DIPLOMA THESIS

Modulation of AKT signalling by the gluconeogenesis enzyme PCK2 in lung cancer cells

submitted by

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Statutory declaration

I, Joscha Pocha, hereby declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material quoted either literally or by content from the used sources.

Zürich, 7th June 2021 Joscha Pocha eh.

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Abbreviations

Acetyl-CoA	Acetyl coenzyme A
AKT/PKB	Protein kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine 5′-triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary desoxyribonucleic acid
Ct	Cycle threshold
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FOXO	Forkhead family transcription factors
GDP	Guanosine diphosphate
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HIF	Hypoxia-inducible factors
IGF-1	Insulin-like growth factor
INSIG	Insulin induced gene
IRS	Insulin receptor substrate
kDa	Kilodalton
mTORC1/2	Mechanistic target of rapamycin complex 1/2
NADP+/H	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NSCLC	Non-small cell lung cancer
NTC	No template control
OAA	Oxaloacetate
PBS	Phosphate buffered saline

PCK1	Phosphoenolpyruvate carboxykinase 1 (cytosol)
PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
PDK1	Phosphoinositide-dependent kinase-1
PD-L1	Programmed death-ligand 1
PEP	Phosphoenolpyruvate
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative real-time polymerase chain reaction
RIPA buffer	Radioimmunoprecipitation assay buffer
RPMI medium	Roswell Park Memorial Institute medium
RT	Reverse Transcriptase
S6K	P70S6 kinase
SCLC	Small cell lung cancer
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween20
TCA cycle	Tricarboxylic acid cycle
ULK1	Unc-51 like autophagy activating kinase 1
WHO	World Health Organisation

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Zusammenfassung

Trotz umfassender Präventionsbemühungen ist Lungenkrebs die häufigste Krebstodesursache weltweit. Hohe Mortalitätsraten bei oft spät erkannten Tumoren verdeutlichen den Bedarf an hochwirksamen Therapeutika. Ein potentieller Ansatz hierbei ist die Modulation des Metabolismus von Tumorzellen. Wechselnde Sauerstoff- und Nährstoffversorgung zwingt sie zu metabolischer Flexibilität. Eine im Jahr 2014 von Leithner et al. veröffentliche Studie, zeigte, dass das Gluconeogenese Schrittmacherenzym Phosphoenolpyruvat Carboxykinase (PCK2) in Nichtkleinzelligen Bronchialkarzinomen (NSCLC) aktiv ist und damit die metabolische Flexibilität erhöht. Ob dadurch auch die Signaltransduktion in den Tumorzellen moduliert wird, ist unbekannt. Ziel der vorliegenden Studie war es, Effekte einer Abschaltung des PCK2 Gens mittels RNA-Interferenz auf für das Zellwachstum wichtige Signaltransduktionswege zu ermitteln. Augenmerk wurde auf den PI3K/AKT/mTORC Signalweg gelegt, da mTORC1 und mTORC2 eine zentrale Rolle als Signalschaltzentralen in der Zelle einnehmen und Überleben, Proliferation, Autophagie, Zellzyklus und Wachstum regulieren. Die beiden NSCLC Zelllinien A549 und H23 wurden mit siRNA transfiziert und unter Bedingungen physiologisch hoher und niedriger Glukose kultiviert. Die Phosphorylierungsgrade von AKT (S473), AKT (T308), AMPK und S6K wurden mittels Western Blot analysiert, weiterhin wurde die Expression verschiedener Membrantransporter sowie des IGF-1 Rezeptors mittels qPCR bestimmt. Es zeigte sich, dass eine Geninaktivierung von PCK2 in H23 Zellen, unter hoher ($p = 0.012$; <0.05) als auch unter niedriger Glukosekonzentration im Medium zu einer vermehrten Phosphorylierung von AKT (S473) führte. Der Einsatz verschiedener Inhibitoren ermöglichte, dies auf eine vermehrte Aktivierung von mTORC2 unter PCK2-Inhibition zurückzuführen. Eindeutige Effekte auf AKT T308, S6K oder AMPK konnten bei Genaktivierung von PCK2 nicht festgestellt werden, es zeigten sich jedoch zelllinienabhängige Effekte der Glucosekonzentration. Mittels quantitativer PCR zeigte sich in H23 Zellen, dass PCK2-Silencing zu einer Überexpression des Glucosetransporters GLUT1 (SLC2A1) und des Glutamin Transporters (SLC1A5) bei hoher Glukose im Medium führte, möglicherweise als Adaptationsmechanismus. Insgesamt deuten die Ergebnisse dieser Studie auf eine Modulation des mTORC2 Signalwegs durch die Aktivität des Gluconeogeneseenzyms PCK2 hin, allerdings in

hoher Abhängigkeit von einzelnen Zellinien und deren Eigenschaften. Eine Mutation am *TP53* Gen könnte ein möglicher Grund für die beobachteten Effekte von AKT (S473) in H23 Zellen sein.

Abstract

Despite extensive preventive measures, lung cancer remains the cancer with the highest mortality, demanding further research for therapeutic targets. One potential therapeutic approach is the remodelling of energy metabolism. Changing supplies of oxygen and nutrients require high metabolic flexibility. Leithner et al. in 2014 showed activity of the gluconeogenesis pacemaker enzyme phosphoenolpyruvate carboxykinase (PCK2) in non-small-cell lung carcinoma (NSCLC) cells, which thereby increases the metabolic flexibility. However, it is unknown whether this modulates signal transduction in tumour cells. This study aimed to evaluate the effects of PCK2 gene silencing via siRNA on signalling pathways affecting cell growth. The PI3K/AKT/mTORC signalling pathway was especially of interest due to the role of mTORC1 and mTORC2 as central signalling hubs and the mediation of signalling related to survival, proliferation, autophagy, cell cycle and growth. Cells of the two NSCLC cell lines A549 and H23 were cultured and transfected with siRNA under high and low glucose conditions. Phosphorylation levels of AKT (S473), AKT (T308), AMPK and S6K were analysed in Western blots. Furthermore, the expression of various membrane transporters and the IGF-1 receptor was evaluated with qPCR. Silencing of PCK2 led to increased phosphorylation of AKT (S473) in H23 under high ($p = 0.012$; <0.05) as well as under low glucose conditions in the medium. The use of different inhibitors allowed to attribute this to increased mTORC2 activation under PCK2 inhibition. No distinct effects on AKT T308, S6K or AMPK could be observed by silencing PCK2, albeit cell line dependent effects of glucose concentration were observed. PCK2 silencing in H23 cells showed overexpression in the qPCR of GLUT1 (SLC2A1) and the glutamine transporter (SLC1A5) under high glucose medium conditions, possibly as a means of adaptation. Altogether, this study's results suggest modulation of the mTORC2 pathway through the activity of the gluconeogenesis enzyme PCK2, although highly dependent on individual cell lines and respective properties. In the H23 cell line, mutant *TP53* could play a role in effects on AKT (S473) observed in the experiments.

1. Introduction

1.1 Epidemiology of lung cancer

A report by the World Health Organisation (WHO) in December 2020 showed that approximately 9.96 million people had died because of malignant neoplasms in that year. The cancer causing the highest absolute number of deaths was lung cancer, leading to 1.7 million deaths in 2020. Only narrowly behind breast cancer, lung cancer currently is the cancer with the highest incidence in both sexes worldwide. The incidence of lung cancer is distinctly higher among males with a ratio of about 2:1. In the year 2000, of all deaths, 2,4% were caused by trachea, bronchus or lung cancer, this number rose to 3% in the year 2016, implicating an ongoing rise in the already very high absolute numbers (1, 2).

In Austria, according to data from the Country Health Profile Report 2017 released by the European Observatory on Health Systems and Policies, the rate of all deaths caused by lung cancer is with 5% even higher. Thus, it is only second to cardiovascular diseases, the leading cause for overall mortality (3). The report from 2019 states that 15% of all deaths can be attributed to tobacco consumption, the single most relevant risk factor for developing lung cancer (4).

For comparison, out of 7.46 billion humans in 2016, a total of 56.8 million people deceased. Of those, 183,000 people were accounted for in the group collective violence and legal intervention. This number represents the casualties of a broad range of armed conflicts, thus receiving extensive media coverage. The mortality rate of lung cancer, however, although much higher, gets less public attention. Even though the suffering arising from one may, for obvious reasons, not be compared to the other, it nevertheless puts the amount of 1.7 million lung cancer deaths per year into perspective and highlights the importance of well-working lung cancer prevention programs as well as extensive research focused on optimal treatment options (1).

1.2 Aetiology

The single most relevant risk factor for lung cancer development is cigarette smoking, being responsible for up to 90% of all lung cancers. There is also a robust causal connection between cigarette smoking and the development of many cancers, including mouth, throat, oesophagus, stomach, liver, pancreas, kidney, urinary bladder, colon, rectum, and cervix cancer. Other ways of smoking tobacco, like pipes and cigars, likewise increase cancer development risk (5).

Tobacco smoke, which forms during the combustion process, contains at least 69 cancerogenic substances and a wide variety of over 7000 chemicals that still might be classified as such in the future. Thus, even inhaling smoke second-hand is proven to be a high-risk factor for the development of lung cancer and increased mortality (5, 6). Another agent with a highly cancerogenic potential for lung cancer development is the radioactive element Radon. Radon is a naturally occurring gas escaping from rocks in the ground and the second most relevant cause for lung cancer (7). Further, well-established risk factors for lung cancer development are workplace exposures to substances like asbestos, arsenic, diesel exhaust, and certain forms of silica and chromium. Other factors include a poor diet, air pollution and genetic susceptibility (8).

The role of e-cigarettes in the development of acute respiratory distress syndrome has recently been a topic of discussion (9); any long-term studies providing credible information about lung cancer development are, however, still pending (10).

1.3 Classification and prognosis

The World Health Organisation categorises bronchial carcinoma based on their histology into the less frequent (15%) small cell lung carcinoma (SCLC) and the more often (85%) occurring non-small cell lung carcinoma (NSCLC). The 2015 newly published WHO classification emphasises the impact of genetic, clinical and radiological improvements. Histological and genetic subcategorisations of NSCLC are becoming increasingly more important with advancements in personalised medicine, enabling physicians to treat patients according to their pathologic tumour subtype (11). Alongside several less common types of NSCLC, the most frequent

NSCLC subtypes are adenocarcinoma, large cell carcinoma and squamous cell carcinoma (12).

The overall 5-year survival rates of lung cancers are low but can in NSCLC in an early stage (IA) be as high as 75-80%. However, as many as 35-40% of all NSCLCs get diagnosed in stage IV and therefore receive primarily palliative treatment due to the tumour's advanced nature. The SCLC, a very aggressively growing tumour with short doubling time and early development of metastases, gets in 60-70% diagnosed in extensive-stage (IV). Thus, patients only in very few instances survive five years post-diagnosis (13, 14).As mentioned, smoking poses the highest risk factor for developing lung cancer, with SCLC being almost exclusively seen in smokers. (8, 12).

1.4 The hallmarks of cancer

For understanding cancer growth, signalling and the elementary principles of cancer treatment, it is vital to understand what in the broad heterogenous group of neoplasms the common features of cancers are. In 2000, the renowned cancer researchers Douglas Hanahan and Robert Weinberg broke the similarities down to 6 easily intelligible traits in an often-cited paper (15). Those traits include self-sufficiency in growth signals, evading growth suppressors, evading programmed cell death, replicative immortality, sustained angiogenesis and invasion of local tissue and metastasis. In 2011, Hanahan and Weinberg proposed the two additional emerging hallmarks reprogramming energy metabolism and evading immune detection (16). Both emerging hallmarks proved to be of great interest to the research community. Especially monoclonal antibodies, also referred to as checkpoint inhibitors, that bind to specific antigens (programmed cell death 1 ligand 1, PD-L1) mediating immune suppression on tumour cells by re-enabling a response by the immune system, were, and increasingly still are, a big success equally in their clinical outcome but also in terms of financial revenue for the pharmaceutical companies investing into their development (17).

Targeting energy metabolism is another promising approach in the development of new tumour therapeutics. Observations suggest that the mutant upregulation of oncogenes or the loss of tumour suppressors lead to increased activation of key

metabolic enzymes in cancer, indicating the possibility of inhibiting these enzymes as a therapeutic approach (18). Essential for any progress in this direction is a deeper understanding of cancer cell metabolism's interconnections and the signalling pathways associated.

1.5 The Warburg effect

A very early discovery in cancer research was the Warburg effect named after the Nobel Prize winner in physiology Otto Heinrich Warburg who published his hypothesis on cancer metabolism in 1924. The effect describes the phenomenon that cancer cells favour aerobic glycolysis over oxidative phosphorylation. One glucose molecule's energy yield is hereby drastically smaller, generating only two molecules of adenosine 5′-triphosphate (ATP) when producing lactate compared to 36 molecule ATP upon complete oxidation (19).

Even though the Warburg effect appears unfavourable to the cell at first glance, the substantial benefit becomes evident when taking the high demand for macromolecules and thereby the high need for biosynthetic precursors into account. Apart from glucose, only glutamine is catabolised in somewhat resembling high quantities in mammalian cells. While studies with metabolic rescue protocols suggest that cancer cells can metabolise a wide variety of precursor molecules, glucose and glutamine are essential to a broad spectrum of metabolic pathways in cancer cells, not only as a source of energy but as precursors for macromolecules such as nucleotides, amino acids, fatty acids etc. (20–22). Lactate and alanine are hereby secreted by cells. The formation of many macromolecules requires relatively high amounts of carbon and nicotinamide adenine dinucleotide phosphate (NADPH) compared to moderate ATP consumption. This reflects how highly beneficial the pathway transforming glutamine to lactate is to the cell’s survival as it yields NADPH and nitrogen for nonessential amino acids. Likewise, the pentose phosphate pathway generates NADPH through the transformation of glucose (23).

Moreover, the high flux through glycolysis ensures the generation of precursors for downstream biosynthetic reactions. For instance, ribose phosphate, generated from glucose through the pentose phosphate pathway, can be used as a precursor for nucleotides. Both acetyl-CoA, needed for the synthesis of fatty acids, and aspartate,

needed for nucleotide synthesis, are generated from tricarboxylic acid cycle (TCA cycle) intermediates. Notably, the TCA cycle and glycolytic intermediates are precursors for the biosynthesis of nonessential amino acids (24). It becomes evident that the optimisation of energy efficiency cannot be the advantage of high glycolytic activity; the overall objective could instead be to provide vital building blocks and reducing agents for maximisation of anabolic growth rate (25). The Warburg effect is not solely occurring in cancer cells but also in other highly proliferative cells**.** For example, the body's immune response or wound healing represent scenarios in which proliferating cells' division rates are massively increased. Most proliferating cells do not face a shortage in nutrient supply in solid tumours. However, the demand for nutrients often exceeds their supply, as will be outlined in the following sections. (19)

1.6 Tumour heterogeneity

Tumours are heterogeneous constructs made of large accumulations of cells with high variabilities in their supplies of essential resources and oxygen (26). Studies have shown a gradual decline in glucose supply from the tumour edge over the vital tumour tissue being lowest around the central necrosis (27). Equally, oxygen is not evenly distributed, leaving some regions of cancer with a low oxygen supply, which leads to the release of hypoxia-inducible factors (HIF) and thereby increases the angiogenesis required for tumour progression (28). However, the early response with the formation of a new vascular network is often insufficient due to leaky vessels and fluctuating blood flow (29).

With uneven resources, but at the same time, high selection pressure on the cells, maximum usage of given supplies is vital. Further investigations into this topic have shown that lactate, previously considered a waste product in cancers, can be used as an alternative energy source (30). Apart from the repurposing of lactate as a fuel, it may also be utilised as an anabolic carbon source. A study released by our research group Leithner et al. in 2015 found strong evidence suggesting that lung cancer cells use some steps of gluconeogenesis to survive harsh metabolic conditions. The focus was hereby on phosphoenolpyruvate carboxykinase 2 (PCK2), the mitochondrial isoform of the gluconeogenesis pacemaker enzyme

phosphoenolpyruvate carboxykinase 1 (PCK1) (31). The same study provided evidence that different NSCLC lines showed a net lactate production while under high glucose conditions. The opposite, a net consumption, occurred under low glucose conditions (31).

1.7 Gluconeogenesis and PCK2

Gluconeogenesis is a well-described pathway present in humans and a broad spectrum of other species. In humans, gluconeogenesis mainly occurs in the liver, the kidneys and the intestines to ensure stable blood levels to supply tissues with a dependency on glucose. The main precursors for the gluconeogenic pathway are lactate, glycerol, alanine and glutamine. A series of other amino acids and substrates from the TCA can also be converted to oxaloacetate and then be channelled into the gluconeogenesis pathway. While many of the gluconeogenesis steps are the reverse reaction of glycolysis, some reactions are specific for gluconeogenesis, partly because the respective steps in glycolysis are exergonic (**figure 1)**. These steps are catalysed by glucose-6-phosphatase, fructose-1,6-bisphosphatase, and PCK1/2. The gluconeogenesis enzymes activity is tightly controlled to avoid uncontrolled channelling of all available resources into the gluconeogenic pathway (32). Feedback loops and stimulating molecules like cyclic adenosine monophosphate (cAMP), acetyl-CoA and citrate lead to the upregulation of gluconeogenesis. By having the exact opposite effect on glycolysis, this regulation ensures that only one of the two pathways is activated at a time. In the case of the enzyme PCK1/2, glucocorticoids, cAMP, and retinoic acid have a stimulating effect (33).

In addition to the control of glycolytic/gluconeogenic enzymes by allosteric effectors, their activity is also controlled by hormones that upregulate or repress the expression of gluconeogenesis enzymes. The hormone glucagon, released by the pancreas, is responsible for inhibiting glycolysis and stimulating gluconeogenesis. Secretion of insulin has the opposite effect (32, 34).

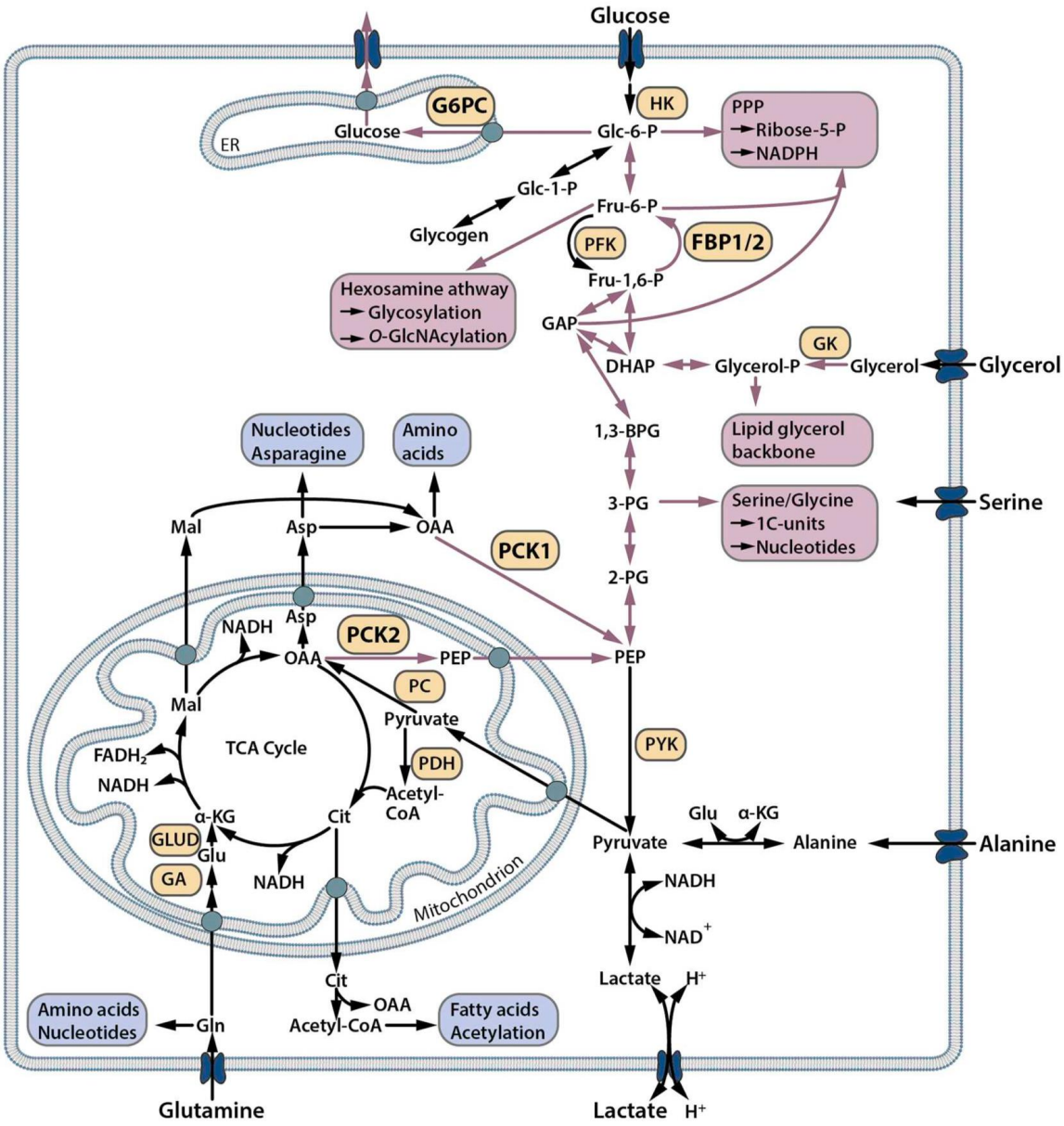

Figure 1: Glycolysis, gluconeogenesis and branching biosynthetic pathways. Glycolysis describes the conversion of glucose into pyruvate and one H+ ion. Pyruvate can then oxygen-dependentely be metabolised in the TCA cycle or be reduced to lactate. Gluconeogenesis, in contrast, results in the formation of glucose from non-carbohydrate precursors like lactate or amino acids, it thereby is at least in some of the reactions the reversal of the glycolysis. The initial step is mediated by PCK1 in the cytosol and by PCK2 in the mitochondria. It is one of three mediated reactions that are not the reversal of the glycolysis. Glycolysis and gluconeogenesis can run partially to allow the formation of cellular building blocks. Figure from Grasmann et al. 2019 (27)

The role of the enzyme PCK1/2 is to convert oxaloacetate (OAA) along with GTP into phosphoenolpyruvate (PEP) while releasing carbon dioxide and GDP. This reaction represents the first step of gluconeogenesis and connects the TCA cycle with the glycolytic pools.

The expression of gluconeogenesis enzymes is not limited to gluconeogenic tissue. PCK2, the mitochondrial isoform of PCK1, is active in gluconeogenic tissue like the liver but has also been found in the pancreas, brain, leukocytes, heart and neurons, in bronchial epithelial, as well as in tumour cells of different origin such as lung, breast and prostate cancer (35, 36). Studies with mammary carcinoma cells showed increased cell death under glutamine deprivation or endoplasmic reticulum (ER) stress conditions when PCK2 was silenced (35). It further has been shown that a knockdown of PCK2 using either siRNA or shRNA in NSCLC cells can reduce cell growth and enhance cell death by diminishing their colony-forming capability under nutrient-low conditions (37). A possible mechanism by which PCK2 expression increases cell survival and cell growth is the de novo synthesis of serine and glycine, which plays a vital role in nucleotide synthesis and cell proliferation. (31, 35, 37). Besides, PCK2 has been shown to mediate glycerol phosphate formation under glucose deprivation, required for the biosynthesis of phospholipids (38). PCK2 ensures the generation of important building blocks in cancer cells under glucose deprivation, eventually promoting proliferation and cell survival (27). PCK2 could, therefore, be an interesting target in therapeutic intervention in lung cancers.

In cancers arising from the primary gluconeogenic organs, liver and kidney, a tumour-suppressive function of fructose-1,6-bisphosphate, one of the other gluconeogenic pacemaker enzymes, and partly also PCK1/2 was found. For reviews see Grasmann et al. (27) and Wang et al. (39). PCK1 has also been shown to directly phosphorylate insulin-induced gene (INSIG) proteins to activate lipolysis in hepatocellular carcinoma cells (40). Glycolytic/gluconeogenic enzymes are increasingly acknowledged to play a role in signalling, partly by so-called moonlighting functions, for example, as transcription factors (41). In a study on colon cancer cells, some of the effects of PCK1 on metabolism have been suggested to be mediated by activation of the signalling node mechanistic target of rapamycin complex 1 (mTORC1) (42). The role of PCK2 in cell signalling, however, remains poorly understood.

1.8 The PI3K/AKT/mTOR signalling pathway

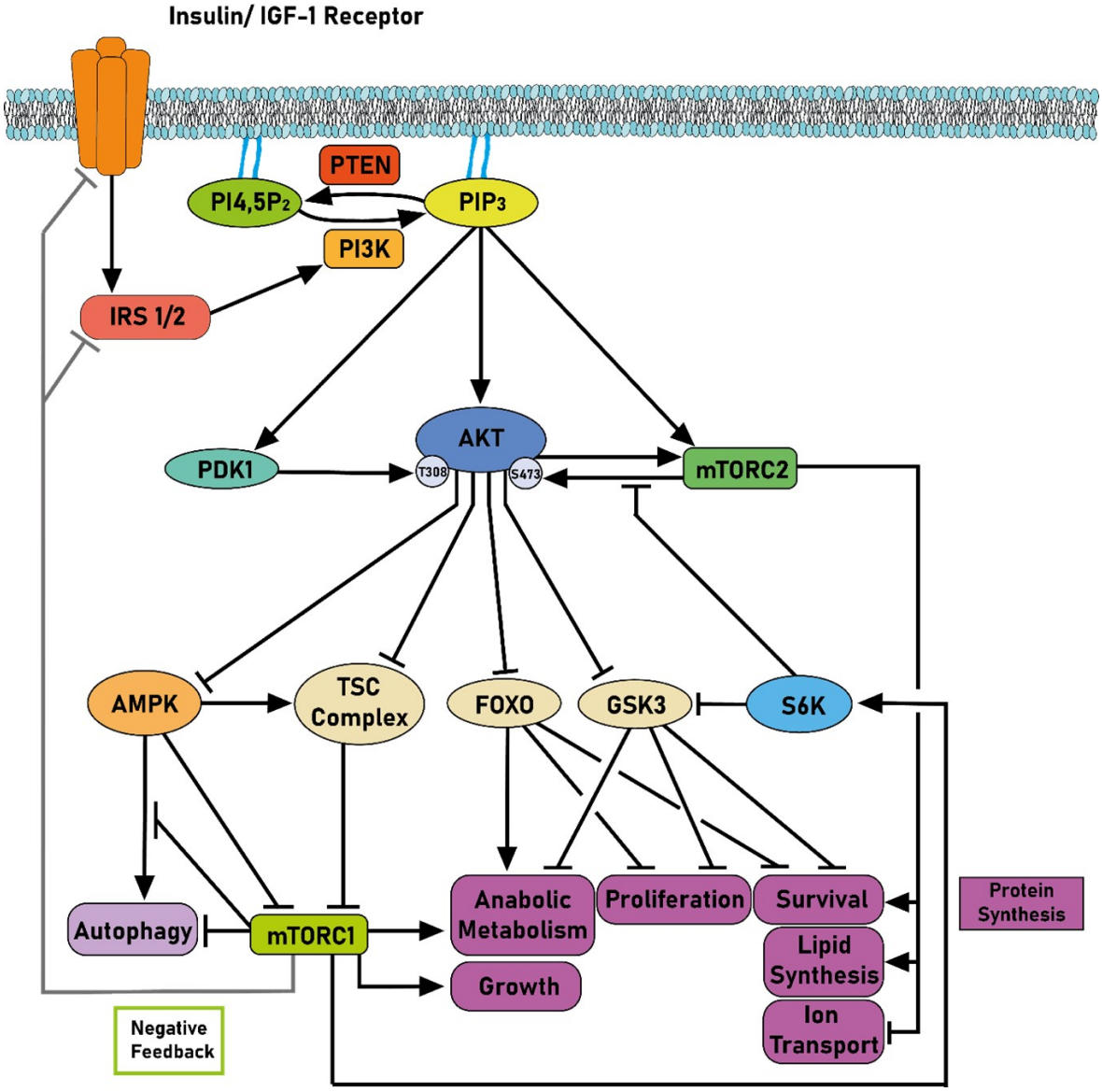

Figure 2: The PI3K/AKT/mTOR signalling pathway. Central to the pathway is the protein kinase B (AKT), which regulates downstream targets leading to a series of metabolic effects. Also, of high relevance are mTORC1/2, as they are directly responsible for the control of many following signalling cascades and feedback loops.

IGF-1, insulin-like growth factor 1; IRS1/2, insulin receptor substrate 1/2; PIP_2, phosphatidylinositol 4,5-bisphosphate; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PIP3, phosphatidylinositol triphosphate; PDK1, phosphoinositide-dependent kinase-1; AKT, protein kinase B; mTORC1/2, mechanistic target of rapamycin complex 1/2; AMPK, AMP-activated protein kinase; TSC, tuberosclerosis complex; FOXO, forkhead box protein O1; GSK3, glycogen synthase kinase 3; S6K, ribosomal protein S6 kinase beta-1.

The primary function of the PI3K/AKT/mTOR signalling pathway (**figure 2**) is the regulation of the cell cycle. The pathway plays a vital role in many cell processes and is closely connected to the signalling related to growth, survival, proliferation, metabolism, autophagy and glucose uptake. Malfunctions of this system can thus lead to decreased cell survival or else the formation of cancer. Mutations leading to cancer formation can both be activating (oncogenes) or inactivating (tumour suppressor genes). The phosphatase and tensin homolog (PTEN) mutation, which is usually responsible for inhibiting the protein kinase B (AKT) activation, is an example of a loss of function mutation. The mutation of a regulatory subunit of AKT and thereby upregulation of AKT is an example of an oncogene mutation. (43, 44)
The phosphoinositide 3-kinase (PI3K) activates AKT and thereby stimulates downstream effectors. Different mechanisms can activate PI3K; one is the signalling downstream of the epidermal growth factor receptor (EGFR). Another pathway leading to downstream activation of PI3K is the insulin and insulin-like growth factor 1 (IGF-1) pathway with the insulin receptor substrates (IRS). Several effectors antagonising the pathway exist. The tumour suppressor phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) inhibits PI3K and thereby negatively regulates the PI3K/AKT pathway. PTEN is frequently mutated in different cancer types, including lung cancer. (43, 45, 46)

Until now, three different isoforms of AKT have been described, Ak1, AKT2 and ATK3. They are proteins of the protein kinase B (PKB) family acting as serine/threonine kinases. AKT has an essential role in cell survival and, for that purpose, regulates various downstream effectors. High AKT activity is associated with proliferation and invasiveness, so tumour cells may even depend on it for survival (47). AKT has thus become the focus of ongoing cancer research.

By inhibiting TSC 2, AKT removes the inhibitory effects of TSC 2 on mTORC1. mTORC1 is an important regulator of cell metabolism and cell growth. It receives the input of a broad network of signalling pathways and influences a variety of effectors in return. Apart from promoting anabolic processes, mTORC1 promotes cell growth by suppressing catabolic processes like autophagy. Another way for AKT to influence proliferation, metabolism and survival is the inhibition of the Forkhead Box O (FOXO) transcription factors and the glycogen synthase kinase 3 (GSK3) (43). Also interesting in this context is the enzyme AMP-activated protein kinase

(AMPK), which activates autophagy, while also suppressing the inhibitory effects of mTORC1 on autophagy, both directly and via activating the TSC complex. In turn, mTORC1, under replete nutrient conditions, inhibits the promoting effects of AMPK on autophagy. This shows how autophagy is regulated by these two proteins depending on the respective cell signalling and the metabolic situation. For analysing the activation level of mTORC1, the downstream target p70S6 kinase (S6K), involved in protein synthesis regulation, can be used as it is directly phosphorylated by mTORC1. (45)

mTORC1 is mainly responsible for the control of mRNA translation, metabolism and protein turnover. mTORC2 controls the cell's metabolism and is responsible for strong survival signalling. The phosphorylation of AKT on Serine 473 and thus activation of AKT is one of the most important functions that have been described so far. However, additional phosphorylation at the kinase domain at Threonine 308 through phosphoinositide-dependent kinase 1 (PDK1) is necessary for maximal activation of AKT. Other functions of mTORC 2 include the regulation of ion transport and lipid biosynthesis. (43, 45)

2. Hypothesis and aims of the study

Much current cancer research focuses on targeting receptors with monoclonal antibodies. Another approach, however, is inhibiting enzymes and signalling pathways to curb the metabolism of cancer cells specifically. As a consequence of rapid growth rates, extensive biomass production and high energy consumption, cancer cells are highly dependent on metabolites. Due to the heterogeneous structure of tumours, not all cancer cells are equally well supplied with resources. Our research group's previous studies suggest that lung cancer cells may overcome metabolic shortages by using at least some gluconeogenesis steps (31). Inhibition of certain gluconeogenesis pacemaker enzymes could be of interest to the development of new targeted therapies. As mentioned in chapter 1.7., PCK1 has been indicated to modulate cell signalling in some cancer cell lines, but only little is known about the role of gluconeogenesis, particularly PCK2, on signalling pathways in lung cancer. We, therefore, investigated the impact of PCK2 on central growth-related signalling pathways in lung cancer cells. This study focused on PCK2 since our group was able to show that this enzyme is the prime isoform expressed in lung cancer (36, 48). The protein kinase AKT is one of the main effectors in cancer cells leading to a series of signalling cascades downstream and hence was the main target of this study. Since simultaneous phosphorylation of Serine 473 and Threonine 308 is a requirement for full activation of the protein kinase, phosphorylation levels of both were analysed to evaluate AKT activity (49). Likewise of relevance to this study were mTORC1 and mTORC2 due to their strong regulatory effects downstream on metabolism and tumour survival as well as their close interdependence with AKT. p70S6 kinase (S6K), a downstream target of mTORC1, served as a marker for activity levels of mTORC1. The enzyme AMP-activated protein kinase (AMPK) was analysed due to its interdependence with mTORC1, for being a significant activator of autophagy and for its role in sensing energy levels. To gain insight into cell-specific differences, the main experiments were conducted with two different NSCLC cell lines, namely A549 and H23. Moreover, because signalling pathways related to growth and metabolism are fundamentally different in a starvation context, all experiments were performed under high and low glucose conditions.

3. Material and methods

3.1 Cell culture

3.1.1 Cell lines

Two different human NSCLC cell lines were used in this study. The first line NCI-H23 (ATCC number CRL-5800), further referred to as H23, was obtained from American Type Culture Collection (ATCC, Manassas, VA). The second line, A549 (Cat. No. 300114), was obtained from Cell Lines Service (Eppelheim, Germany). Roswell Park Memorial Institute 1640 (RPMI) medium (Gibco) with additional 10% fetal bovine serum (Biowest, Nuaillé, France), L-glutamine (Gibco) and antibiotics was used to culture H23 cells. Equally, A549 cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Carlsbad, CA), which was supplemented with 10% fetal bovine serum (Biowest,) L-glutamine (Gibco), and antibiotics (100 µg/ml streptomycin and 100 units/ml penicillin). Regular testing ruled out mycoplasma contamination.

3.1.2 Transfection

Of the two cell lines, each was plated in two 6-well plates with 2 ml per well of the aforementioned media (further referred to as “normal growth media”) with a concentration of 200 000 cells per well. The cell numbers in all experiments were always measured using the CASY® cell counter (Schärfe System, Reutlingen, Germany). After 24 hours, the media were removed and replaced with fresh media. Cells were transfected with smart pool non-silencing siRNA (Csi, Dharmacon) or smart pool PCK2 siRNA (Dharmacon). In addition, untransfected cells were included. The transfection was performed with the Jet Prime® kit (Polyplus-Transfection, New York, USA) according to the manufacturer. Another 24 hours later, the media were changed again.

3.1.3 Low/high glucose treatment

On the fourth day, each well was washed twice with PBS (phosphate-buffered saline). After washing, the cells received treatment with 2 ml/well DMEM medium

(A549) or RPMI medium (H23) containing low glucose (0.2 mM) or high glucose (10 mM) concentrations. Low and high glucose media were prepared by adding appropriate amounts of stock solutions of glucose (1 M) or glutamine (200 mM) to serum-free media lacking glucose and glutamine. Stock solutions of glucose and glutamine in water were filtered through 0.2 µm filters. Aliquots were stored at -20°C. Antibiotics were added to the media as described above. Treatments were performed for 24 hours. An overview of low/high glucose treatments can be seen in **table 1**.

Table 1: Low/high glucose treatment of PCK2 silenced/control cells

Treatment	*Non-transfected*	*Csi*	*PCK2si*
High glucose (10 mM)	*Sample 1*	*Sample 2*	*Sample 3*
Low glucose (0.2 mM)	*Sample 4*	*Sample 5*	*Sample 6*

Note: Csi, non-silencing siRNA; PCK2si, PCK2 siRNA.

3.1.4 Collection

On the final day of the experiment, all wells were washed with phosphate-buffered saline (PBS). Cells were lysed on ice, for protein analysis, in radioimmunoprecipitation assay buffer (RIPA buffer) (Sigma-Aldrich) containing phosphatase and protease inhibitors and were subsequently stored for further analysis by Western blot. For RNA isolation, cells were lysed in RNA lysis buffer (PeqLab) at room temperature and stored for subsequent analysis by qPCR.

3.1.5 Rapamycin/Torin

One variation of the experiment was carried out only with the H23 cell line. Instead of regular high/low glucose treatment on the fourth day of the experiment, the cells received additional Rapamycin (100 nM, Selleckchem.com), Torin1 (50 nM, Selleckchem.com) or dimethyl sulfoxide (DMSO) treatment as a control. The treatment was carried out as follows. Cells were transfected with PCK2 siRNA or non-silencing siRNA as described above. 48h after transfection, cells were washed

twice and treated with low or high glucose medium containing Torin1, Rapamycin or DMSO as vehicle control. An overview of the different treatments resulting in the following setup can be seen in **table 2**.

Table 2: Rapamycin or Torin1 treatment in H23 cells

Treatment	*DMSO (Control)*	*Rapamycin*	*Torin 1*
Csi siRNA	*Sample 1*	*Sample 2*	*Sample 3*
PCK2 siRNA	*Sample 4*	*Sample 5*	*Sample 6*

Note: Csi, non-silencing siRNA; PCK2si, PCK2 siRNA; DMSO, dimethyl sulfoxide.

The complex mTORC2 is responsible for the phosphorylation of AKT at the locus Serin 473. Therefore, this experiment was used as proof of principle to evaluate whether mTORC2 was responsible for potentially observable changes in phosphorylation levels and whether they changed when mTORC2 was inhibited via Torin. The aim was to inhibit each mTOR individually. There is, however, no specific inhibitor targeting mTORC2 which is why Torin as a dual inhibitor was used instead. Rapamycin, in contrast, is a relatively specific inhibitor for mTORC1.

3.1.6 Insulin-like growth factor 1 (IGF-1) treatment

A separate experiment was conducted, including both cell lines A549 and H23, utilising Insulin-like growth factor 1 for treatment. The media used was the same as in the experiments before. H23 cells were plated at 400,000 cells per well in standard growth medium and A549 in DMEM containing 300,000 cells per well. After 24 h, all wells received fresh media. Cells were treated with 1 µl recombinant IGF-1 (Thermo Fisher Scientific) dissolved in 0.1% bovine serum albumin (BSA) in PBS or left untreated. After an incubation period of 45 minutes, all wells were washed with PBS and collected for protein with 75 µl RIPA buffer per well containing phosphatase inhibitor and proteinase inhibitor.

3.2. BCA protein assay

The collected samples' protein concentration was analysed using the BCA Protein Assay Kit (Thermo Scientific™). Since whole cells were collected in the cell culture, samples were first sonicated (Hielscher Ultrasonics, Teltow, Germany, ultrasonic processor UP50H) twice for 3 seconds (cycle 1, amplitude 80%) to break open the cell compartments to allow reliable protein measurement. Lysates were centrifuged at 4°C and 13,000 rpm for 10 minutes to remove insoluble cell debris. Five µl of the sample was diluted with 20 µl of RIPA Buffer and applied to the 96 well plates. Further on, 200 µl of a prepared mixture of BCA solution and copper sulphate (1:50) was added. A linear calibration curve was created using 25 µl RIPA Buffer and additionally 25 µl of a protein standard resulting in a serial dilution with decreasing concentration. All samples and all protein standards were measured in duplicates; air bubbles were well avoided. Absorption was measured in 96 well plates using the Spectramax Plus 384 (Molecular Devices, Orleans Drive Sunnyvale, CA) at 562 nm.

3.3. Western blot

Table 3: Buffers for electrophoresis and Western blot

Running buffer 10x	*Transfer buffer 10x*	*TBS 10x*	*TBS-T*
30 g Tris Base	*28 g Trizma Base*	*31.5 g Tris HCL*	*100 mL 10x TBS*
144 g Glycine	*143 g Glycine*	*80 g NaCl*	*1000 µL Tween 20*
100 mL SDS			
Ad 1000 mL with A.D. adjustment to pH 7.5	*Ad 1000mL with A.D. adjustment to pH 7.5*	*Ad 1000 mL with A.D. adjustment to pH 7.5*	*Ad 1000 mL with A.D.*

Note: The pH was adjusted by titrating to a pH of 7.5 with 1 M NaOH and eventually filling up to 1 L with Aqua destillata (A.D.) The Running Buffer 10x was diluted with A.D. before use in the ratio of 1:10. 1x Transfer Buffer (2000 mL) was prepared using 200 mL 10x Transfer Buffer, 400 ml methanol and 1400 mL A.D.

Table 4: Antibodies used

Primary antibody	*Dilution*	*Catalogue no.*	*Manufacturer*
P-AKT(S473)	1:1000	*#4058*	Cell Signaling
P-AKT(T308)	1:1000	#5106	Cell Signaling
T-AKT	1:1000	*#9272*	Cell Signaling
P-AMPK	1:1000	*#2535*	Cell Signaling
T-AMPK	1:1000	*#2532*	Cell Signaling
P-S6K	1:1000	*#9205S*	Cell Signaling
T-S6K	1:1000	*#9202S*	Cell Signaling

Note: Bovine serum albumin (BSA) was used for all antibodies as a blocking reagent except for β-actin for which milk was used.

3.3.1 Electrophoresis

For electrophoresis RIPA buffer and 5x Laemmli buffer containing mercaptoethanol were added to all samples to a final concentration of 1x Laemmli buffer. Thereafter, all was heated up to 95°C for 10 min. The samples containing 20 µg of protein were then loaded onto 10% acrylamide gels and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using the Mini-PROTEAN® electrophoresis unit (BioRad, Hercules, CA) in 1x running buffer. Protein standards (BioRad) were included in a separate lane. Running conditions were 160 volts for 60-90 minutes. The composition of the buffers and reagents is shown in **table 3.**

3.3.2 Blotting and immunodetection

Subsequently, the protein was transferred onto PVDF membranes (BioRad) previously activated for 15 seconds in methanol. The transfer was done at 400 mA constant electric current for 120 minutes in a transfer buffer containing 20% methanol. After this step, the membranes were blocked in 5% BSA in tris-buffered saline with Tween20 (TBS-T) for one hour at room temperature. Following this, the membranes were incubated with the respective primary antibody in 5% BSA

overnight at 4°C. All antibodies and antibody dilutions are listed in **table 3**. The following day, membranes were washed with TBS-T three times for 10 minutes and then incubated with the secondary antibody diluted in TBS-T for 1 hour at room temperature. Horseradish peroxidase linked anti-mouse or anti-rabbit antibodies were used as the secondary antibodies at a dilution of 1:2,000 or 1:3,000, respectively. The protein immunodetection substrate (SuperSignal® West Pico chemiluminescent substrate, Thermo Scientific) or the ECL Prime Western Blotting Detection Reagent (GE Healthcare) was then applied to the membranes and the chemiluminescence detected with the ChemiDoc Touch (BioRad).

3.3.3 Quantification of Western blots

Quantification of the immunodetection signal in ChemiDoc images was done with Image Lab. Firstly, the samples were normalised to the control sample (untreated, high glucose). Subsequently, a ratio of the phosphorylated AKT (Ser473) to the total AKT was calculated.

3.4 Quantitative real-time polymerase chain reaction (qPCR)

3.4.1 RNA isolation and reverse transcription

Total RNA (1µg) was extracted and cleared of all DNAs from the obtained samples using the peqGOLD Total RNA Kit (VWR, Vienna, Austria). The amount of RNA was then measured with the NanoDrop (Thermo Scientific, Fremont, USA). The cleaned-up RNA was thereafter pipetted into PCR strips altogether with nuclease-free water, qScript Reaction Mix and qScript Reverse Transcriptase (all from the qScript™ cDNA Synthesis Kit, QuantaBio), resulting in a total of 20 µl. cDNA synthesis was performed using the My Cycler™ thermal cycler (Bio-Rad) according to the manufacturer's protocol. All steps were performed on ice.

Table 5: qPCR primer sequences

qPCR Primer	*Manufacturer*	*Forward sequence/ reverse sequence*
IGF1R	*Eurofins*	*F 5' – AAAAGGAATGAAGTCTGGCTCC –3'* *R 5'– GCCCGCAGATTTCTCCACTC – 3'*
SLC2A1	*Eurofins*	*F 5' – TGGCATCAACGCTGTCTTCT –3'* *R 5'– AGCCAATGGTGGCATACACA – 3'*
SLC7A1	*Eurofins*	*F 5' – TCTGTCTGTTCGCGATCCT –3'* *R 5'– TGCTGTTCAGAGCTGGAATATG – 3'*
SLC1A5	*Eurofins*	*F 5' – TGGACTGGCTAGTCGACCG –3'* *R 5'– GGGCAGCTCACTCTTCACTT – 3'*
ACTB	*Eurofins*	*F 5' – ATTGCCGACAGGATGCAGGAA –3'* *R 5'– GCTGATCCACATCTGCTGGAA – 3'*

3.4.2 Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed in the LightCycler 480 (Roche) in 384 well plates utilising the QuantiNova SYBR® Green PCR Kit. Master mixes including primers for qPCR primer sequences (see **table 5**) were prepared using 0.5 µl of a primer mix (containing forward and reverse primers diluted 1:10 in water) and 2.5 µl of SYBR® Green Master Mix per well. The master mix and primers were combined with 2 µl/ sample of 1:16 diluted cDNA. 4 µl of the mix was added per well. All measurements were done in duplicates. RT minus controls and NTC samples containing no template were included.

3.4.3 Primer testing

RNA from untreated A549 cells was reverse transcribed to cDNA as described before. Master mixes were prepared utilising the QuantiNova SYBR® Green PCR Kit with respective primers. Different dilutions of the cDNA in the ratio of 1:4, 1:8, 1:16 and 1:256 were prepared and added to the PCR 384-well plate, which was then placed in the LightCycler 480 (Roche, Vienna, Austria) for quantitative real-time PCR. For control purposes, an RT minus control (1:16, without reverse

transcriptase in the RT reaction) and an NTC sample (no template control, aqua dest. instead of cDNA) were included for each primer.

3.5 Statistical analysis

Statistical analysis was performed using Excel 2019. Group comparisons between the Control siRNA and the PCK2 siRNA were performed using a two-sided, unpaired Student´s t-test. Results came from at least three independent experiments in all cases but A549 (S473) due to the low quality of the signal on the PVDF membranes caused by air bubbles during the transfer of the protein. P-values smaller than 0.05 were considered significant. All graphs were made using Excel 2019.

For the qPCR's statistical analysis, the average of the Ct value of all duplicates was calculated. Following this, ΔCt was calculated by subtracting the Ct value of the gene of interest from the Ct value of the housekeeping gene ACTB, which was chosen due to its stable gene expression. The ΔΔCt was then determined by subtracting the ΔCt value of the control group from the ΔCt values of the treated PCK2 siRNA sample. The n-fold gene expression was obtained by using the $2^{-\Delta\Delta Ct}$ formula.

4. Results

4.1 PCK2 silencing

The initially most crucial finding was to evaluate whether the silencing of PCK2 with the commercially available PCK2 siRNA (smart pool siRNA, Dharmacon) was effective. This verification was a basic requirement to make any further assertions. Expression levels of PCK2 determined by Western blots were controlled with our loading control β-actin to ensure proper loading of the blots. The loading proved to be equal (**figure 3**), and decreased intensity both visually and quantitatively (Image Lab) confirmed that the silencing was successful since less PCK2 was detectable.

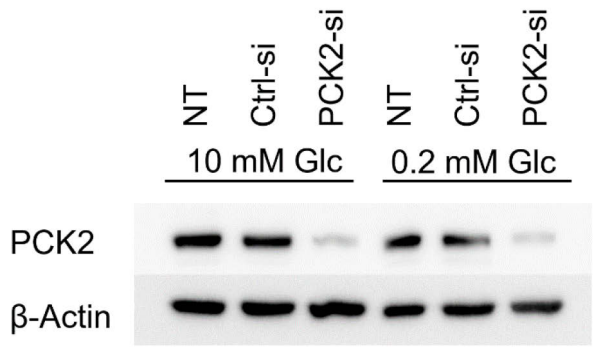

Figure 3: Proof of successful PCK2 silencing. Comparison between PCK2 and β-actin. The third lanes of the blot in high as well as low glucose media show decreased intensity when stained with PCK2 antibodies. When stained with β-actin, all lanes show a high intensity indicating proper loading.

4.2 Effects on AKT Serine 47

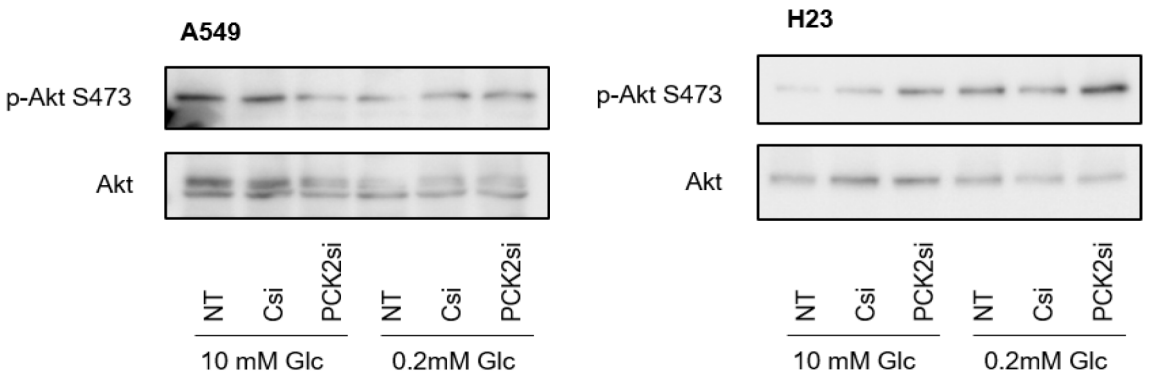

Figure 4: AKT Ser473 phosphorylation. Representative Western blots are shown. AKT phosphorylation was measured in A549 and H23 cells under high and low glucose conditions. NT, non-transfected; Csi, non-silencing siRNA; PCK2si, PCK2 siRNA; Glc, glucose.

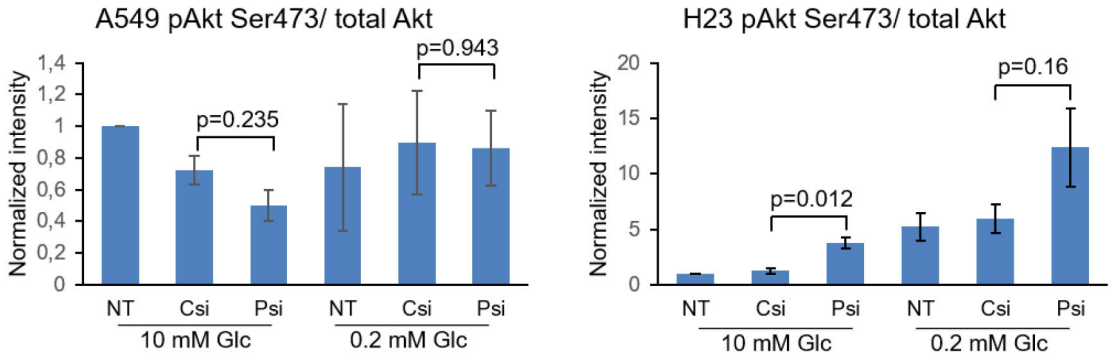

Figure 5: Semiquantitative assessment of AKT S473 phosphorylation. Results of densitometric analysis of AKT Serine 473 phosphorylation levels in A549 and H23 cells. All samples were normalised to the non-transfected (NT) low glucose sample and to the non-phosphorylated form. Results are shown as a mean +/- SEM from two (A549) or three (H23) independent experiments. NT, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA; Glc, Glucose.

This study's focus was to analyse the effect the silencing of the gluconeogenesis pacemaker enzyme PCK2 would have on the activation levels of AKT. Activation levels were determined using Western blots and analysing the phosphorylation levels of the locus AKT Serine 473. Effects, both under the conditions of starvation and non-starvation, were compared.

In H23 cells, PCK2 silencing led to a clearly increased phosphorylation of AKT S473 (**figure 4** and **figure 5**). This effect could be observed under high (10 mM) and low (0.2 mM) glucose conditions. Using densitometric analysis, the phosphorylation level of AKT in the high glucose group with silenced PCK2 (Psi) was significantly ($p = 0.012$) higher compared to the non-silenced siRNA control (Csi) (**figure 5**). The increase was not statistically significant under low glucose conditions, probably due to high variability (**figure 5**). These results suggest an impact of PCK2 silencing on AKT activation in H23 cells. The A549 cell line Western blots' results were somewhat inconclusive and suggested a slight decline in phosphorylation of the AKT locus Ser473 (**figure 4** and **figure 5**). However, due to technical reasons, the data on AKT Ser473 phosphorylation in A549 cells could be obtained only from two independent experiments.

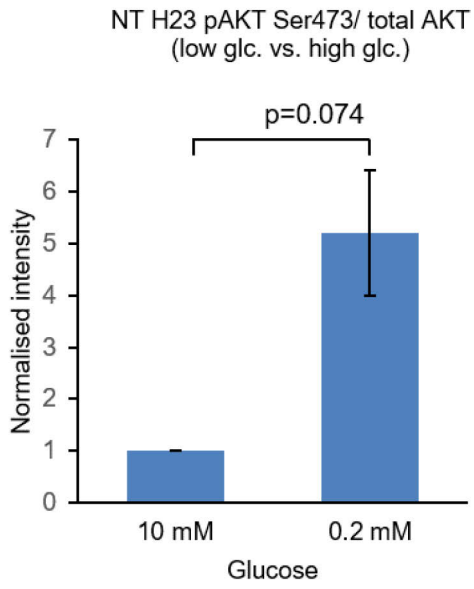

Figure 6: Semiquantitative assessment of H23 AKT Serine 473 phosphorylation levels in low glucose (0.2 mM) vs. high glucose (10 mM) conditions. Phospho-AKT Ser473 in high glucose treated untransfected cells was normalised to the low glucose treated cells. Results are shown as a mean +/- SEM from three (H23) independent experiments. NT, non-transfected; Glc, glucose.

Another interesting finding (**figure 6**) was that the phosphorylation level of AKT was much higher in H23 cells treated with low glucose than in the cells treated with high glucose medium. These findings were not statistically significant ($p = 0.074$) but showed a strong tendency indicating that the phosphorylation level of AKT might be

directly connected to the available amount of glucose in H23 cells, with increased activity of AKT under low glucose conditions in H23 cells.

4.3 Rapamycin-Torin

The Western blots (**figure 7**) of the Rapa-Torin experiment served the purpose of proving that mTORC 2 was the complex responsible for mediating AKT phosphorylation.

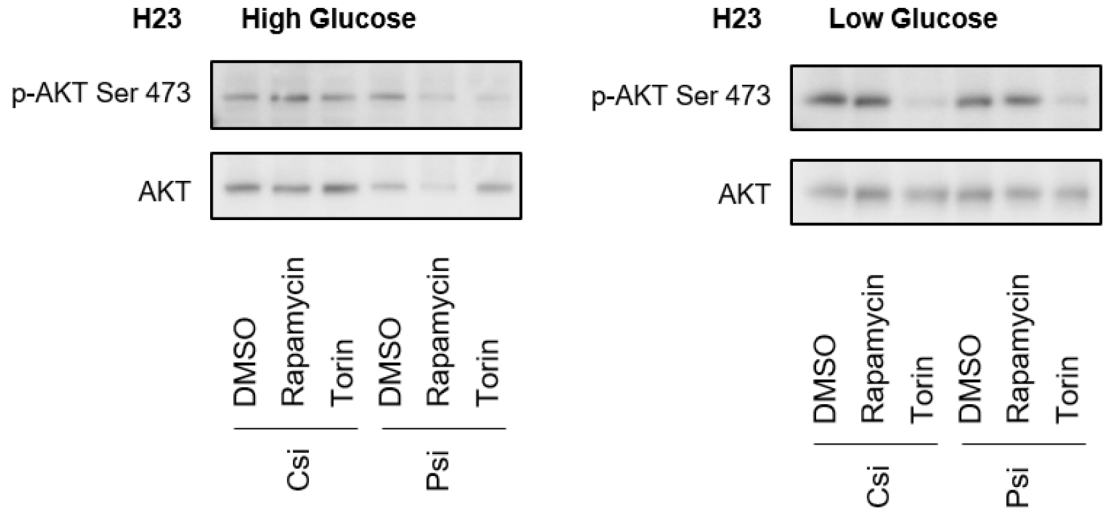

Figure 7: Phosphorylation level of AKT Serin 473 in H23 cells under high and low glucose conditions. Representative Western blots are shown. DMSO was used as a vehicle control. The total AKT is depicted as a reference to present the loading of the sample. All phosphorylation levels must be observed relative to the total AKT loaded. DMSO, Dimethylsulfoxide; Csi, non-silencing siRNA; Psi, PCK2 siRNA.

Under high and low glucose conditions, treatment with Torin, a dual mTORC1 and mTORC2 inhibitor, led to a visually observable decrease of phosphorylated AKT. This effect could be seen both in the PCK2 silenced (Psi) and in the control group (Csi). DMSO, which served as a vehicle control showed no influence on the phosphorylation level. Rapamycin, a selective mTORC1 inhibitor, did not cause any noticeable change in the degree of phosphorylation of no group whatsoever. Phosphorylation levels were determined relative to the total AKT levels. These data suggest that the mTORC2 rather than the mTORC1 inhibiting property of Torin caused changes in AKT phosphorylation levels.

4.4 Effects on AKT Threonine 308

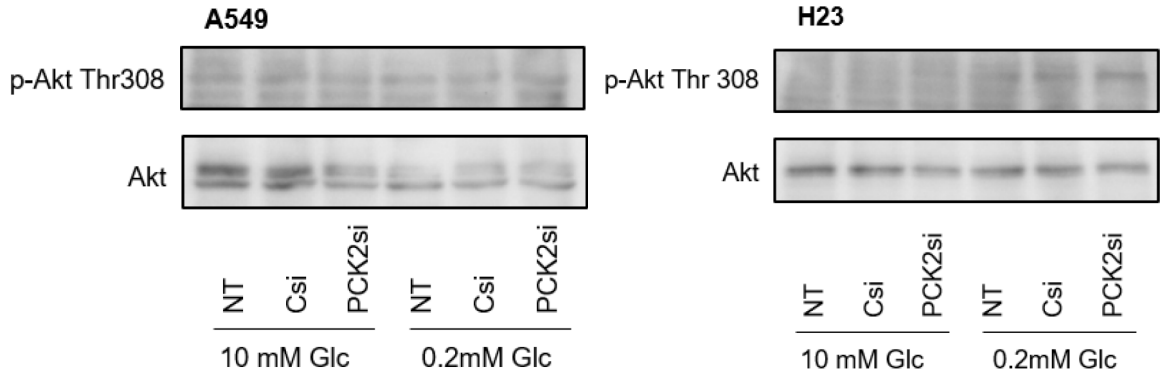

Figure 8: Phosphorylation levels of AKT Threonine 308 in A549 and H23 cells under high and low glucose conditions. Representative Western blots are shown. NT, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA; Glc, glucose.

The Western blot of the second AKT phosphorylation site, Threonine 308 (**figure 8**), showed very little phosphorylation. Unspecific bands also hampered the analysis. No impact of PCK2 silencing or glucose levels could be observed.

4.5 IGF-1

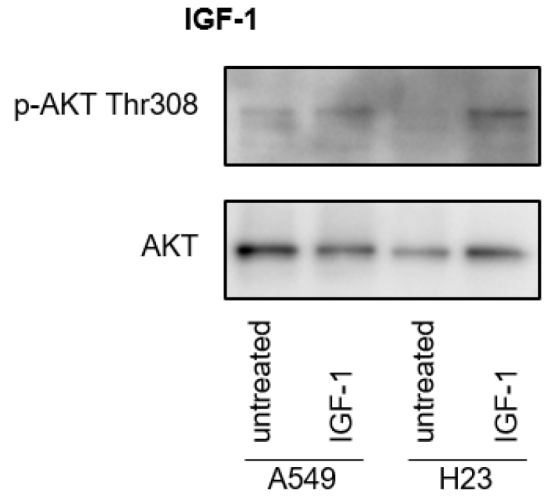

Figure 9: Phosphorylation level of p-AKT Threonine 308 in A549 and H23 cells with and without IGF-1 treatment. IGF-1, Insulin-like growth factor 1; After the plating of 400,000 cells/ well on the first day, all wells received fresh media on the second day and one of each cell line was treated for 45 min with 1µl IGF-1 (Gibco) before collection.

To generate a positive control for the p-AKT (Threonine 308) antibody, A549 and H23 cells were treated with insulin-like growth factor 1 (IGF-1), a known activator of AKT Threonine 308 phosphorylation.

The results of the performed Western blot can be seen in **figure 9**. Both cell lines showed increased signalling of the treated p-AKT lanes compared to the untreated taking the loading control of the total AKT into account. This confirms the assumption that activation of the Insulin-like growth factor 1 receptor leads, further downstream, to activation of AKT at locus Threonine 308 and further confirms the functionality of the antibody used.

4.6 Effects on S6K

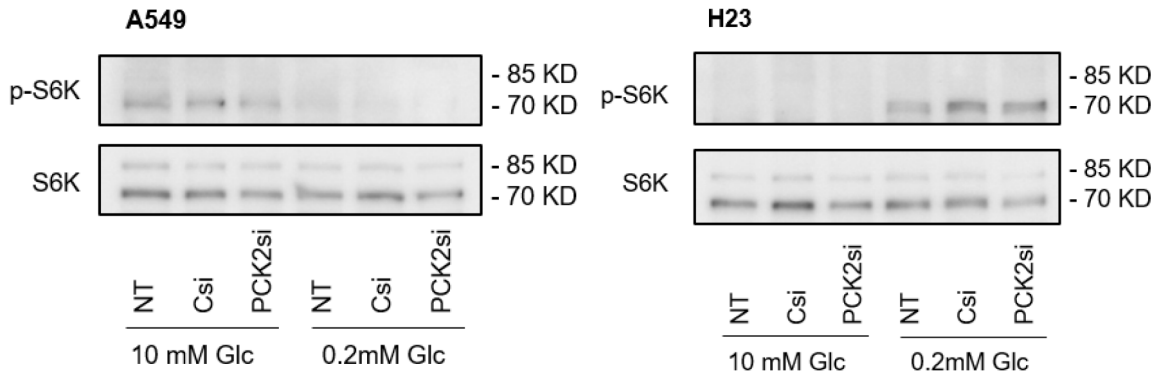

Figure 10: Phosphorylation level of S6K in A549 and H23 cells under high and low glucose conditions. Representative Western blots are shown. The antibodies used for p-S6k and S6K respectively, target both isoforms of S6K at 70 kDa and 85 kDa. NT, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA; Glc, glucose.

Another enzyme of interest was the p70S6 kinase (S6K). Since S6K is a downstream target of mTORC1, it can be used as an indicator of its activation levels. This experiment was thus performed to explore the effects of PCK2 silencing under starvation and non-starvation conditions on mTORC1 and its downstream targets.

Both cell lines showed no definable differences in phosphorylation of S6K between the non-transfected, the control and the PCK2 silenced group (**figure 10**). There were, however, cell line-specific effects depending on the glucose concentration of cells. The A549 cells showed increased activation of S6K in a high glucose

environment, which appeared to be a reasonable outcome since mTORC1 and S6K are both part of signalling pathways leading to increased translation and metabolic turnover, therefore requiring filled energy storages.

Less intuitive was the cell-specific outcome of the H23 cell line as it showed contrary effects. A low concentration of glucose increased phosphorylation levels of S6K. This effect will be further elaborated on in the discussion.

4.7 Effects on AMPK

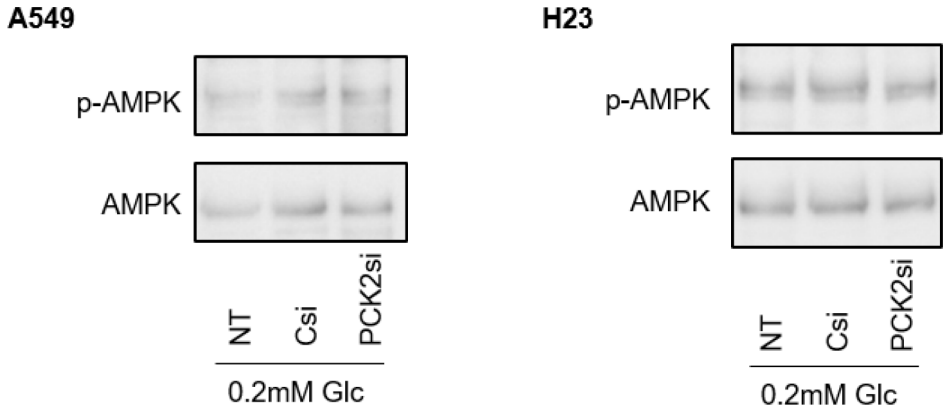

Figure 11: Phosphorylation level of AMPK in A549 and H23 cells under low glucose conditions. Representative Western blots are shown. NT, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA; Glc, glucose.

The effect on the AMP-activated protein kinase was examined due to AMPK's role in inhibiting the mTORC1 complex and its role in controlling protein synthesis and the cell cycle. High activation levels of AMPK are a marker for low energy levels within cells, indicating the activation of pathways that generate energy through autophagy (50). Preliminary experiments (**figure 11**) suggest that PCK2 silencing does not affect phosphorylation of AMPK under low glucose conditions in the cell lines examined. Moreover, no difference could be observed between the control group and the PCK2 silenced samples. As AMPK was not the focus of this study, no further experiments were performed to confirm this assumption or investigate any interactions between AMPK and PCK2. The Western blot representing the high glucose samples was not included as a result of poor quality. However, the results

suggested decreased phosphorylation compared with the low glucose samples in the H23 cell line. The A549 cell line did not show any observable differences between the high and low glucose samples.

4.8 qPCR

Along with protein samples, RNA was collected of the H23 cell line during the main experiment, which allowed for analysis via quantitative polymerase chain reaction (qPCR). High glucose samples with non-silencing siRNA (Csi) and silenced PCK2 (Psi) were normalised to the non-transfected sample. The same was done in the low glucose group.

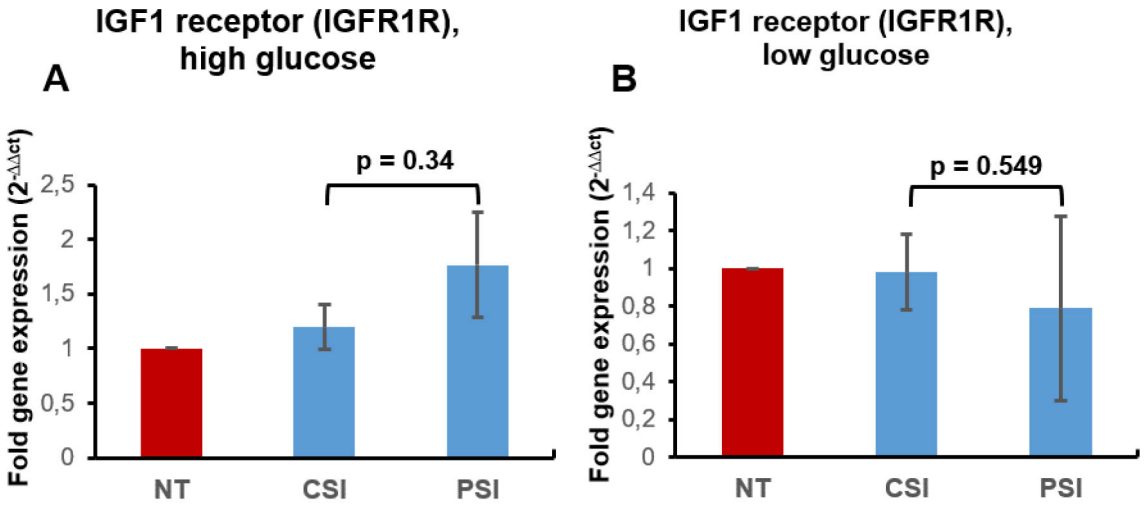

Figure 12: Expression of the IGF1 receptor (IGFR1R) in (A) high glucose and (B) low glucose conditions in H23 cells. Expression analysis was assessed with qPCR. All samples were normalised to the non-transfected sample. Results are shown as a mean +/- SEM from three different experiments. Nt, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA.

Since lung cancer cells have been reported to secrete IGF-1 and express IGF-1 receptors resulting in autocrine activation of the IGF-1/AKT pathway (51), we were quite interested in the expression levels of the IGF-1 receptor.

No differences in gene expression between PCK2 siRNA and control siRNA transfected cells could be observed (**figure 12**). In high glucose conditions (**A**), a slight increase in the IGF-1 receptor gene expression was noted, albeit this finding

was not statistically significant. Thus, no indications suggest that any difference in the IGF-1 receptor expression is responsible for enhanced AKT Serine 473 phosphorylation.

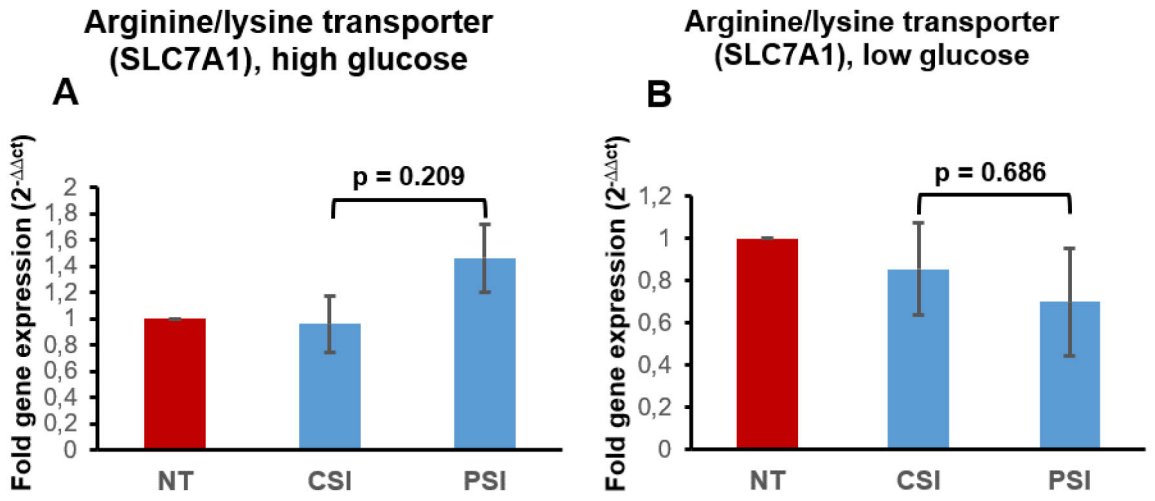

Figure 13: Expression of the arginine/lysine transporter (SLC7A1) for (A) high glucose and (B) low glucose in H23 cells. Expression analysis was assessed with qPCR. All samples were normalised to the non-transfected sample. Results are shown as a mean +/- SEM from three different experiments. Nt, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA.

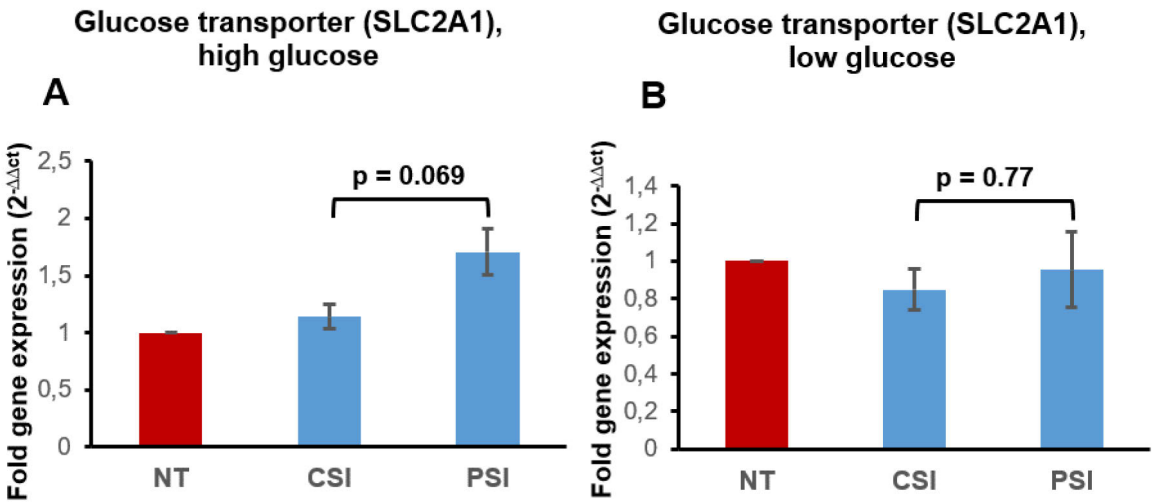

Figure 14: Expression of the glucose transporter (SLC2A1 (GLUT1)) for (A) high glucose and (B) low glucose in H23 cells. Expression analysis was assessed with qPCR. All samples were normalised to the non-transfected sample. Results are shown as a mean +/- SEM from three different experiments. Nt, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA.

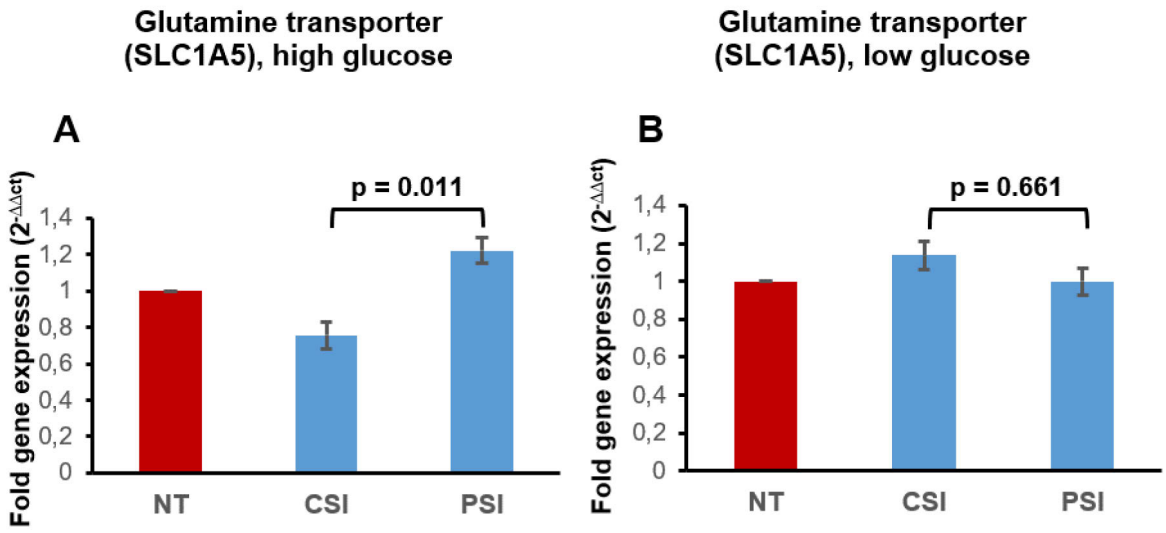

Figure 15: Expression of the glutamine transporter (SLC1A5) for (A) high glucose and (B) low glucose in H23 cells. Expression analysis was assessed with qPCR. All samples were normalised to the non-transfected sample. Results are shown as a mean +/- SEM from three different experiments. Nt, non-transfected; Csi, non-silencing siRNA; Psi, PCK2 siRNA.

Growth factor pathways are known to modulate the expression of several nutrient transporters. Analysis was, therefore, performed of a series of different nutrient transporters that could provide information about the metabolic implications of ongoing cell signalling. The examined transporters were for glucose (SLC2A1), glutamine (SLC1A5), and arginine/lysine (SLC7A1).

Under low glucose conditions (**figures 13-15, B**), none of the three transporters showed evidence of being different in their gene expression compared to the non-transfected sample or the control group. No statistical differences could be obtained.

However, in high glucose treated cells (**figures 11-13, A**), all three evaluated transporters showed increased expression of PCK2siRNA treated cells compared to the control group. In the case of the glutamine transporter (SLC1A5), this difference was statistically significant ($p = 0.011$).

5. Discussion

5.1 Discussion of results

The complexity and sheer amount of possible interactions of different signalling pathways activated in cancer cells is evident. Most pathways are closely entangled and resemble more a network than a flow chart. Their regulation by the metabolic state of the cell just recently came into focus. Here, the aim was to assess the interaction of glucose availability and gluconeogenesis activation with AKT, a signalling pathway with immediate relevance for cancer growth.

After evaluating the results, some notable differences between the control and the PCK2-silenced group were observed. In respect of our hypothesis and research interest, the most prominent result was the increased phosphorylation of AKT at (S473) in H23 PCK2 silenced cells (**figure 5**), indicating increased AKT activation of PCK2 silenced cells, leading further downstream to signalling related to proliferation and survival. This result was especially visible in the high glucose group (10 mM) with a statistical significance of $p = 0.012$. The low glucose group (0.2 mM) had a higher standard error of the mean (SEM) and showed no statistical significance but reflected the same tendency.

5.2 Impact of PCK2 on signalling pathways

Using Rapamycin and Torin, we were able to identify mTORC2 as the upstream kinase leading to AKT phosphorylation. However, the underlying mechanisms leading to the reduction of phosphorylated AKT Ser473 by PCK2 silencing are at present unknown. A study that could help us understand the mechanisms involved was recently published and focused on the modulation of AMPK and Unc-51 like autophagy activating kinase 1 (ULK1) dependent autophagy (52). The study found significant suppression of AMPK mediated autophagy in mesenchymal stem cells when PCK2 was deficient. According to this study, if applicable to our NSCLC cells, the silencing of PCK2 could have suppressed the signalling of AMPK (**see figure 16**). AMPK inhibits mTORC1 through the TSC-complex as well as directly and could,

therefore, via S6K or the insulin receptor substrate (IRS) negative feedback loop, influence AKT S473 phosphorylation levels (53, 54).

However, in contrast to this study, which was performed on mesenchymal stem cells (hMSCs), we found no apparent effect of PCK2 silencing on AMPK (**figure 11**). Thus, the enhanced AKT phosphorylation observed in our study does not appear to be mediated by altered AMPK signalling. Cell type-specific attributes appear to be responsible for the differences observed.

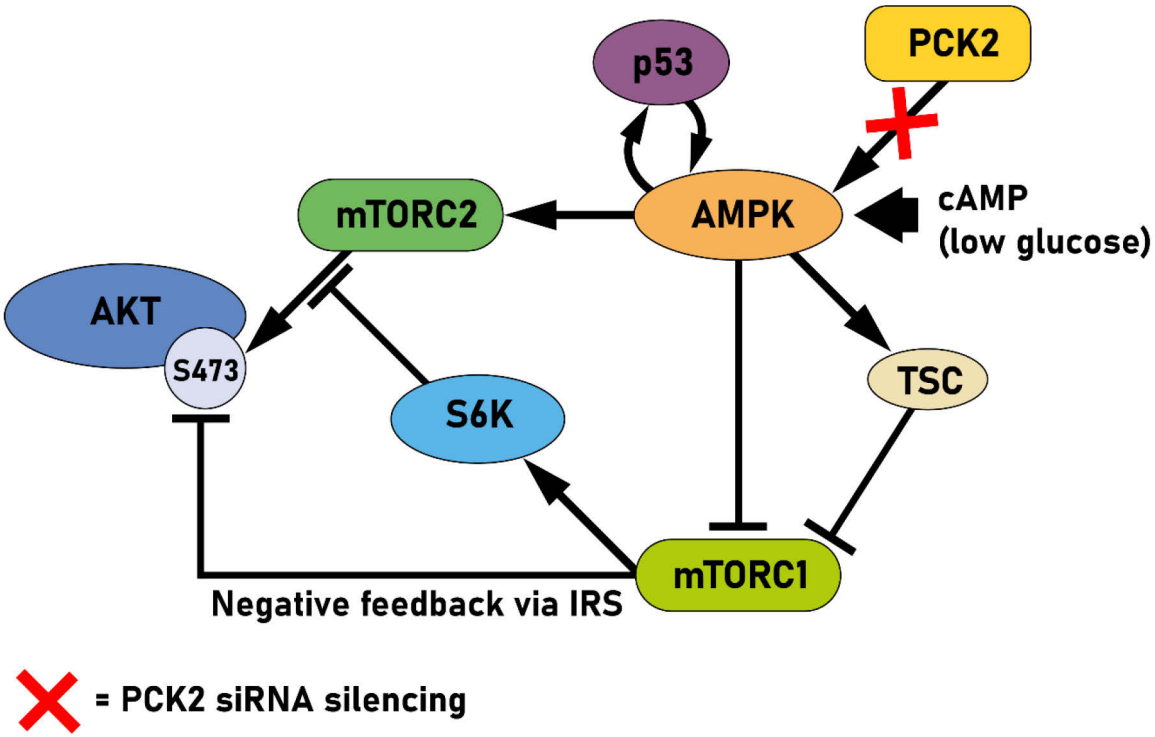

Figure 16: Interconnections of AMPK in the signalling pathway. This draft outlines the most relevant connections needed to explain our results. Some pathways were simplified for reasons of clarity and comprehensibility.

5.3 p53 – responsible for increased AKT phosphorylation under PCK2 silencing?

One notable difference between our two cell lines was the tumour protein p53 expressed as wildtype in A549 but as missense mutant in H23 cells. The tumour protein p53 is responsible for regulating apoptosis, controlling cell cycle arrest, and initiating DNA repair should the cell be confronted with stressors. The cell cycle arrest at the glucose-dependent checkpoint G1/S is induced by AMPK phosphorylating p53. A recent study on the role of p53 in cancer by Marco Cordani

et al. (55) found that p53, when mutant, was able to suppress the activity of AMPK. According to these studies, both the silencing of PCK2 and a missense mutation of p53 inhibit the activity of AMPK. Studies are pointing towards AMPK directly activating mTORC2 and thereby AKT S473, which would let us expect an even lower degree of AKT S473 activation and not the increase in phosphorylation we observed in the PCK2 silenced H23 cells (56, 57). Moreover, no impact of PCK2 siRNA on AMPK phosphorylation was found. Other enzymes regulated by p53 include PTEN and TSC2, both negatively regulate the IGF-1/AKT/mTOR pathways after stress (58). The exact regulatory mechanisms remain ominous, but an influence of mutant p53 on AKT activation by silencing PCK2 could certainly be possible.

5.4 AMPK – explanation for increased low glucose AKT phosphorylation?

The Western blots evaluating phosphorylation levels of AMPK showed no differences, neither between treated and non-treated cells nor in-between cell lines, which would suggest no direct link between AMPK and PCK2 (**figure 11**).

When, however, taking the AMPK high glucose Western blots, not included due to poor quality, into account, it showed that AMPK reacted differently in the two cell lines. The H23 Western blot had, as we expected, increased phosphorylation of the low glucose medium samples, whereas the A549 cell line had no delimitable differences between the high and low glucose medium samples' phosphorylation levels, indicating an inability to sense low glucose levels via AMPK in the A549 cell line.

This result reflects parts of our main AKT S473 experiment's outcome. Although the difference was not significant ($p = 0.074$), we observed in non-transfected H23 cell samples higher AKT S473 phosphorylation levels in the low glucose group compared to the high glucose group (**figure 6**). This might also explain why in contrast A549 had results that showed no distinguishable difference between high and low glucose treated cells.

The benefit for this potential activation under low glucose conditions is not entirely clear. Since AKT is responsible for downstream effects like proliferation and

survival, the question arises why survival signalling should be elevated under starvation. A potential answer to this question could be an increase in chances of survival. An adequate response to starvation in non-cancerous cells should be a shutdown of metabolic functions and cell cycle arrest, potentially even apoptotic cell death induced by AMPK while dependent on p53 (59).

5.5 Effects on AKT Threonine 308?

Neither PCK2 silencing nor different glucose levels resulted in any observable difference in AKT Threonine 308 phosphorylation (**figure 8**). According to the literature, negative feedback via mTORC1 inhibits IRS and its downstream target PDK1, responsible for the phosphorylation of AKT T308. Although, this may not be of major importance under our experimental conditions. However, the antibodies and phosphorylation mechanism's functionality could be confirmed by stimulating the PI3K/AKT signalling pathway with IGF-1 (**figure 9**). A reason for the absence of changes in AKT T308 could be a too short starvation period, with some studies suggesting the effects of feedback loops being dependent on acute or chronic stimuli (54).

5.6 The two faces of mTORC1

Western blots of S6K1 phosphorylation, of which we hoped to gain insight into the state of mTORC1 activation, showed impressive results (**figure 10**). No quantification of the Western blot was done since no apparent differences in the transfection groups could be perceived visually. It was, however, notable that glucose levels caused distinct changes in activation levels independent of transfection. A decrease of S6K1 phosphorylation, as mTORC1 activity marker, under low glucose conditions, as could be observed in A549 cells, was in line with the present literature since AMPK is responsible for sensing energy levels in the cytoplasm and inhibiting mTORC1 when glucose levels are low (60, 61). However, in H23 the opposite effect, i.e., the increase of S6K1 phosphorylation under low glucose levels, was observed. This finding is contrary to our previous findings regarding AMPK and especially surprising since our results suggest proper low

glucose sensing by AMPK in H23 but not in A549 cells. As previously discussed, this may be attributed to specific mutations in H23 cells, e.g., in genes regulating this pathway.

5.7 Transporter regulation

Amino acid and glucose transporters are frequently overexpressed in cancer following activation of pro-proliferative signalling pathways (62, 63). Therefore, we analysed the gene expression of the GLUT1 glucose transporter (SLC2A1) and the two amino acid transporters for arginine/lysine (SLC7A1) and glutamine (SLC1A5) under conditions of PCK2 silencing in different glucose levels using qPCR. Furthermore, we analysed the expression of the IGF-1 receptor (IGFR1R) since altered levels of this AKT upstream effector may potentially explain the increased AKT phosphorylation under PCK2 siRNA and low glucose treatment. Because RNA was only collected from H23 cells, no statement for the A549 cell line can be made, and no comparison between cell lines drawn. The gene expression of the IGF-1 receptor in PCK2 silenced cells was not clearly distinguishable from the control group in this study. However, all three other transporters showed increased gene expression under PCK2 silencing in the high glucose group.

In cancer cells, GLUT1 is upregulated by different oncogenes, e.g. by myc and HIF-1α (64). GLUT1 expression was increased in the PCK2 silenced sample. This outcome showed a clear tendency in the high glucose group but was not statistically significant. A study with a focus on the relation between PCK2 and GLUT1 was recently published by our group (E. Smolle et al., (48)). The study found an inverse correlation between PCK2 and GLUT1, which matches our observed results. The complete mechanism responsible for the regulation of amino acid transporters is not yet entirely understood. Recent papers suggest a dual function as transporters and signalling receptors (65). Our results showed increased expression of the arginine/lysine (SLC7A1) transporter and a statistically significant ($p = 0.011$) increase in the expression of the glutamine (SLC1A5) transporter in PCK2 silenced cells under high glucose conditions. High translation levels of transporters may be attributed to changes in signalling regarding the metabolic system, in this case, induced by the silencing of PCK2. As mTORC1 is the central signalling hub for

regulation of growth, while being equally dependent on growth factors and nutrients for activation, it likely is involved in the process of controlling the translation of transporters (60). However, whether mTORC2 activation is involved in these transporters' up-regulation should be clarified in future studies.

5.8 Limitations

Due to limited time, only three, in some cases only two, independent experiment replications were performed. Obtained results might have shown more explicit results with a higher chance for statistical significance had more separate repetitions been included in the statistical analysis. Another aspect to consider was the silencing of PCK2. Even though we could confirm our siRNA's efficacy in our experiments, only one siRNA was used in the experiments. The chance of possible off-target effects could have been further minimised by using different siRNA pools. The same is true for the antibodies; although our group had used and tested the antibodies beforehand, the possibility of unspecific reactions still exists. To gain further insight into the reasons for cell line-specific differences and to precisely explain their causes, more cell lines with similar mutation and property profiles will need to be included in future research.

The influence of PCK2 silencing on only a limited number of enzymes was evaluated. Many more could be affected or have shared interdependences. Another question that has not been fully answered is the effect of varying glucose concentrations between our used concentrations of 0.2 mM and 10 mM since correlations may not necessarily be linear. This approach, as well as the effects of different glutamine concentrations, could be further evaluated in future studies. All results were generated using in vitro methods. No in vivo approach was pursued whatsoever, which implicates that all results of this study can only be applied to in vivo situations to a very limited extent.

5.9 Conclusion

This study generated novel results that give insight into glucose-modulated activation of the PI3K/AKT/mTOR pathway and potential connections to PCK2 and gluconeogenesis. Although still preliminary, the study suggests that the up-regulation of PCK2 under low glucose limits mTORC2 activity in a cell-line dependent manner. Another important finding of the study is that modulation of the PI3K/AKT/mTOR pathway in response to low glucose conditions diverged fundamentally in the evaluated cell lines. The cell line A549 showed no impact on AKT S473 by PCK2 silencing. H23 cells, on the other hand, showed an increase in AKT S473 through PCK2 silencing. Regulatory mechanisms appear to be complex, and cell line-specific aspects will need to be considered in future research on this question. Using multiple cell lines with well-researched properties may be a feasible approach to study the heterogeneity in signalling pathway activation and attribute them to established cell line properties. Combining the analysis of related signalling pathways with assessments of growth and survival in cell culture may also be crucial to clarify whether activation of certain signalling pathways influences cell survival or growth under glucose starvation. Future investigations considering cell line-specific features will help to elucidate the role of PCK2 and gluconeogenesis in cancer.

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