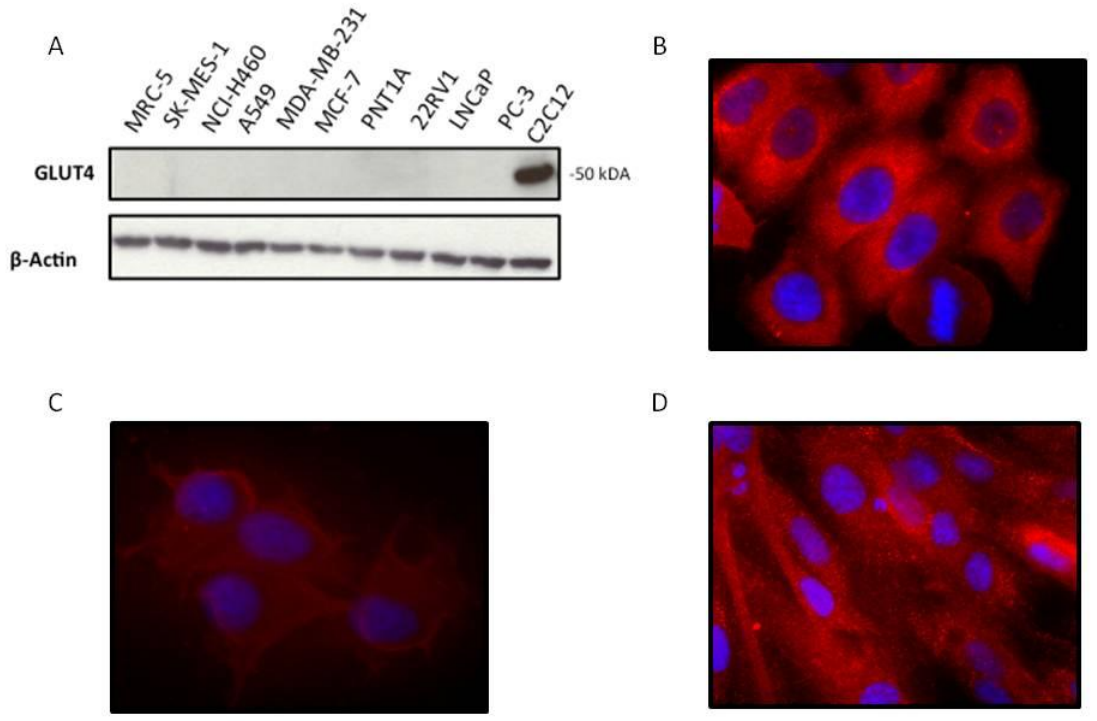

Figure 20. GLUT4 protein expression in cancer cells. A) Whole cell lysates were used in immunoblotting with a specific antibody that recognizes GLUT4. This protein was not detected in the human cancer cell lines, however the antibody recognized GLUT4 in a C2C12 mouse skeletal muscle sample. A representative immunoblot is shown. A549 (B), MCF-7 (C) and L6 rat myotube cells (D) were immunostained with GLUT4 and images were obtained from a widefield fluorescent microscope. A representative section from each sample is shown. Results are representative of two independent experiments.

positive control and found to also display strong cytoplasmic GLUT4 staining (**Figure 20 D**).

3.4 GLUT12 Expression in Cancer Cells

3.4.1 mRNA Expression

GLUT12 mRNA expression is found in insulin-sensitive tissues as well as the small intestine, prostate and placenta and mammary gland[41,42]. The SK-MES-1 lung cancer cell line showed higher levels of GLUT12 mRNA compared to the MRC-5 cell line (3.22 ±

1.26, NS) (**Figure 21 A**). The H460 (0.124 ± 0.05, NS), A549 (0.44 ± 0.13, NS) and H1299 (0.002 ± 0.001, NS) cell lines were found to express low GLUT12 mRNA levels compared to MRC-5 cells.

In comparison to the 184B5 cell line, MDA-MB-231 (3.42 ± 0.26, $p<0.001$) breast cancer cells showed 3-fold higher levels of GLUT12 mRNA expression (**Figure 21 B**). The GLUT12 transporter was first described in MCF-7 breast cancer cells, consistent with these reports GLUT12 mRNA was detected in the MCF-7 (0.07 ± 0.006, $p<0.05$) cell line, however, this was at lower levels than in the 184B5 cell line.

Within the prostate cancer cell lines, 22Rv1 (0.16 ± 0.065, NS) cells expressed lower GLUT12 mRNA levels than the PNT1A epithelial cell line (**Figure 21 C**). Both the LNCaP (8.58 ± 2.88, $p<0.05$) and PC-3 (2.47 ± 0.68, NS) cell lines showed higher GLUT12 mRNA expression at approximately 8- and 2-fold higher than the noncancerous PNT1A cells, respectively.

3.5 GLUT Expression in Xenograft Tumours

In order to determine the *in vivo* expression of GLUT proteins, tumours from Balb/c nude mice xenografted with H1299, A549 and PC-3 human cancer cells were immunohistochemically examined for the presence of GLUT1 and GLUT3. Faint cytoplasmic and discontinuous membrane staining of GLUT1 was observed in the H1299 tumours (**Figure 22 A**). This staining appeared to be stronger in the A549 and PC tumours (**Figure 22 B-C**). GLUT3 staining was detected in all three tumour types (**Figure 23 A-C**). The H1299 and A549 tumours showed signs of both faint cytoplasmic and discontinuous membrane staining (**Figure 23 A-B**) while the GLUT3 protein in the

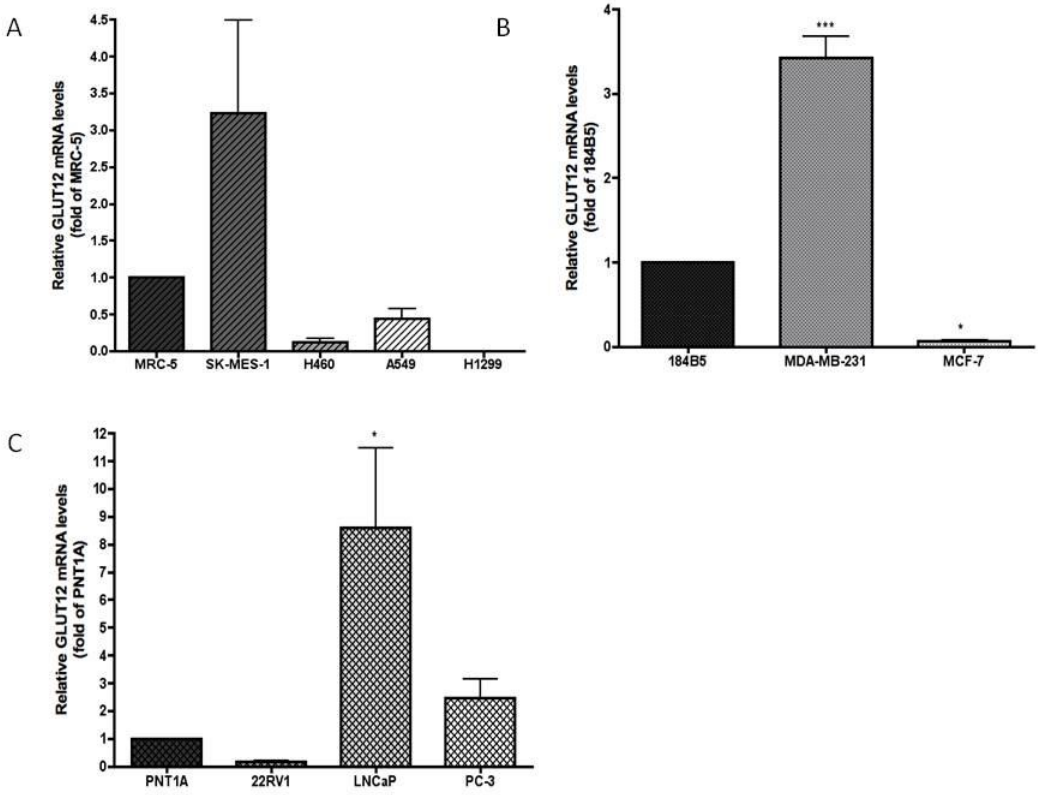

Figure 21. GLUT12 mRNA expression in cancer cells. A) Basal GLUT12 mRNA levels were determined in lung cancer cell lines by Real Time PCR and are expressed as a fold change of the lung fibroblast cell line MRC-5. B) Basal GLUT12 mRNA levels were determined in breast cancer cell lines by Real Time PCR and are expressed as a fold change of the mammary epithelial cell line 184B5. C) Basal GLUT12 mRNA levels were determined in prostate cancer cell lines by Real Time PCR and are expressed as a fold change of the prostate epithelial cell line PNT1A. Results represent the mean ± SEM of three to four independent experiments. (*$p<0.05$,***$p<0.001$).

PC-3 tumours appeared to be mainly cytoplasmic (**Figure 23 C**). There was no signal detected in the slides that were not incubated in primary antibody as a control (**Figures s.3-4**). The specificity of immunohistochemistry staining was confirmed by a clinically trained pathologist.

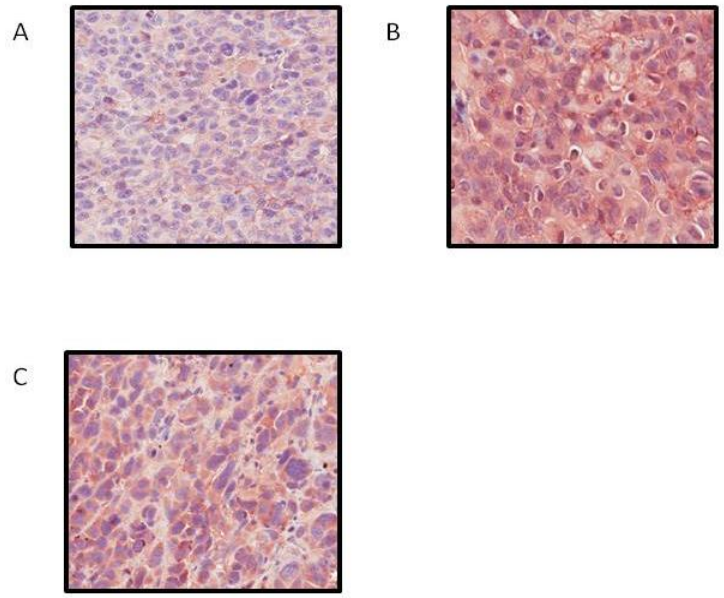

Figure 22. GLUT1 expression in tumours from nude mice xenografted with cancer cell lines. Paraffin-embedded sections of H1299 (A), A549 (B) and PC-3 (C) tumours were incubated with anti-GLUT1 antibody and analyzed by immunohistochemistry . Representative images of three independent experiments are shown. Magnification=18.4X

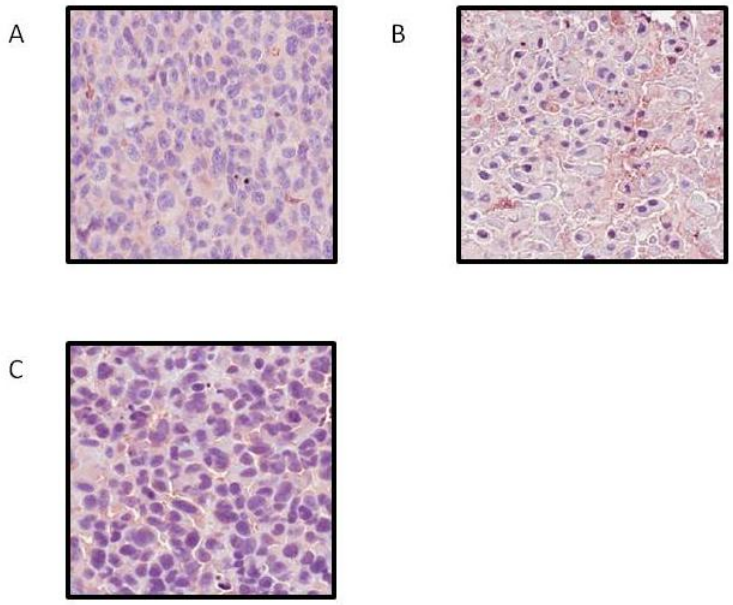

Figure 23. GLUT3 expression in tumours from nude mice xenografted with cancer cell lines. Paraffin-embedded sections of H1299 (A), A549 (B) and PC-3 (C) tumours were incubated with anti-GLUT3 antibody and analyzed by immunohistochemistry. Representative images of three independent experiments are shown. Magnification=18.4X

Table. 7 Summary of GLUT Expression in Cancer Cell Lines by Mutation Status			
Mutation status	**Cell Lines**	**mRNA Expression***	**Protein Expression****
p53 null	**SK-MES-1** (Lung squamous cell carcinoma)	GLUT1 ($p<0.01$) GLUT3 GLUT4 ($p<0.01$) GLUT12	GLUT1 ($p<0.01$) GLUT3
	H1299 (Lung adenocarcinoma)	GLUT1 GLUT3 GLUT4 ($p<0.01$) GLUT12	GLUT1 ($p<0.05$) GLUT3 ($p<0.001$)
	MDA-MB-231 (Breast adenocarcinoma)	GLUT1 GLUT3 GLUT4 GLUT12 ($p<0.001$)	GLUT1 GLUT3
	22Rv1 (Prostate adenocarcinoma)	GLUT1 GLUT3 ($p<0.05$) GLUT4 GLUT12	GLUT1 ($p<0.05$) GLUT3
	PC-3 (Prostate adenocarcinoma)	GLUT1 ($p<0.001$) GLUT3 GLUT4 GLUT12	GLUT1 GLUT3
KRAS mutant	**H460** (Lung large cell carcinoma)	GLUT1 GLUT3 ($p<0.01$) GLUT4 GLUT12	GLUT1 GLUT3 ($p<0.01$)
	A549 (Lung adenocarcinoma)	GLUT1 GLUT3 ($p<0.05$) GLUT4 ($p<0.05$) GLUT12	GLUT1 GLUT4
	MDA-MB-231 (Breast adenocarcinoma)	GLUT1 GLUT3 GLUT4 GLUT12 ($p<0.001$)	GLUT1 GLUT3
PIK3CA mutant	**H460** (Lung large cell carcinoma)	GLUT1 GLUT3 ($p<0.01$) GLUT4 GLUT12	GLUT1 GLUT3 ($p<0.01$)
	MCF-7 (Breast adenocarcinoma)	GLUT1 GLUT3 ($p<0.001$) GLUT4 GLUT12 ($p<0.05$)	GLUT1 GLUT3 GLUT4

PTEN null	**LNCaP** (Prostate adenocarcinoma)	GLUT1 GLUT3 GLUT4 ($p<0.001$) GLUT12 ($p<0.05$)	GLUT1 GLUT3
	PC-3 (Prostate adenocarcinoma)	GLUT1 ($p<0.001$) GLUT3 GLUT4 GLUT12	GLUT1 GLUT3
*If no p value provided, the GLUT mRNA was detected in the cell line but not significantly different than the corresponding noncancerous cell line ** If no p value provided, the GLUT protein was detected in the cell line but not significantly different than the corresponding noncancerous cell line			

CHAPTER 4: DISCUSSION

Given that cancer cells display an increased rate of glucose uptake and consumption compared to healthy cells, it is important to understand the role of glucose transporter proteins in cancer cell metabolism. Although previous studies have suggested that GLUTs are over expressed in cancer, GLUT expression in a number of cell lines, with different mutations and histologies, has not been characterized. The current study revealed a number of new findings regarding GLUT detection in cancer cell lines and confirmed the findings of previously reported GLUT expression.

4.1 GLUT1 Expression in Cancer Cells

In support of previous research that has highlighted an important role of GLUT1 in the increased glucose uptake by cancer cells[16,70,132], GLUT1 was found to be the most highly expressed GLUT at the mRNA level in six out of the nine cancer cell lines studied (**Figures s.9-11**). In comparison to the MRC-5 lung fibroblast line, higher GLUT1 expression was observed at the mRNA and protein level in SK-MES lung cancer cells and at the protein level in the H1299 cell line (**Figure 13**). This is consistent with previous reports of elevated GLUT1 expression in primary lung tumours as compared to normal lung tissue[168-170]. Suzawa et al. (2011) found a higher degree of GLUT1 protein overexpression in squamous cell carcinomas than adenocarinomas[171]. While the SK-MES squamous lung cancer cell line in this study had significantly increased GLUT1 mRNA and protein levels, the A549 adenocarcinoma cell line had comparatively lower expression and the H1299 adenocarcinoma showed significant GLUT1 overexpression only at the protein level. Therefore, some types of lung adenocarcinomas may have a

greater degree of GLUT1 upregulation than others. Indeed, a study by Rastogi et al. also demonstrated higher GLUT1 expression in the H1299 cell line than in A549 cells[164]. Elevated GLUT1 levels are also apparent in large cell lung cancer as the H460 cell line showed increased mRNA and protein levels compared to MRC-5 cells, this is supported by a study showing higher GLUT1 mRNA expression in the H460 cell line as compared to a control cell pool[128]. Therefore, GLUT1 overexpression was detected in all types of lung cancer studied suggesting this transporter may play an important role in providing nutrients to lung cancers.

Despite the lack of GLUT1 detection in breast cancer tumours in previous studies[129-131], both breast cancer cell lines investigated were found to express GLUT1 at the mRNA and protein level (**Figure 15**). The more aggressive estrogen-independent breast cancer cell line MDA-MB-231 was found to express greater GLUT1 mRNA and protein than the estrogen receptor positive cell line MCF-7. This was also noted in the study by Grover-McKay et al. 1998 in which cell surface GLUT1 expression was found to be associated with the invasive ability of the breast cancer cell lines studied[172]. This suggests GLUT1 expression may be useful in identifying more aggressive and invasive types of breast cancer.

The present study provides the first comparison of glucose transporter expression between a noncancerous prostate epithelial cell line and various cancer cell lines. The finding that GLUT1 mRNA expression was elevated in all of the prostate cancer cell lines compared to PNT1A suggests that upregulation of this transporter may be characteristic of cancerous prostate cells (**Figure 16**). These findings are in accordance with previous reports of GLUT1 mRNA detection in LNCaP and PC-3 cells[139,142]. While

studies of malignant prostate tissue have been negative for GLUT1[139-141], here the protein was detected at variable levels in all of the cancer cell lines. Interestingly, GLUT1 expression at the mRNA and protein level was highest in the androgen-insensitive line PC-3 compared to the other cell lines that are better differentiated. This is supported by Effert et al. (2004) who also found higher GLUT1 mRNA expression in the PC-3 cell line than LNCaP cells[142]. In derivatives of LNCaP cells, however, no relationship was found between tumour progression and the level of GLUT1 expression[139]. Therefore, more work is needed to clarify if GLUT1 expression increases with the degree of prostate cancer malignancy.

4.2 GLUT3 Expression in Cancer Cells

Several studies have reported GLUT3 protein expression in lung cancer tumours[140,170,171,173]. Unlike the results of Suzawa et al. (2011) that showed a significantly higher expression of GLUT3 in lung squamous cell carcinomas than adenocarcinomas, in this study the SK-MES cell line was found to have lower mRNA and protein expression of this transporter compared to the adenocarcinoma cell lines (**Figure 17**). Evidence for use of GLUT3 as an indicator of cancer can be seen by the absence of the GLUT3 protein in the noncancerous MRC-5 cell line and its consistent expression in the lung cancer cell lines. The differences in GLUT3 protein between MRC-5 cells and H460 and H1299 cell lines were striking at 21-fold or 70-fold higher, respectively. Interestingly, the H1299 cell line was shown to possess more GLUT3 than GLUT1 mRNA (**Figure s.9**), suggesting an important role of this transporter in the cell line. The limited data available regarding GLUT3 expression in lung cancer cell lines supports the finding of significantly elevated

GLUT3 mRNA in the H460 and A549 cell lines as Cao et al. (2007) reported a 1.5-1.7 fold mRNA increase in H460 cells compared to a control cell pool and increased expression in A549 cells[128].

Fewer studies have examined GLUT3 expression in breast cancer tumours. The reports consist of low levels of expression in these tumours or a failure to detect the protein[136,140,154]. In contrast, this study detected GLUT3 at the protein level in both breast cancer cell lines investigated (**Figure 18 B**). In support of these results, Grover-McKay et al. (1998) also found GLUT3 protein to be present in the MDA-MB-231 and MCF-7 cell lines[172]. Although this group did not observe a correlation between GLUT3 expression and cell line invasiveness, Krzeslak et al. (2012) found a significantly higher expression in poorly differentiated tumours[136]. Protein expression between the MDA-MB-231 and MCF-7 cell lines was found to be similar in this study suggesting potential differences in GLUT3 regulation between breast cancer cells *in vitro* and *in vivo*.

The present study is the first to report mRNA and protein expression of GLUT3 in prostate cancer cell lines. Discrepancies between the mRNA and protein data were apparent, as the 22Rv1 cell line had significantly elevated GLUT3 mRNA and negligible protein expression and the PC-3 cell line showed elevated protein, but not mRNA, expression (**Figure 19**). GLUT3 does not appear to be consistently overexpressed in different types of prostate cancer.

4.3 GLUT4 Expression in Cancer Cells

A growing body of evidence links circulating insulin levels to the development of cancer[174]. In order to better understand the relationship between insulin and cancer

progression, the expression of the insulin-sensitive glucose transporter GLUT4 must be established in various cancers to determine if the metabolism of these cells may include insulin-mediated glucose uptake. Although GLUT4 has been sporadically detected in lung cancer tissue[140,152], previous studies have not determined the expression of this transporter in cancer cell lines. A number of cell lines displayed increased mRNA expression of GLUT4 in this study. In the lung cancer cell lines, both A549 and H1299 adenocarcinoma cell lines and the squamous cell line SK-MES had approximately 3-fold higher expression of GLUT4 (**Figure 20 A**). As well, the 22Rv1 and LNCaP prostate cancer cell lines had 7- and 31-fold increases in GLUT4 mRNA compared to the noncancerous prostate cell line, respectively. The upregulation of this gene in a number of cancer types suggests tissues that are typically not considered insulin-sensitive may express this transporter when they become malignant. The GLUT4 protein was not detected through western blotting in any of the cell lines (**Figure 21 A**), this is likely due to the nature of this technique and the difficulty of the antibody recognizing the linear form of the epitope of the protein. Immunofluorescence microscopy showed cytoplasmic staining of GLUT4 in both A549 and MCF-7 cells indicating that GLUT4 is also present at the protein level in these cells. Although GLUT4 protein expression in the remaining cell lines was not determined, this is an interesting finding and the phenomenon of GLUT4 expression at the mRNA and protein level in tissues that are not known to be insulin sensitive should be further investigated. The role of growth factors in carcinogenesis is well established but the influence of growth factors on GLUT expression and activity in cancer cells is less clear. Whether growth factors influence

proliferation of cancer cells through upregulation and translocation of GLUTs remains to be examined.

4.4 GLUT12 Expression in Cancer Cells

The GLUT12 transporter was first identified and cloned in the MCF-7 breast cancer cell line and has subsequently been shown to be expressed more strongly in breast carcinoma than benign tissue[41,42]. To date, few studies have examined the expression of GLUT12 in cancer tissues and cell lines. The results of the current study reveal increased mRNA expression of GLUT12 in select cancer cell lines, those being SK-MES, MDA-MB-231, LNCaP and PC-3 (**Figure 22**). Consistent with the reports of Rogers et al. (2002) GLUT12 mRNA was detected in the MCF-7 cell line[41]. Given that previous findings have shown GLUT12 protein expression to be increased in response to estrogen, it is interesting that the estrogen receptor negative MDA-MB-231 breast cancer cell line showed a marked increase in GLUT12 mRNA expression. The protein expression of this transporter was not determined in this study due to the lack of a commercially reliable GLUT12 antibody. In the study by Macheda et al. (2005)[16], GLUT12 protein was not detected in the MDA-MB-231 cell line, suggesting that while breast cancer cells show increased mRNA expression of GLUT compared to healthy breast cells, translation of this mRNA may be dependent on sex hormones. This has implications for therapeutic treatment as hormone therapies for cancers may modulate glucose uptake by GLUT12. The androgen sensitive LNCaP cell line had the highest GLUT12 mRNA expression over its corresponding noncancerous cell line with an 8-fold increase over PNT1A. The study by Chandler et al. (2003) also examined GLUT12 expression in this cell line and found it

to be detected at the mRNA and protein level[107]. The protein expression of GLUT12 in these cells lines, and the correlation between mRNA and protein levels, is an important issue to address in future research.

4.5 Discrepancies between GLUT mRNA and Protein Expression

Although the mRNA and protein expression of the glucose transporters were associated in most instances, there are some cell lines in which discrepancies exist. Many studies have found the correlation between mRNA and protein expression to be weak[175, 176]. The most recent large scale study of more than 5,000 mammalian genes by Schwanhausser et al. (2011) suggests that approximately 40% of the variance in protein levels can be explained by mRNA levels[177]. This study found that protein levels are best predicted by translation efficiency rather than transcription rates. Thus, in cell lines that showed abundant mRNA expression of a specific transporter, regulation of translation may have prevented the mRNA from ever being translated into protein resulting in low or absent protein expression. In the case of GLUT3 it is known that in most tissues mRNA is expressed but not translated into protein[178], therefore it is possible this also occurs with other glucose transporters in cancer. In cell lines that show relatively low GLUT mRNA expression with high protein levels (ie. H1299) with respect to GLUT1 and GLUT3 (**Figures 14 and 17**), high translation rates may account for this discrepancy. Schwanhausser's study found that proteins are approximately 900 times more abundant than corresponding transcripts, therefore small changes in mRNA expression may result in significant differences at the protein level.

4.6 The Cell Culture Environment

Although there are a number of advantages to using a cell culture model to determine expression patterns in cancer, there are certain limitations that should be considered when interpreting results. Cells grown in vitro have an unlimited supply of nutrients and oxygen, both of which have been shown to affect glucose transporter expression. Cell culture studies have shown that GLUT1 expression is higher in cells that are deprived of glucose[179], and hypoxia, which frequently occurs in solid tumours, has been shown to upregulate the expression of GLUT1 and GLUT3[85]. Therefore, the degree of GLUT expression in cancer may be underestimated in this study compared to tumours in which inadequate blood supply, and a lack of glucose and oxygen, would be common.

4.7 GLUT Expression *In Vivo*

Despite the potential limitations of using a cell culture model, *in vivo* data supported the findings of the present study regarding GLUT protein expression *in vitro*. The expression of GLUT1 and GLUT3 in tumours from nude mice xenografted with H1299, A549 and PC-3 cancer cells demonstrated that glucose transporter expression in cancer cell lines is not a phenomenon only seen in cell culture. The GLUT1 protein was found to be localized to the cytoplasm and the plasma membrane in the lung and prostate tumours. The discontinuous membrane staining observed is likely due to areas of hypoxia and differing vascularization within the tumour. Future examination of hypoxia and angiogenesis markers in these samples would provide a clearer understanding of glucose transporter expression patterns in tumours. In contrast to the western blotting results of the lung cancer cell lines, the lowest GLUT1 expression appeared in the H1299 tumours, It is possible other glucose transporters play a more prominent role for these cancer cells

in vivo. Indeed, H1299 tumours had roughly equal expression of GLUT1 and GLUT3. This protein was also detected in the cytoplasm and plasma membrane of the cancer cells. A549 and PC-3 tumours appeared to have a lower extent of GLUT3 expression compared to GLUT1, this is supported by the mRNA data for these cell lines (**Figures s.9 D and s.11 D**). Few studies have looked at GLUT expression in xenograft models and based on findings from this research, this may be a useful model for studying GLUT proteins.

4.8 Additional Nutrients Used by Cancer Cells

The GLUT family has an ability to transport substrates other than glucose, therefore, it is possible overexpression of GLUTs in cancer may be due to increased needs for other sugars. For example, GLUT1 transports glucose as well as galactose, mannose, glucosamine and dehydroascorbic acid (**Table 1**). The glucose uptake of the cancer cell lines in this study was not measured, therefore it cannot be determined whether an increase in the expression of specific GLUTs correlates with an increase in glucose uptake. The findings of elevated GLUT expression in cancers that have high FDG-PET accumulation suggests a similar relationship may be seen in cancer cell lines, however this should be addressed in future research.

There is evidence that tumours have increased demands for nutrients other than glucose. Cancer cells appear to take up high levels of essential and non-essential amino acids, specifically the amino acid glutamine[180]. In addition, changes in fatty acid metabolism have been observed in some cancers. It has been demonstrated that fatty acid uptake is dominant over glucose uptake in the prostate cancer cell lines LNCaP and PC-3[181]. Furthermore, FDG-PET has been shown to have limited accuracy in the diagnosis

and staging of prostate cancer, indicating glucose uptake may not be more important for prostate cancer than healthy tissue[182]. In this study, significant differences in GLUT expression were found between the prostate cancer cell lines and the noncancerous PNT1A cell line and high GLUT levels were also seen in a comparison with the lung and breast cancer cell lines (**Figures s.4-7**). Given the limited clinical applicability of FDG-PET in prostate cancer it is unclear why GLUTs are upregulated in prostate cancer cell lines. Whether the expressed glucose transporters may be used to transport other hexoses, or whether they have limited transport activity, remains to be examined.

4.9 Mutation Status and Expression of GLUTs

Given what is known about the potential oncogenic regulation of GLUTs and suppression of these transporter genes by tumour suppressors, it is expected that cell lines with the same mutation status will show similar patterns of GLUT expression. The tumour suppressor p53 has been previously reported to downregulate the expression of GLUT1, GLUT3, GLUT4 and GLUT12[81,90,105]. In the cancer cell lines examined, those harbouring a mutation in p53 (SK-MES, H1299, MDA-MB-231 and PC-3) were shown to overexpress GLUT1 in comparison to the corresponding noncancerous cell line of that tissue.

The regulation of GLUTs appears to be complex, not all cancer cell lines with p53 mutations had upregulation of the same GLUTs and those with elevated mRNA levels did not always show the same pattern at the protein level. This was seen in the 22Rv1 cell line that had significantly increased GLUT3 mRNA levels without a subsequent increase in protein expression. It is possible that while loss of p53 function can relieve the inhibitory effects on GLUT3 transcription, there are other factors influencing whether

translation of this mRNA will occur. Likewise, GLUT12 mRNA expression was seen in two of the p53 mutant cell lines studied, the SK-MES and MDA-MB-231 cell lines, and Macheda et al. (2005) have previously shown that MDA-MB-231 cells do not express the GLUT12 protein[16].

In support of the evidence implicating an inhibitory effect of p53 on the transcriptional activity of GLUT4, significant increases in GLUT4 mRNA expression were seen in the p53 mutant cell lines SK-MES and H1299, and the 22Rv1 cell line showed a 7-fold increase in mRNA expression. In this study the expression of GLUT4 protein in these cell lines was not measured, however, this remains an important issue to address to determine if pharmacological reactivation of p53 may be useful for preventing the growth of these cancer cells.

Both mutations in the *PIK3CA* gene and the tumour suppressor PTEN lead to the kinase Akt being constitutively activated. Akt has been shown to increase expression of both GLUT1 and GLUT3 and translocation of GLUT4[46-48]. In the H460 cell line, which harbours a PIK3CA mutation, GLUT1 and GLUT3 were increased at both the mRNA and protein level. In comparison, the MCF-7 cell line showed elevated GLUT1 mRNA and GLUT3 protein. Similar effects on the expression of GLUT1 and GLUT3 would therefore be expected in the PTEN mutant cell lines LNCaP and PC-3. Interestingly, these cell lines both show increased GLUT12 mRNA levels and GLUT4 mRNA expression was significantly elevated in the LNCaP line. Whether GLUT12 and GLUT4 gene expression is also suppressed by PTEN is worthy of investigating, it is possible that tumour suppressors play an important role in down-regulating GLUT expression in healthy tissue.

Additionally, cancer cells with K-ras mutations have been shown to have elevated glucose uptake and upregulation of GLUT1 mRNA[80]. Of the three Kras mutant cell lines studied, increased GLUT1 mRNA and protein levels were observed in the H460 and MDA-MB-231 cell lines. The breast cancer MDA-MB-231 cell line had the highest GLUT1 mRNA expression of all cancer cell lines studied (**Figure s.5**) suggesting that the presence of both p53 and K-ras mutations in this cell line may lead to substantial upregulation of this transporter. An increase in GLUT3 mRNA was seen in the K-ras mutant lung cancer cell lines A549 and H460, with a significant increase in GLUT3 protein in the H460 cell line. The potential regulation of GLUT3 by K-ras mutations in cancer should be further explored.

Another mutation that may be relevant to the GLUT expression observed in cancer cells studied is loss of the tumor suppressor LKB1. This protein was originally identified as a tumor suppressor in humans because it is mutated in Peutz-Jeghers syndrome (PJS). Subjects with PJS develop benign tumors in the gastrointestinal tract and have an increased risk of developing malignant tumors at other sites[91]. The LKB1 protein is the major upstream kinase responsible for regulating AMPK[91]. Given that AMPK is involved in regulation of the transcription and localization of several GLUTs, loss of LKB1 may lead to deregulation of GLUT expression in cancer. The lung cancer cell lines A549 and H460 lack functional LKB1 and show high levels of GLUT3 at the mRNA and protein level. Although AMPK has been demonstrated to cause translocation of GLUT3 to the plasma membrane and increase its activity[92,93], its role in regulating this transporter in lung cancer cells is unclear. Whether the elevated expression of GLUT3 in these cell

lines is due to decreased AMPK activation because of the loss of LKB1 remains to be examined.

The regulation of multiple GLUTs by the same mutations and pathways may explain the redundancy that is seen in some cancer cell lines where high expression levels of more than one GLUT is seen. Studies determining whether all of the glucose transporters expressed in the cancer cell line are active in taking up glucose, and if this happens simultaneously or if some GLUTs are preferred under certain conditions, will aid in our understanding of the potential role of GLUT proteins in cancer growth and survival.

4.10 Significance of the Present Study

Previous research has indicated that expression of certain GLUTs may be upregulated in cancer. While the majority of these studies have used tumour models, the present study examined GLUT expression *in vitro* using a number of cancer cell lines with different mutations and histologies. Additionally, this study included the comparison of a noncancerous cell line of the corresponding tissue type in order to determine GLUT expression patterns that were specific to cancer cell lines.

The findings of this study can serve as a reference for authors wishing to select an appropriate *in vitro* model to study GLUT1, GLUT3, GLUT4 or GLUT12. It also provides evidence that GLUTs are expressed in the cell lines studied both *in vitro* and *in vivo* as expression of GLUT1 and GLUT3 were detected in xenografts grown in immunodeficient mice. The results presented provide a baseline for further investigation of GLUTs as biomarkers of cancer progression and potential targets for therapeutics.

4.11 Future Directions

Recent attempts to inhibit GLUT-mediated glucose uptake in cancer cells have focused on GLUT1. The present study suggests that GLUT3, GLUT4 and GLUT12 may also serve as potential candidates for targeting in cancer. Once the expression of the glucose transporters within a specific cancer/tumour has been established, future research should focus on determining the activity of these transporters and whether they play a functional role in providing energy and nutrients to the cancer cell. This will provide stronger evidence to target GLUTs for anti-cancer therapy than expression patterns alone.

Additional studies are required to better understand the complex regulation of GLUTs and to explain the discrepancies observed in this study between mRNA and protein expression of some GLUTs. The relationship between tumour suppressor genes and the regulation of GLUTs should be explored in detail to determine if p53 inhibits aerobic glycolysis by downregulating the expression of multiple GLUTs and if PTEN may play a similar role.

The potential modulation of GLUT expression by current cancer therapeutics has not been studied and should be investigated to better understand how these therapies affect cancer cell metabolism. Using agents that decrease GLUT translocation and/or expression while also acting on pathways to inhibit proliferation may represent attractive strategies for the treatment of cancer. Furthermore, GLUTs could serve as biomarkers of tumour response to these agents.

Expression patterns of additional members of the GLUT family should be examined in cancer cell lines and tumour samples. Potential GLUTs of interest may include the fructose transporters GLUT2, GLUT5, GLUT7 and GLUT11 since increased rates of

fructose uptake have been observed in some cancers[183,184] and GLUT8 as this transporter has been suggested to be important during development[185] and has recently been described in multiple myeloma and endometrial cancers[186,187].

CHAPTER 5: CONCLUSIONS

1) The present study examined the expression of the glucose transporters GLUT1, GLUT3, GLUT4 and GLUT12 at the mRNA level in lung, breast and prostate cancer cell lines as well as the corresponding non-cancerous cell lines of that tissue. SK-MES-1 lung cancer cells and PC-3 prostate cancer cells were found to have higher GLUT1 mRNA expression than non-cancerous cells of that tissue. H460 and A549 lung cancer cells, and 22Rv1 prostate cancer cells had high levels of GLUT3 mRNA, while in MCF-7 breast cancer cells the levels were lower than that found in non-cancerous cells. High GLUT4 mRNA expression was found in SK-MES-1, A549 and H1299 lung cancer, and LNCaP prostate cancer cells. The MDA-MB-231 breast and LNCaP prostate cancer cell lines were found to have high levels of GLUT12 mRNA compared to non-cancerous cells.
2) Next, the present examined the protein expression of GLUT1, GLUT3, GLUT4 and GLUT12 in the aforementioned cancer cell lines. SK-MES-1 and H1299 lung cancer cells were found to have higher GLUT1 protein expression than non-cancerous lung fibroblast cells, while 22Rv1 prostate cancer cells expressed GLUT1 at lower levels than non-cancerous prostate epithelial cells. The lung cancer cells H460 and H1299, and the breast cancer cells MDA-MB-231 and MCF-7 had higher GLUT3 protein expression compared to the lung fibroblast cell line MRC-5. GLUT4 protein was not recognized in human cancer cell lines using western blotting, however, when selecting the cancer cell lines A549 and MCF-7 for use in immunofluorescence microscopy,

GLUT4 protein was detected in the cytoplasm of these cells. GLUT12 protein expression was not examined due to the unavailability of a specific antibody.

3) In order to determine the *in vivo* expression of GLUTs in cancer cells, expression of GLUT1 and GLUT3 was examined in tumours from nude mice xenografted with cancer cell lines. In agreement with the expression of GLUT1 and GLUT3 protein observed in tissue culture models of H1299, A549 and PC-3 cells, both transporters were also detected in tumour xenografts of these cells lines.

In summary, the present study revealed new findings of GLUT detection in cancer cells and describes basal GLUT expression patterns in a number of cancer cells with different mutations and histologies. The study of GLUT protein expression in cancer is important for understanding cancer metabolism and may lead to identification of biomarkers of cancer progression and development of target therapies.

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011;144(5):646-674.

2. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer 2011;11(2):85-95.

3. Rich PR. The molecular machinery of keilin's respiratory chain. Biochem Soc Trans 2003;31(Pt 6):1095-1105.

4. Warburg O. The metabolism of tumours. 1930. London: Constable and Company.

5. Schwartz MK. Enzymes as prognostic markers and therapeutic indicators in patients with cancer. Clin Chim Acta 1992;206(1-2):77-82.

6. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 2006;9(6):425-434.

7. Bonnet S, Archer SL, Allalunis-Turner J et al. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell 2007;11(1):37-51.

8. Weinberg F, Hamanaka R, Wheaton WW et al. Mitochondrial metabolism and ROS generation are essential for kras-mediated tumorigenicity. Proc Natl Acad Sci U S A 2010;107(19):8788-8793.

9. Fogal V, Richardson AD, Karmali PP et al. Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. Mol Cell Biol 2010;30(6):1303-1318.

10. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: The metabolic requirements of cell proliferation. Science 2009;324(5930):1029-1033.

11. Stern R, Shuster S, Neudecker BA, Formby B. Lactate stimulates fibroblast expression of hyaluronan and CD44: The warburg effect revisited. Exp Cell Res 2002;276(1):24-31.

12. Baumann F, Leukel P, Doerfelt A et al. Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. Neuro Oncol 2009;11(4):368-380.

13. Wilson-O'Brien AL, Patron N, Rogers S. Evolutionary ancestry and novel functions of the mammalian glucose transporter (GLUT) family. BMC Evol Biol 2010;10:152.

14. Wright EM, Loo DD, Hirayama BA. Biology of human sodium glucose transporters. Physiol Rev 2011;91(2):733-794.

15. Uldry M, Thorens B. The SLC2 family of facilitated hexose and polyol transporters. Pflugers Arch 2004;447(5):480-489.

16. Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 2005;202(3):654-662.

17. Olsowski A, Monden I, Krause G, Keller K. Cysteine scanning mutagenesis of helices 2 and 7 in GLUT1 identifies an exofacial cleft in both transmembrane segments. Biochemistry 2000;39(10):2469-2474.

18. Schurmann A, Doege H, Ohnimus H et al. Role of conserved arginine and glutamate residues on the cytosolic surface of glucose transporters for transporter function. Biochemistry 1997;36(42):12897-12902.

19. Sato M, Mueckler M. A conserved amino acid motif (R-X-G-R-R) in the Glut1 glucose transporter is an important determinant of membrane topology. J Biol Chem 1999;274(35):24721-24725.

20. Garcia JC, Strube M, Leingang K et al. Amino acid substitutions at tryptophan 388 and tryptophan 412 of the HepG2 (Glut1) glucose transporter inhibit transport activity and targeting to the plasma membrane in xenopus oocytes. J Biol Chem 1992;267(11):7770-7776.

21. Schurmann A, Keller K, Monden I et al. Glucose transport activity and photolabelling with 3-[125I]iodo-4-azidophenethylamido-7-O-succinyldeacetyl (IAPS)-forskolin of two mutants at tryptophan-388 and -412 of the glucose transporter GLUT1: Dissociation of the binding domains of forskolin and glucose. Biochem J 1993;290 (Pt 2)(Pt 2):497-501.

22. Mori H, Hashiramoto M, Clark AE et al. Substitution of tyrosine 293 of GLUT1 locks the transporter into an outward facing conformation. J Biol Chem 1994;269(15):11578-11583.

23. Wandel S, Schurmann A, Becker W et al. Substitution of conserved tyrosine residues in helix 4 (Y143) and 7 (Y293) affects the activity, but not IAPS-forskolin binding, of the glucose transporter GLUT4. FEBS Lett 1994;348(2):114-118.

24. Joost HG, Thorens B. The extended GLUT-family of sugar/polyol transport facilitators: Nomenclature, sequence characteristics, and potential function of its novel members (review). Mol Membr Biol 2001;18(4):247-256.

25. Asano T, Takata K, Katagiri H et al. The role of N-glycosylation in the targeting and stability of GLUT1 glucose transporter. FEBS Lett 1993;324(3):258-261.

26. Augustin R. The protein family of glucose transport facilitators: It's not only about glucose after all. IUBMB Life 2010;62(5):315-333.

27. Mueckler M, Weng W, Kruse M. Glutamine 161 of Glut1 glucose transporter is critical for transport activity and exofacial ligand binding. J Biol Chem 1994;269(32):20533-20538.

28. Doege H, Schurmann A, Ohnimus H et al. Serine-294 and threonine-295 in the exofacial loop domain between helices 7 and 8 of glucose transporters (GLUT) are involved in the conformational alterations during the transport process. Biochem J 1998;329 (Pt 2)(Pt 2):289-293.

29. Calvo MB, Figueroa A, Pulido EG et al. Potential role of sugar transporters in cancer and their relationship with anticancer therapy. Int J Endocrinol 2010;2010:205357. Epub 2010 Jul 18.

30. Zhao FQ, Keating AF. Functional properties and genomics of glucose transporters. Curr Genomics 2007;8(2):113-128.

31. Thorens B, Mueckler M. Glucose transporters in the 21st century. Am J Physiol Endocrinol Metab 2010;298(2):E141-5.

32. Hebert DN, Carruthers A. Glucose transporter oligomeric structure determines transporter function. reversible redox-dependent interconversions of tetrameric and dimeric GLUT1. J Biol Chem 1992;267(33):23829-23838.

33. Uldry M, Ibberson M, Hosokawa M, Thorens B. GLUT2 is a high affinity glucosamine transporter. FEBS Lett 2002;524(1-3):199-203.

34. Bell GI, Burant CF, Takeda J, Gould GW. Structure and function of mammalian facilitative sugar transporters. J Biol Chem 1993;268(26):19161-19164.

35. Rumsey SC, Kwon O, Xu GW et al. Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. J Biol Chem 1997;272(30):18982-18989.

36. Takata K, Kasahara T, Kasahara M et al. Erythrocyte/HepG2-type glucose transporter is concentrated in cells of blood-tissue barriers. Biochem Biophys Res Commun 1990;173(1):67-73.

37. Zhao FQ, Glimm DR, Kennelly JJ. Distribution of mammalian facilitative glucose transporter messenger RNA in bovine tissues. Int J Biochem 1993;25(12):1897-1903.

38. Maher F, Davies-Hill TM, Simpson IA. Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons. Biochem J 1996;315 (Pt 3)(Pt 3):827-831.

39. Haber RS, Weinstein SP, O'Boyle E, Morgello S. Tissue distribution of the human GLUT3 glucose transporter. Endocrinology 1993;132(6):2538-2543.

40. Rumsey SC, Daruwala R, Al-Hasani H et al. Dehydroascorbic acid transport by GLUT4 in xenopus oocytes and isolated rat adipocytes. J Biol Chem 2000;275(36):28246-28253.

41. Rogers S, Macheda ML, Docherty SE et al. Identification of a novel glucose transporter-like protein-GLUT-12. Am J Physiol Endocrinol Metab 2002;282(3):E733-8.

42. Rogers S, Docherty SE, Slavin JL et al. Differential expression of GLUT12 in breast cancer and normal breast tissue. Cancer Lett 2003;193(2):225-233.

43. Wong KK, Engelman JA, Cantley LC. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev 2010;20(1):87-90.

44. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 2009;8(8):627-644.

45. Samuels Y, Wang Z, Bardelli A et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004;304(5670):554.

46. Hajduch E, Alessi DR, Hemmings BA, Hundal HS. Constitutive activation of protein kinase B alpha by membrane targeting promotes glucose and system A amino acid transport, protein synthesis, and inactivation of glycogen synthase kinase 3 in L6 muscle cells. Diabetes 1998;47(7):1006-1013.

47. Barthel A, Okino ST, Liao J et al. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. J Biol Chem 1999;274(29):20281-20286.

48. Wang Q, Somwar R, Bilan PJ et al. Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. Mol Cell Biol 1999;19(6):4008-4018.

49. Gottlob K, Majewski N, Kennedy S et al. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev 2001;15(11):1406-1418.

50. Deprez J, Vertommen D, Alessi DR et al. Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J Biol Chem 1997;272(28):17269-17275.

51. Manning BD, Cantley LC. AKT/PKB signaling: Navigating downstream. Cell 2007;129(7):1261-1274.

52. Majumder PK, Febbo PG, Bikoff R et al. mTOR inhibition reverses akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med 2004;10(6):594-601.

53. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell Metab 2008;7(1):11-20.

54. Iyer NV, Kotch LE, Agani F et al. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;12(2):149-162.

55. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3(10):721-732.

56. Obach M, Navarro-Sabate A, Caro J et al. 6-phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. J Biol Chem 2004;279(51):53562-53570.

57. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metab 2006;3(3):177-185.

58. Karnoub AE, Weinberg RA. Ras oncogenes: Split personalities. Nat Rev Mol Cell Biol 2008;9(7):517-531.

59. Castellano E, Downward J. RAS interaction with PI3K: More than just another effector pathway. Genes Cancer 2011;2(3):261-274.

60. Chiaradonna F, Sacco E, Manzoni R et al. Ras-dependent carbon metabolism and transformation in mouse fibroblasts. Oncogene 2006;25(39):5391-5404.

61. Dang CV. Rethinking the warburg effect with myc micromanaging glutamine metabolism. Cancer Res 2010;70(3):859-862.

62. Osthus RC, Shim H, Kim S et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-myc. J Biol Chem 2000;275(29):21797-21800.

63. Shim H, Dolde C, Lewis BC et al. c-myc transactivation of LDH-A: Implications for tumor metabolism and growth. Proc Natl Acad Sci U S A 1997;94(13):6658-6663.

64. Laptenko O, Prives C. Transcriptional regulation by p53: One protein, many possibilities. Cell Death Differ 2006;13(6):951-961.

65. Mathupala SP, Heese C, Pedersen PL. Glucose catabolism in cancer cells. the type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53. J Biol Chem 1997;272(36):22776-22780.

66. Kawamura M, Yamashita T, Segawa K et al. The 273rd codon mutants of p53 show growth modulation activities not correlated with p53-specific transactivation activity. Oncogene 1996;12(11):2361-2367.

67. Bensaad K, Tsuruta A, Selak MA et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 2006;126(1):107-120.

68. Stambolic V, MacPherson D, Sas D et al. Regulation of PTEN transcription by p53. Mol Cell 2001;8(2):317-325.

69. Matoba S, Kang JG, Patino WD et al. P53 regulates mitochondrial respiration. Science 2006;312(5780):1650-1653.

70. Ganapathy V, Thangaraju M, Prasad PD. Nutrient transporters in cancer: Relevance to warburg hypothesis and beyond. Pharmacol Ther 2009;121(1):29-40.

71. Younes M, Brown RW, Stephenson M et al. Overexpression of Glut1 and Glut3 in stage I nonsmall cell lung carcinoma is associated with poor survival. Cancer 1997;80(6):1046-1051.

72. Sommermann TG, O'Neill K, Plas DR, Cahir-McFarland E. IKKbeta and NF-kappaB transcription govern lymphoma cell survival through AKT-induced plasma membrane trafficking of GLUT1. Cancer Res 2011;71(23):7291-7300.

73. Wieman HL, Wofford JA, Rathmell JC. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Mol Biol Cell 2007;18(4):1437-1446.

74. Wofford JA, Wieman HL, Jacobs SR et al. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of akt to support T-cell survival. Blood 2008;111(4):2101-2111.

75. Taha C, Liu Z, Jin J et al. Opposite translational control of GLUT1 and GLUT4 glucose transporter mRNAs in response to insulin. role of mammalian target of rapamycin, protein kinase b, and phosphatidylinositol 3-kinase in GLUT1 mRNA translation. J Biol Chem 1999;274(46):33085-33091.

76. Taha C, Tsakiridis T, McCall A, Klip A. Glucose transporter expression in L6 muscle cells: Regulation through insulin- and stress-activated pathways. Am J Physiol 1997;273(1 Pt 1):E68-76.

77. Rivenzon-Segal D, Boldin-Adamsky S, Seger D et al. Glycolysis and glucose transporter 1 as markers of response to hormonal therapy in breast cancer. Int J Cancer 2003;107(2):177-182.

78. Kozlovsky N, Rudich A, Potashnik R et al. Transcriptional activation of the Glut1 gene in response to oxidative stress in L6 myotubes. J Biol Chem 1997;272(52):33367-33372.

79. Hayashi M, Sakata M, Takeda T et al. Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells. J Endocrinol 2004;183(1):145-154.

80. Yun J, Rago C, Cheong I et al. Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. Science 2009;325(5947):1555-1559.

81. Schwartzenberg-Bar-Yoseph F, Armoni M, Karnieli E. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res 2004;64(7):2627-2633.

82. Piper RC, Hess LJ, James DE. Differential sorting of two glucose transporters expressed in insulin-sensitive cells. Am J Physiol 1991;260(3 Pt 1):C570-80.

83. Bilan PJ, Mitsumoto Y, Ramlal T, Klip A. Acute and long-term effects of insulin-like growth factor I on glucose transporters in muscle cells. translocation and biosynthesis. FEBS Lett 1992;298(2-3):285-290.

84. Wilson CM, Mitsumoto Y, Maher F, Klip A. Regulation of cell surface GLUT1, GLUT3, and GLUT4 by insulin and IGF-I in L6 myotubes. FEBS Lett 1995;368(1):19-22.

85. Zelzer E, Levy Y, Kahana C et al. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J 1998;17(17):5085-5094.

86. Badr GA, Zhang JZ, Tang J et al. Glut1 and glut3 expression, but not capillary density, is increased by cobalt chloride in rat cerebrum and retina. Brain Res Mol Brain Res 1999;64(1):24-33.

87. Vannucci SJ, Reinhart R, Maher F et al. Alterations in GLUT1 and GLUT3 glucose transporter gene expression following unilateral hypoxia-ischemia in the immature rat brain. Brain Res Dev Brain Res 1998;107(2):255-264.

88. Baumann MU, Zamudio S, Illsley NP. Hypoxic upregulation of glucose transporters in BeWo choriocarcinoma cells is mediated by hypoxia-inducible factor-1. Am J Physiol Cell Physiol 2007;293(1):C477-85.

89. Rajakumar A, Thamotharan S, Raychaudhuri N et al. Trans-activators regulating neuronal glucose transporter isoform-3 gene expression in mammalian neurons. J Biol Chem 2004;279(25):26768-26779.

90. Kawauchi K, Araki K, Tobiume K, Tanaka N. p53 regulates glucose metabolism through an IKK-NF-kappaB pathway and inhibits cell transformation. Nat Cell Biol 2008;10(5):611-618.

91. Hardie DG. AMP-activated protein kinase: A cellular energy sensor with a key role in metabolic disorders and in cancer. Biochem Soc Trans 2011;39(1):1-13.

92. Weisova P, Concannon CG, Devocelle M et al. Regulation of glucose transporter 3 surface expression by the AMP-activated protein kinase mediates tolerance to glutamate excitation in neurons. J Neurosci 2009;29(9):2997-3008.

93. Cidad P, Almeida A, Bolanos JP. Inhibition of mitochondrial respiration by nitric oxide rapidly stimulates cytoprotective GLUT3-mediated glucose uptake through 5'-AMP-activated protein kinase. Biochem J 2004;384(Pt 3):629-636.

94. Liu ML, Olson AL, Moye-Rowley WS et al. Expression and regulation of the human GLUT4/muscle-fat facilitative glucose transporter gene in transgenic mice. J Biol Chem 1992;267(17):11673-11676.

95. Oshel KM, Knight JB, Cao KT et al. Identification of a 30-base pair regulatory element and novel DNA binding protein that regulates the human GLUT4 promoter in transgenic mice. J Biol Chem 2000;275(31):23666-23673.

96. Knight JB, Eyster CA, Griesel BA, Olson AL. Regulation of the human GLUT4 gene promoter: Interaction between a transcriptional activator and myocyte enhancer factor 2A. Proc Natl Acad Sci U S A 2003;100(25):14725-14730.

97. Garvey WT, Maianu L, Hueckstead TP et al. Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. J Clin Invest 1991;87(3):1072-1081.

98. Sinha MK, Raineri-Maldonado C, Buchanan C et al. Adipose tissue glucose transporters in NIDDM. decreased levels of muscle/fat isoform. Diabetes 1991;40(4):472-477.

99. Ren JM, Semenkovich CF, Gulve EA et al. Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle. J Biol Chem 1994;269(20):14396-14401.

100. Kraniou Y, Cameron-Smith D, Misso M et al. Effects of exercise on GLUT-4 and glycogenin gene expression in human skeletal muscle. J Appl Physiol 2000;88(2):794-796.

101. Zheng D, MacLean PS, Pohnert SC et al. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 2001;91(3):1073-1083.

102. Eguez L, Lee A, Chavez JA et al. Full intracellular retention of GLUT4 requires AS160 rab GTPase activating protein. Cell Metab 2005;2(4):263-272.

103. Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273(6 Pt 1):E1107-12.

104. Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 1999;48(8):1667-1671.

105. Zawacka-Pankau J, Grinkevich VV, Hunten S et al. Inhibition of glycolytic enzymes mediated by pharmacologically activated p53: Targeting warburg effect to fight cancer. J Biol Chem 2011;286(48):41600-41615.

106. Flessner LB, Moley KH. Similar [DE]XXXL[LI] motifs differentially target GLUT8 and GLUT12 in chinese hamster ovary cells. Traffic 2009;10(3):324-333.

107. Chandler JD, Williams ED, Slavin JL et al. Expression and localization of GLUT1 and GLUT12 in prostate carcinoma. Cancer 2003;97(8):2035-2042.

108. Stuart CA, Howell ME, Zhang Y, Yin D. Insulin-stimulated translocation of glucose transporter (GLUT) 12 parallels that of GLUT4 in normal muscle. J Clin Endocrinol Metab 2009;94(9):3535-3542.

109. Wilson-O'Brien AL, Dehaan CL, Rogers S. Mitogen-stimulated and rapamycin-sensitive glucose transporter 12 targeting and functional glucose transport in renal epithelial cells. Endocrinology 2008;149(3):917-924.

110. Butler M. Mammalian cell biotechnology: A practical approach. USA: Oxford University Press, 1991.

111. Primrose SB. Molecular biotechnology. Oxford, United Kingdom: Blackwell Scientific Publications, 1991.

112. Jacobs JP, Jones CM, Baille JP. Characteristics of a human diploid cell designated MRC-5. Nature 1970;227(5254):168-170.

113. Fough J. Human tumor cells in vitro. New York:Plenum Press, 1975.

114. Banks-Schlegel SP, Gazdar AF, Harris CC. Intermediate filament and cross-linked envelope expression in human lung tumor cell lines. Cancer Res 1985;45(3):1187-1197.

115. Giard DJ, Aaronson SA, Todaro GJ et al. In vitro cultivation of human tumors: Establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973;51(5):1417-1423.

116. Phelps RM, Johnson BE, Ihde DC et al. NCI-navy medical oncology branch cell line data base. J Cell Biochem Suppl 1996;24:32-91.

117. Stampfer MR, Bartley JC. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci U S A 1985;82(8):2394-2398.

118. Cailleau R, Young R, Olive M, Reeves WJ,Jr. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 1974;53(3):661-674.

119. Soule HD, Vazguez J, Long A et al. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973;51(5):1409-1416.

120. Cussenot O, Berthon P, Berger R et al. Immortalization of human adult normal prostatic epithelial cells by liposomes containing large T-SV40 gene. J Urol 1991;146(3):881-886.

121. Sramkoski RM, Pretlow TG,2nd, Giaconia JM et al. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell Dev Biol Anim 1999;35(7):403-409.

122. Murphy GP, ed. Models for prostate cancer. New York:Liss, 1980.

123. Kaighn ME, Narayan KS, Ohnuki Y et al. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 1979;17(1):16-23.

124. Ogawa J, Inoue H, Koide S. Glucose-transporter-type-I-gene amplification correlates with sialyl-lewis-X synthesis and proliferation in lung cancer. Int J Cancer 1997;74(2):189-192.

125. Kurata T, Oguri T, Isobe T et al. Differential expression of facilitative glucose transporter (GLUT) genes in primary lung cancers and their liver metastases. Jpn J Cancer Res 1999;90(11):1238-1243.

126. Rastogi S, Banerjee S, Chellappan S, Simon GR. Glut-1 antibodies induce growth arrest and apoptosis in human cancer cell lines. Cancer Lett 2007;257(2):244-251.

127. Ito S, Fukusato T, Nemoto T et al. Coexpression of glucose transporter 1 and matrix metalloproteinase-2 in human cancers. J Natl Cancer Inst 2002;94(14):1080-1091.

128. Cao X, Fang L, Gibbs S et al. Glucose uptake inhibitor sensitizes cancer cells to daunorubicin and overcomes drug resistance in hypoxia. Cancer Chemother Pharmacol 2007;59(4):495-505.

129. Younes M, Brown RW, Mody DR et al. GLUT1 expression in human breast carcinoma: Correlation with known prognostic markers. Anticancer Res 1995;15(6B):2895-2898.

130. Kang SS, Chun YK, Hur MH et al. Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. Jpn J Cancer Res 2002;93(10):1123-1128.

131. Ravazoula P, Batistatou A, Aletra C et al. Immunohistochemical expression of glucose transporter Glut1 and cyclin D1 in breast carcinomas with negative lymph nodes. Eur J Gynaecol Oncol 2003;24(6):544-546.

132. Godoy A, Ulloa V, Rodriguez F et al. Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: Ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. J Cell Physiol 2006;207(3):614-627.

133. Alo PL, Visca P, Botti C et al. Immunohistochemical expression of human erythrocyte glucose transporter and fatty acid synthase in infiltrating breast carcinomas and adjacent typical/atypical hyperplastic or normal breast tissue. Am J Clin Pathol 2001;116(1):129-134.

134. Brown RS, Wahl RL. Overexpression of glut-1 glucose transporter in human breast cancer. an immunohistochemical study. Cancer 1993;72(10):2979-2985.

135. Zamora-Leon SP, Golde DW, Concha II et al. Expression of the fructose transporter GLUT5 in human breast cancer. Proc Natl Acad Sci U S A 1996;93(5):1847-1852.

136. Krzeslak A, Wojcik-Krowiranda K, Forma E et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. Pathol Oncol Res 2012.

137. Grover-McKay M, Walsh SA, Seftor EA et al. Role for glucose transporter 1 protein in human breast cancer. Pathol Oncol Res 1998;4(2):115-120.

138. Chan KK, Chan JY, Chung KK, Fung KP. Inhibition of cell proliferation in human breast tumor cells by antisense oligonucleotides against facilitative glucose transporter 5. J Cell Biochem 2004;93(6):1134-1142.

139. Chandler JD, Williams ED, Slavin JL et al. Expression and localization of GLUT1 and GLUT12 in prostate carcinoma. Cancer 2003;97(8):2035-2042.

140. Godoy A, Ulloa V, Rodriguez F et al. Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: Ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues. J Cell Physiol 2006;207(3):614-627.

141. Reinicke K, Sotomayor P, Cisterna P et al. Cellular distribution of glut-1 and glut-5 in benign and malignant human prostate tissue. J Cell Biochem 2012;113(2):553-562.

142. Effert P, Beniers AJ, Tamimi Y et al. Expression of glucose transporter 1 (glut-1) in cell lines and clinical specimens from human prostate adenocarcinoma. Anticancer Res 2004;24(5A):3057-3063.

143. Hatanaka M. Transport of sugars in tumor cell membranes. Biochim Biophys Acta 1974;355(1):77-104.

144. Isselbacher KJ. Sugar and amino acid transport by cells in culture--differences between normal and malignant cells. N Engl J Med 1972;286(17):929-933.

145. Khan MA, Combs CS, Brunt EM et al. Positron emission tomography scanning in the evaluation of hepatocellular carcinoma. J Hepatol 2000;32(5):792-797.

146. Ma WW, Jacene H, Song D et al. 18F]fluorodeoxyglucose positron emission tomography correlates with akt pathway activity but is not predictive of clinical outcome during mTOR inhibitor therapy. J Clin Oncol 2009;27(16):2697-2704.

147. Riedl CC, Akhurst T, Larson S et al. 18F-FDG PET scanning correlates with tissue markers of poor prognosis and predicts mortality for patients after liver resection for colorectal metastases. J Nucl Med 2007;48(5):771-775.

148. Gu J, Yamamoto H, Fukunaga H et al. Correlation of GLUT-1 overexpression, tumor size, and depth of invasion with 18F-2-fluoro-2-deoxy-D-glucose uptake by positron emission tomography in colorectal cancer. Dig Dis Sci 2006;51(12):2198-2205.

149. Higashi K, Ueda Y, Sakurai A et al. Correlation of glut-1 glucose transporter expression with. Eur J Nucl Med 2000;27(12):1778-1785.

150. Yen TC, See LC, Lai CH et al. 18F-FDG uptake in squamous cell carcinoma of the cervix is correlated with glucose transporter 1 expression. J Nucl Med 2004;45(1):22-29.

151. Kurokawa T, Yoshida Y, Kawahara K et al. Expression of GLUT-1 glucose transfer, cellular proliferation activity and grade of tumor correlate with [F-18]-fluorodeoxyglucose uptake by positron emission tomography in epithelial tumors of the ovary. Int J Cancer 2004;109(6):926-932.

152. Brown RS, Leung JY, Kison PV et al. Glucose transporters and FDG uptake in untreated primary human non-small cell lung cancer. J Nucl Med 1999;40(4):556-565.

153. Avril N, Menzel M, Dose J et al. Glucose metabolism of breast cancer assessed by 18F-FDG PET: Histologic and immunohistochemical tissue analysis. J Nucl Med 2001;42(1):9-16.

154. Younes M, Lechago LV, Somoano JR et al. Immunohistochemical detection of Glut3 in human tumors and normal tissues. Anticancer Res 1997;17(4A):2747-2750.

155. Sung JY, Kim GY, Lim SJ et al. Expression of the GLUT1 glucose transporter and p53 in carcinomas of the pancreatobiliary tract. Pathol Res Pract 2010;206(1):24-29.

156. Haber RS, Rathan A, Weiser KR et al. GLUT1 glucose transporter expression in colorectal carcinoma: A marker for poor prognosis. Cancer 1998;83(1):34-40.

157. Cantuaria G, Fagotti A, Ferrandina G et al. GLUT-1 expression in ovarian carcinoma: Association with survival and response to chemotherapy. Cancer 2001;92(5):1144-1150.

158. Kang SS, Chun YK, Hur MH et al. Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma. Jpn J Cancer Res 2002;93(10):1123-1128.

159. Hoskin PJ, Sibtain A, Daley FM, Wilson GD. GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: Relationship with vascularity and proliferation as predictors of outcome of ARCON. Br J Cancer 2003;89(7):1290-1297.

160. Tohma T, Okazumi S, Makino H et al. Overexpression of glucose transporter 1 in esophageal squamous cell carcinomas: A marker for poor prognosis. Dis Esophagus 2005;18(3):185-189.

161. Ayala FR, Rocha RM, Carvalho KC et al. GLUT1 and GLUT3 as potential prognostic markers for oral squamous cell carcinoma. Molecules 2010;15(4):2374-2387.

162. Baer S, Casaubon L, Schwartz MR et al. Glut3 expression in biopsy specimens of laryngeal carcinoma is associated with poor survival. Laryngoscope 2002;112(2):393-396.

163. Chung FY, Huang MY, Yeh CS et al. GLUT1 gene is a potential hypoxic marker in colorectal cancer patients. BMC Cancer 2009;9:241.

164. Rastogi S, Banerjee S, Chellappan S, Simon GR. Glut-1 antibodies induce growth arrest and apoptosis in human cancer cell lines. Cancer Lett 2007;257(2):244-251.

165. Wood TE, Dalili S, Simpson CD et al. A novel inhibitor of glucose uptake sensitizes cells to FAS-induced cell death. Mol Cancer Ther 2008;7(11):3546-3555.

166. Melstrom LG, Salabat MR, Ding XZ et al. Apigenin inhibits the GLUT-1 glucose transporter and the phosphoinositide 3-kinase/Akt pathway in human pancreatic cancer cells. Pancreas 2008;37(4):426-431.

167. Liu Y, Zhang W, Cao Y et al. Small compound inhibitors of basal glucose transport inhibit cell proliferation and induce apoptosis in cancer cells via glucose-deprivation-like mechanisms. Cancer Lett 2010;298(2):176-185.

168. Kurata T, Oguri T, Isobe T et al. Differential expression of facilitative glucose transporter (GLUT) genes in primary lung cancers and their liver metastases. Jpn J Cancer Res 1999;90(11):1238-1243.

169. Younes M, Lechago LV, Somoano JR et al. Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. Cancer Res 1996;56(5):1164-1167.

170. Younes M, Brown RW, Stephenson M et al. Overexpression of Glut1 and Glut3 in stage I nonsmall cell lung carcinoma is associated with poor survival. Cancer 1997;80(6):1046-1051.

171. Suzawa N, Ito M, Qiao S et al. Assessment of factors influencing FDG uptake in non-small cell lung cancer on PET/CT by investigating histological differences in expression of glucose transporters 1 and 3 and tumour size. Lung Cancer 2011;72(2):191-198.

172. Grover-McKay M, Walsh SA, Seftor EA et al. Role for glucose transporter 1 protein in human breast cancer. Pathol Oncol Res 1998;4(2):115-120.

173. Kurata T, Oguri T, Isobe T et al. Differential expression of facilitative glucose transporter (GLUT) genes in primary lung cancers and their liver metastases. Jpn J Cancer Res 1999;90(11):1238-1243.

174. Pollack MN. Insulin, insulin-like growth factors, insulin resistance, and neoplasia. Am J Clin Nutr 2007;86(3):s820-2.

175. Maier T, Guell M, Serrano L. Correlation of mRNA and protein in complex biological samples. FEBS Lett 2009;583(24):3966-3973.

176. de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. Mol Biosyst 2009;5(12):1512-1526.

177. Schwanhausser B, Busse D, Li N et al. Global quantification of mammalian gene expression control. Nature 2011;473(7347):337-342.

178. Simpson IA, Dwyer D, Malide D et al. The facilitative glucose transporter GLUT3: 20 years of distinction. Am J Physiol Endocrinol Metab 2008;295(2):E242-53.

179. Klip A, Tsakiridis T, Marette A, Ortiz PA. Regulation of expression of glucose transporters by glucose: A review of studies in vivo and in cell cultures. FASEB J 1994;8(1):43-53.

180. Tennant DA, Duran RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. Nat Rev Cancer 2010;10(4):267-277.

181. Liu Y, Zuckier LS, Ghesani NV. Dominant uptake of fatty acid over glucose by prostate cells: A potential new diagnostic and therapeutic approach. Anticancer Res 2010;30(2):369-374.

182. Apolo AB, Pandit-Taskar N, Morris MJ. Novel tracers and their development for the imaging of metastatic prostate cancer. J Nucl Med 2008;49(12):2031-2041.

183. Levi J, Cheng Z, Gheysens O et al. Fluorescent fructose derivatives for imaging breast cancer cells. Bioconjug Chem 2007;18(3):628-634.

184. Liu H, Huang D, McArthur DL et al. Fructose induces transketolase flux to promote pancreatic cancer growth. Cancer Res 2010;70(15):6368-6376.

185. Pinto AB, Carayannopoulos MO, Hoehn A et al. Glucose transporter 8 expression and translocation are critical for murine blastocyst survival. Biol Reprod 2002;66(6):1729-1733.

186. McBrayer SK, Cheng JC, Singhal S et al. Multiple myeloma exhibits novel dependence on GLUT4, GLUT8, and GLUT11: Implications for glucose transporter-directed therapy. Blood 2012.

187. Goldman NA, Katz EB, Glenn AS et al. GLUT1 and GLUT8 in endometrium and endometrial adenocarcinoma. Mod Pathol 2006;19(11):1429-1436.

APPENDIX

Supplemental Figures

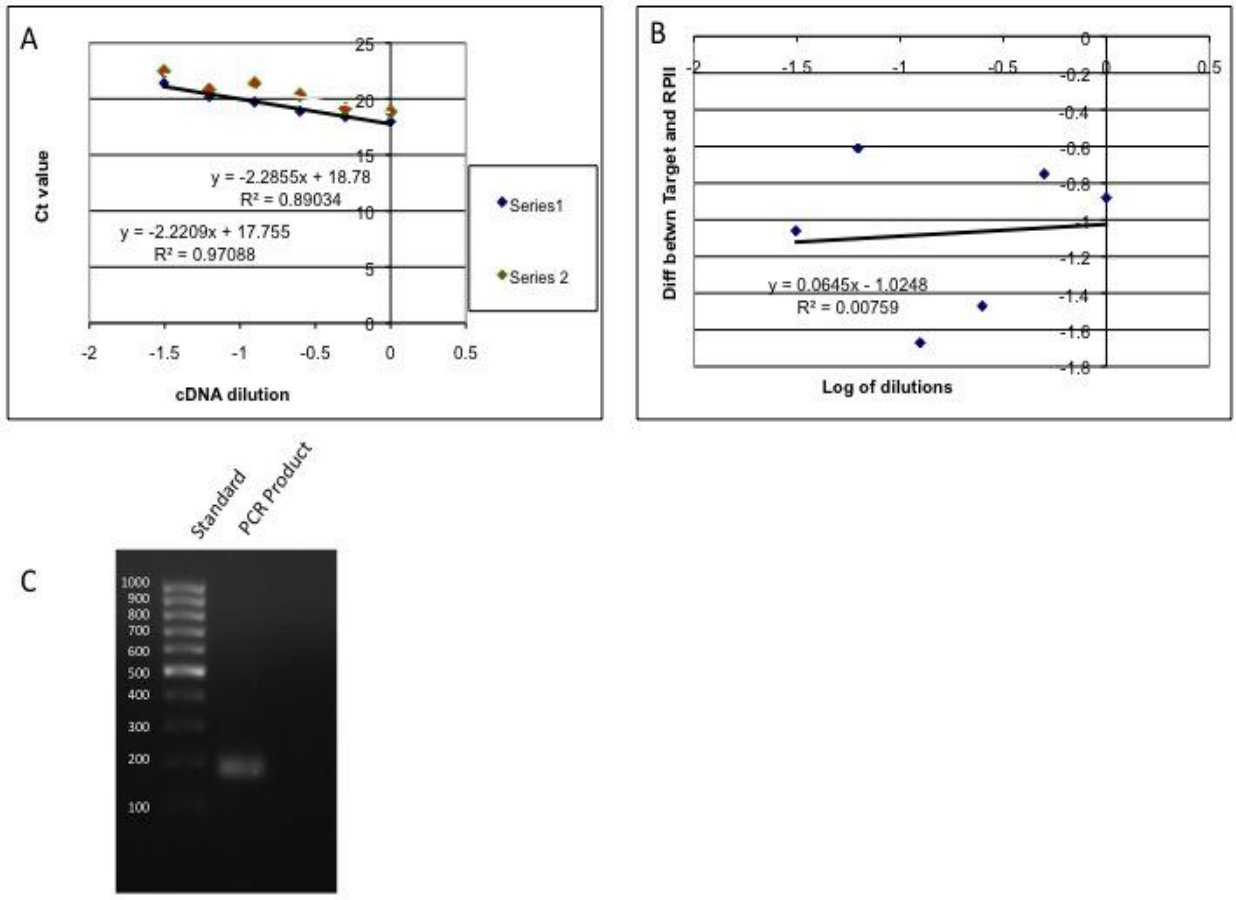

Figure s.1. Efficiency testing of GLUT1 primer. A) Amplification of cDNA synthesized from different amounts of RNA. Serial dilutions of cDNA were amplified by real-time PCR to determine the efficiency of amplification of the GLUT1 gene and internal control (RPII-2). The Ct values at each cDNA dilution were plotted for each gene and slope of the line was determined to be similar, indicating that the GLUT1 primer has a similar efficiency to the reference gene primer and the $2^{-[\Delta][\Delta]Ct}$ calculation for the relative quantification of target may be used. B) The differences between the Ct value of GLUT1 and RPII-2 were plotted against the log of the concentration of cDNA samples. A delta Ct slope of less than 0.1 indicates similar efficiencies of the target and reference genes, therefore, since the delta Ct slope was determined to be 0.0645 it can be inferred that the GLUT1 and RPII-2 primers have similar efficiencies. C) The amplified PCR product collected when using the GLUT1 primer was run on an agarose gel and a strong band appeared under the 200 bp standard marker. This is consistent with the expected size of the product of 189 bp.

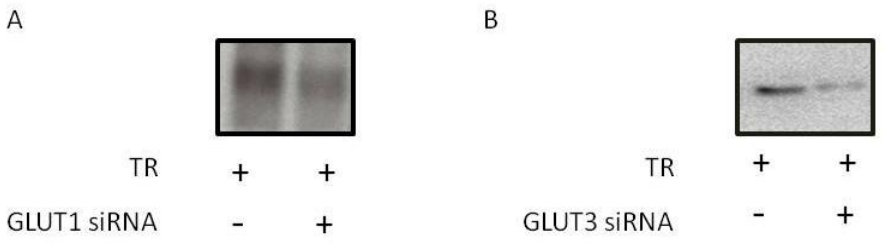

Figure s.2. siRNA knockdown of GLUT1 and GLUT3 in H1299 cells. H1299 cells were incubated with transfection reagent (TR) or transfection reagent + GLUT1 (A) or GLUT3 (B) siRNA to transiently knock down these transporters. The specificity of the GLUT antibodies was examined by immunoblotting with whole cell lysates from each group.

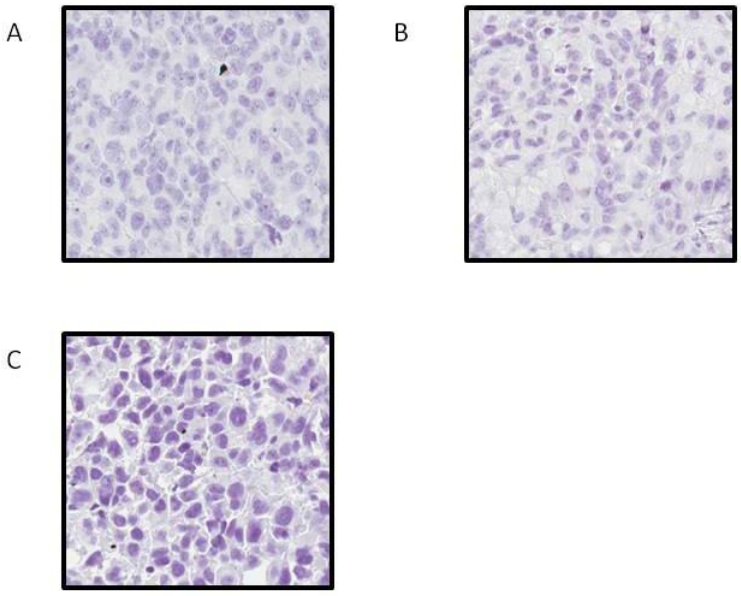

Figure s.3. Immunohistochemistry analysis of no-primary GLUT1 controls. Parrafin-embedded sections of H1299 (A), A549 (B) and PC-3 (C) tumours were not incubated with an anti-GLUT1 antibody and then analyzed by immunohistochemistry . No signal was detected in these tumours. Representative images of three independent experiments are shown. Magnification=18.4X

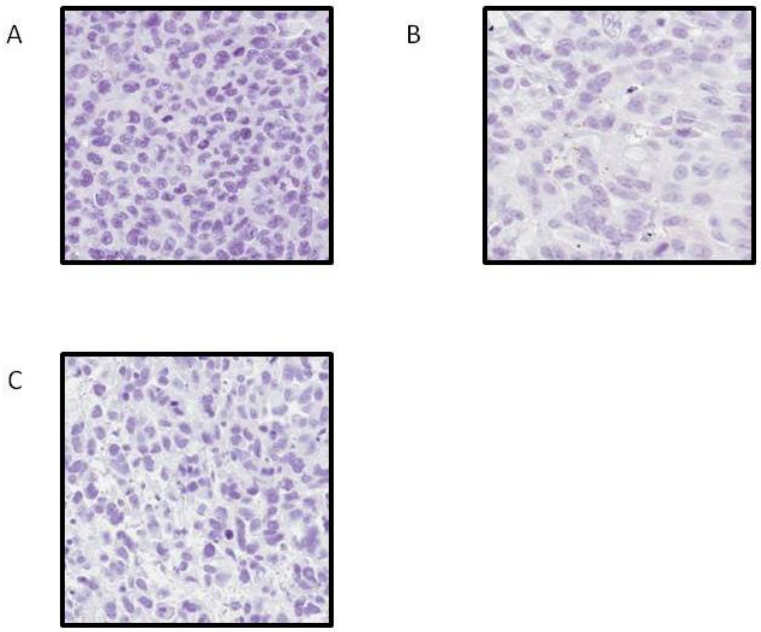

Figure s.4. Immunohistochemistry analysis of no-primary GLUT3 controls. Parrafin-embedded sections of H1299 (A), A549 (B) and PC-3 (C) tumours were not incubated with an anti-GLUT3 antibody and then analyzed by immunohistochemistry . No signal was detected in these tumours. Representative images of three independent experiments are shown. Magnification=18.4X

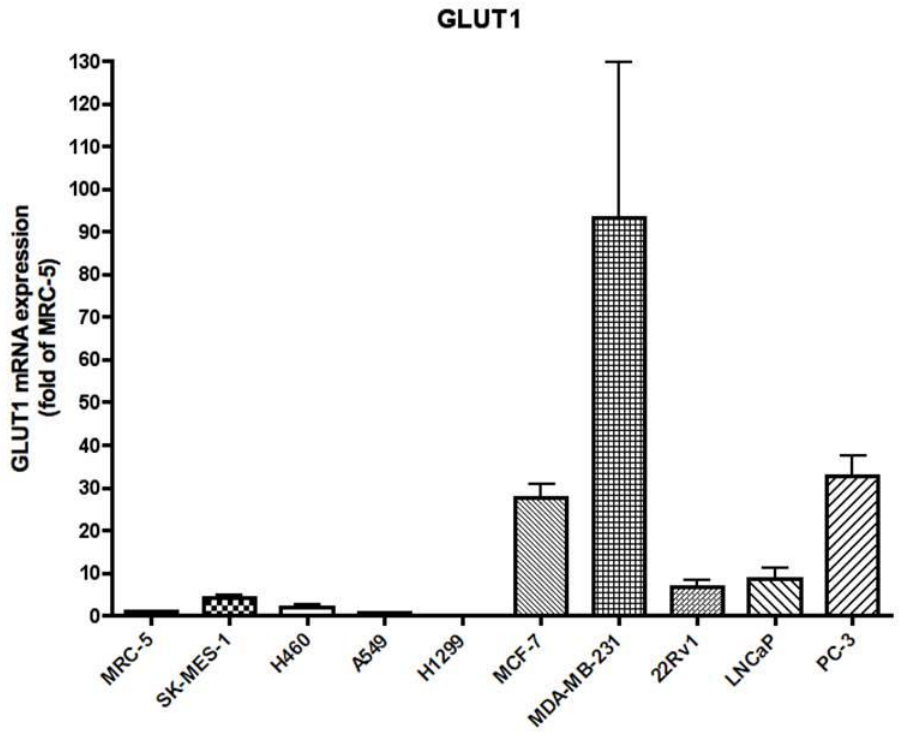

Figure s.5. GLUT1 mRNA expression in cancer cell lines normalized to MRC-5. Basal GLUT1 mRNA levels were determined in lung, breast and prostate cancer cell lines by Real Time PCR and expressed as a fold change of the lung fibroblast cell line MRC-5. Results represent the mean ± SEM of three to four independent experiments.

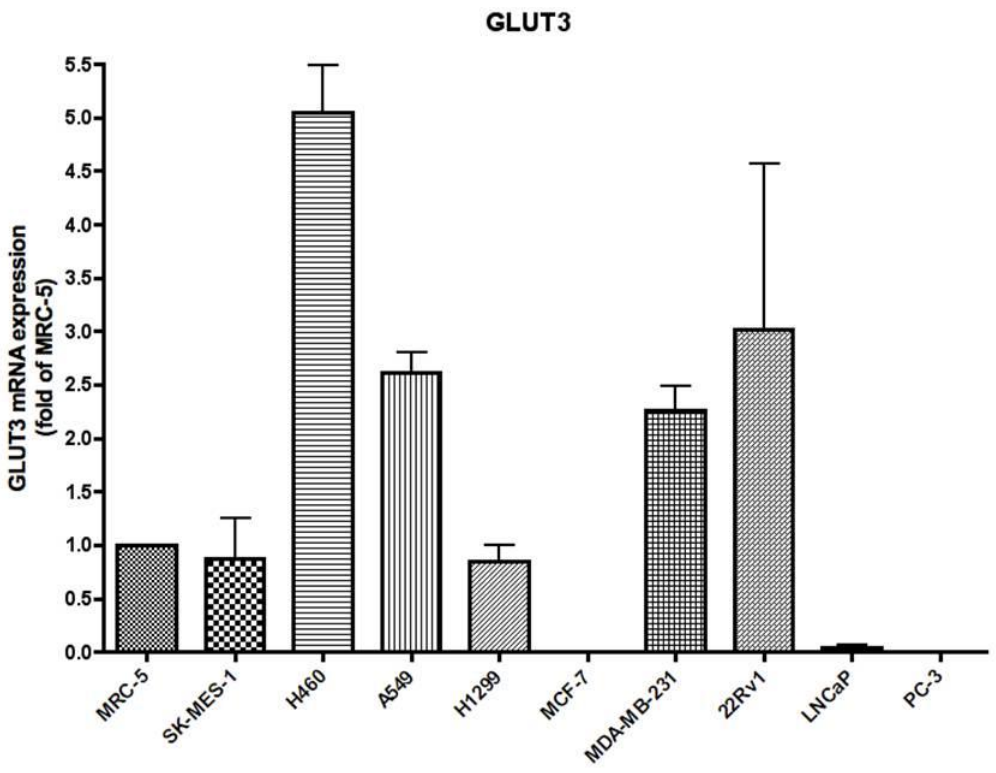

Figure s.6. GLUT3 mRNA expression in cancer cell lines normalized to MRC-5. Basal GLUT3 mRNA levels were determined in lung, breast and prostate cancer cell lines by Real Time PCR and expressed as a fold change of the lung fibroblast cell line MRC-5. Results represent the mean ± SEM of three to four independent experiments.

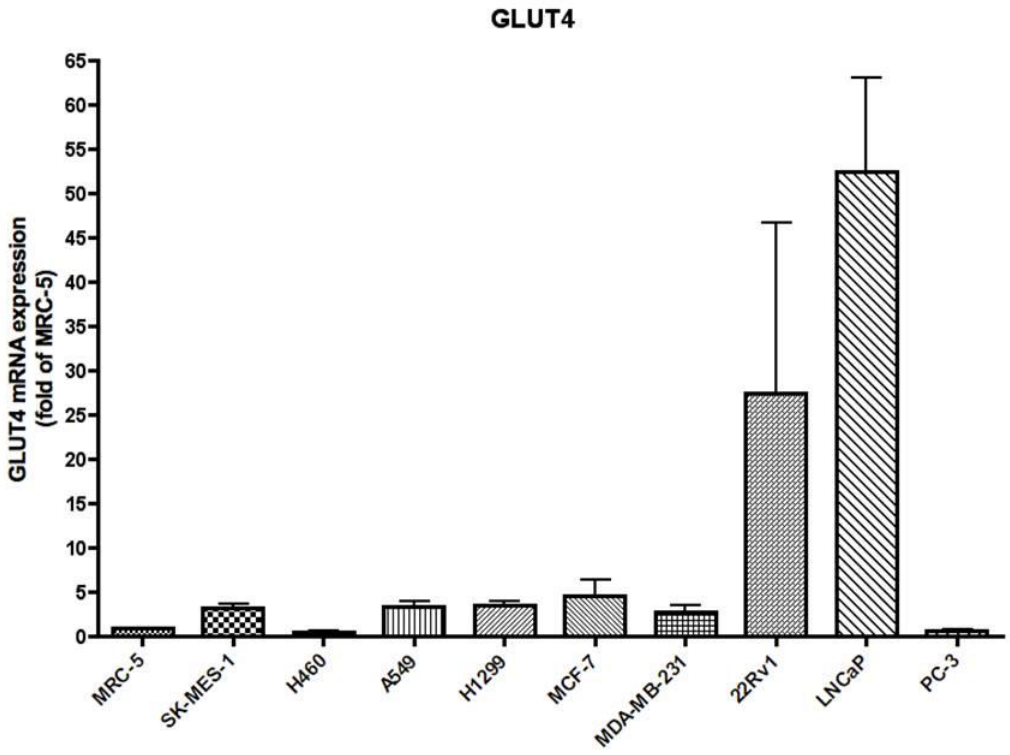

Figure s.7. GLUT4 mRNA expression in cancer cell lines normalized to MRC-5. Basal GLUT4 mRNA levels were determined in lung, breast and prostate cancer cell lines by Real Time PCR and expressed as a fold change of the lung fibroblast cell line MRC-5. Results represent the mean ± SEM of three to four independent experiments.

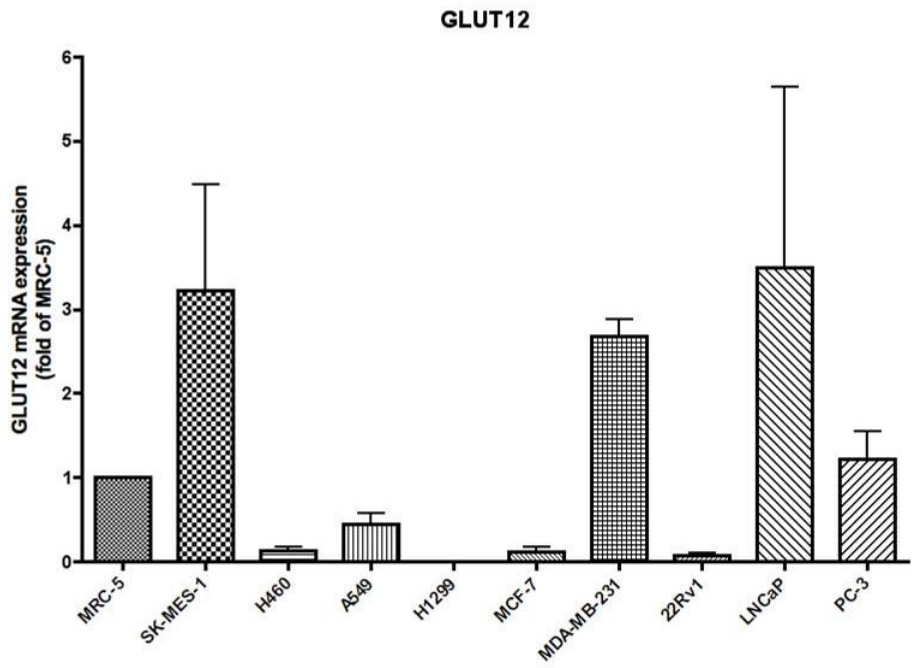

Figure s.8. GLUT12 mRNA expression in cancer cell lines normalized to MRC-5. Basal GLUT12 mRNA levels were determined in lung, breast and prostate cancer cell lines by Real Time PCR and expressed as a fold change of the lung fibroblast cell line MRC-5. Results represent the mean ± SEM of three to four independent experiments.

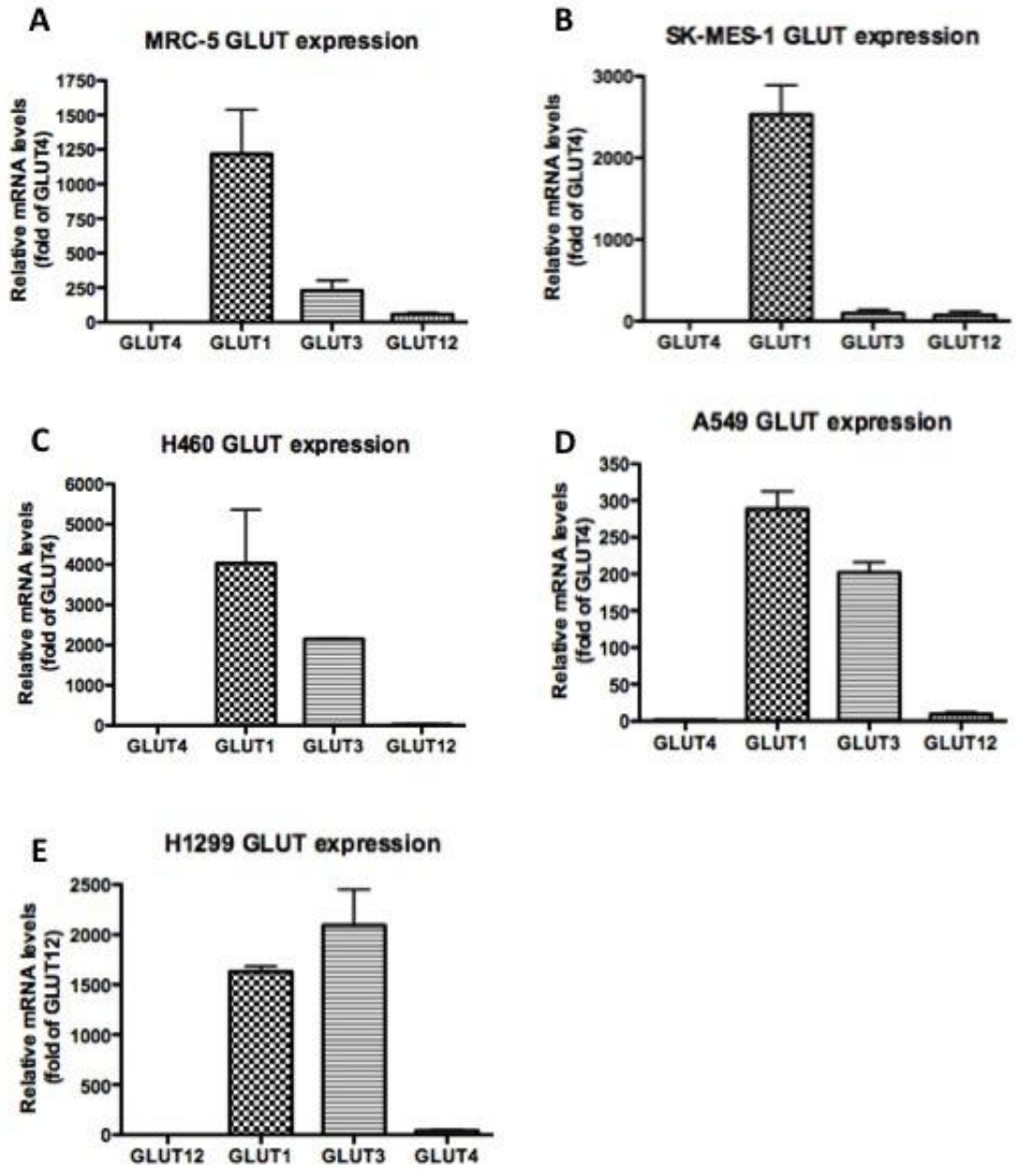

Figure s.9. GLUT expression within each lung cell line normalized to the lowest expressed GLUT. A) GLUT expression in the MRC-5 cell line normalized to GLUT4. B) GLUT expression in the SK-MES-1 cell line normalized to GLUT4. C) GLUT expression in the H460 cell line normalized to GLUT4. D) GLUT expression in the A549 cell line normalized to GLUT4. E) GLUT expression in the H1299 cell line normalized to GLUT12.

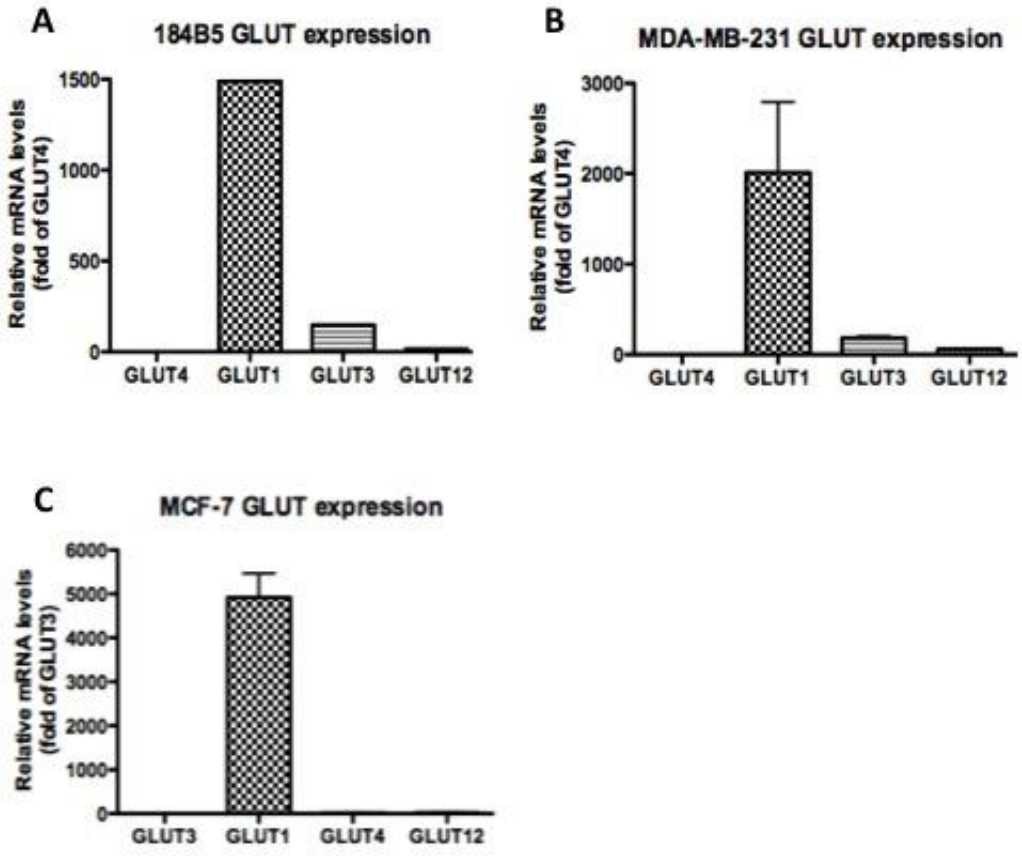

Figure s.10. GLUT expression within each breast cell line normalized to the lowest expressed GLUT. A) GLUT expression in the 184B5 cell line normalized to GLUT4. B) GLUT expression in the MDA-MB-231 cell line normalized to GLUT4. C) GLUT expression in the MCF-7 cell line normalized to GLUT3.

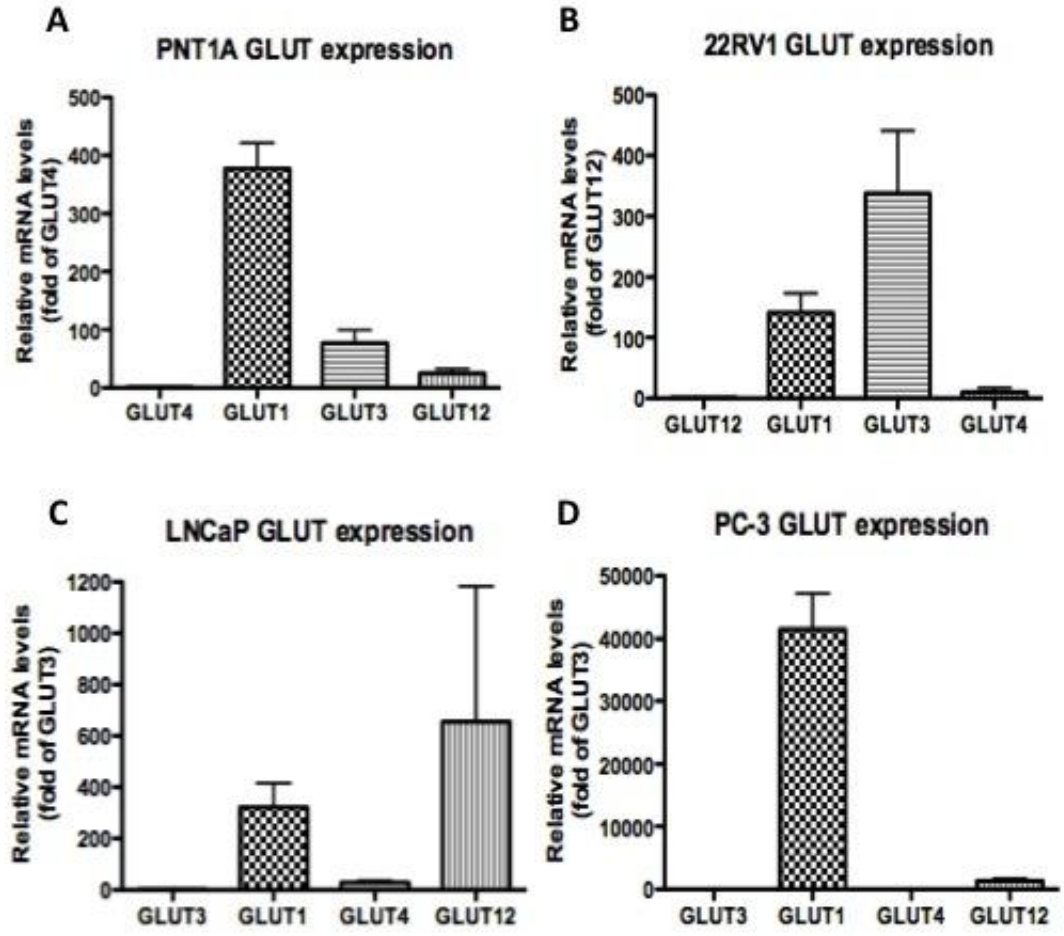

Figure s.11. GLUT expression within each prostate cell line normalized to the lowest expressed GLUT. A) GLUT expression in the PNT1A cell line normalized to GLUT4. B) GLUT expression in the 22Rv1 cell line normalized to GLUT12. C) GLUT expression in the LNCaP cell line normalized to GLUT3. D) GLUT expression in the PC-3 cell line normalized to GLUT3.