Hypoxic regulation and selective silencing of pyruvate kinase isoforms PKM1 and PKM2 by siRNA

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III. Abbreviations

ATP	Adenosine triphosphate
ATCC	American type culture collection
ADP	Adenosine diphosphate
aa	Amino acid
AP	Alkaline phosphatase
ARNT	Aryl hydrocarbon receptor nuclear translocator
AMV	Avian Myeloblastosis Virus
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
bp	Base pair
bHLH	Basic helix loop helix
°C	Celsius
CBP	CREB binding protein
cDNA	Complementary deoxyribonucleic acid
ChoRE	Carbohydrate-response element
CTAD	C-terminal transactivation domain
cAMP	Cyclic adenosine monophosphate
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
dNTP	Deoxyribose nucleotide triphosphate
DNA	Deoxyribonucleic acid
ddNTP	Di-deoxyribose nucleotide triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
DEPC	Diethylpyrocarbonate
EDTA	Ethylendinitrilo-N,N,N',N',-tetra-acetate
ESS	Exonic splicing silencers
ECL	Enhanced chemi luminescence
FCS	Fetal calf serum
FGFR1	Fibroblast growth factor receptor 1
FIH-1	Factor inhibiting HIF-1

FBP	Fructose-1, 6-bisphosphate
FLuc	Firefly luciferase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GLUT	Glucose transporter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HIF-1α	Hypoxia-inducible factor-1α
HIF-2α	Hypoxia-inducible factor-2α
HRP	Horseradish peroxidase
HRE	Hypoxia response element
HnRNP	Heterogeneous nuclear ribonucleo protein
HPV16	Human papillomavirus type 16
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ID	Inhibitory domain
ISS	Intronic splicing silencers
Kb	Kilo base
kDa	Kilo dalton
Km	Michaelis constant
LDHA	Lactate dehydrogenase A
mm	Millimetre
min	Minute
ml	Milliliter
MCT	Monocarboxylate transporter
mRNA	Messenger RNA
M-MuLV	Moloney murine leukemia virus
Mw	Molecular weight
NTAD	N-terminal transactivation domain
NaCl	Sodium chloride
ODD	Oxygen-dependent degradation
Opa	Opacity associated proteins
Oct-4	Octamer-binding transcription factor 4
PBGD	Porphibilinogen deaminase
PBS	Phosphate buffer saline
PK	Pyruvate kinase

PKM	Pyruvate kinase m
PKM1	Pyruvate kinase m1
PKM2	Pyruvate kinase m2
PKL	Pyruvate kinase L
PCR	Polymerase chain reaction
PDK1	Pyruvate dehydrogenase kinase isozyme 1
PGK	Phosphoglycerate kinase
PVDF	Polyvinylidene fluoride
PEP	Phosphoenol pyruvate
pTyr	Phosphor tyrosine
PTB	Polypyrimidine tract binding protein
pO_2	Partial pressure of O_2
PKC	Protein kinase C
PHD	Prolyl hydroxylase domain
PML	Promyelocytic leukemia
PAS	Per-ARNT-Sim
P300	E1A binding protein p300
RT-PCR	Reverse transcription-PCR
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Rotations per minute
RPMI	Roswell park memorial institute
RT	Room temperature
RLU	Relative light units
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOSC3	Suppressor of cytokine signaling 3
ShRNA	Short hairpin RNA
SMP	Slim milk powder
SP1	Specificity protein 1
SP3	Specificity protein 3
si-RNA	Small interfering RNA
Rsv	Rous sarcoma virus
s	Second

TEMED	N',N',N',N'-Tetra methyl diamine
Tris	Tris-(hydroxy methyl)-amino methane
TBST	Tris-buffered saline and tween 20
TMB	Tetramethyl benzidine
UV	Ultra violet
USF	Upstream stimulating factor
V	Volte
V/V	Volume to volume
VHL	Von hippel-lindau

1. Introduction

1.1 Classification of pyruvate kinase enzymes

Pyruvate kinase (PK) catalyzes the dephosphorylation of phosphoenol pyruvate (PEP) to pyruvate, generating ATP in the last step of glycolysis. Active PK is a homotetramer composed of four identical subunits. Each subunit consists of four domains: the A, B and C domains and the N-terminal domain [1]. In mammalian cells, PK activity is regulated at the level of expression through allosteric regulation [1]. PK is expressed as four different isoforms named: type M1 (PKM1), type M2 (PKM2), type L (Liver) and type R (Red blood cell). PKM1 and PKM2 represent two splice variants of the PKM gene. Type L and type R isoforms represent two splice variants of the PKL gene that are regulated by different tissue specific promoters [1], [2], [3], [4], [5], [6]. Pyruvate kinase isoform R is expressed in erythrocytes, while pyruvate kinase isoform L is expressed in tissues with high gluconeogenesis, such as liver and kidney [7], [8].

1.1.2 PKM gene

The PKM gene consists of 12 exons and 11 introns. PKM1 splice variant contains exon 9 and the PKM2 splice variant contains exon 10 (Figure 1). The PKM gene expression is regulated under a variety of conditions [4]. PKM1 is expressed in tissues with high energy demand, such as skeletal muscle and brain. It has the highest affinity to its substrate PEP and its not allosterically regulated or phosphorylated [9].

PKM2 is expressed in tissues with a high rate of nucleic acid synthesis, such as fetal tissues. It was discovered in a hepatoma cell line and its elevated levels have been observed in numerous cancerous cells [6], [10], [11]. The PEP affinity and activity of PKM2 depends on its quaternary structure and is regulated by metabolic intermediates and oncogenes [9].

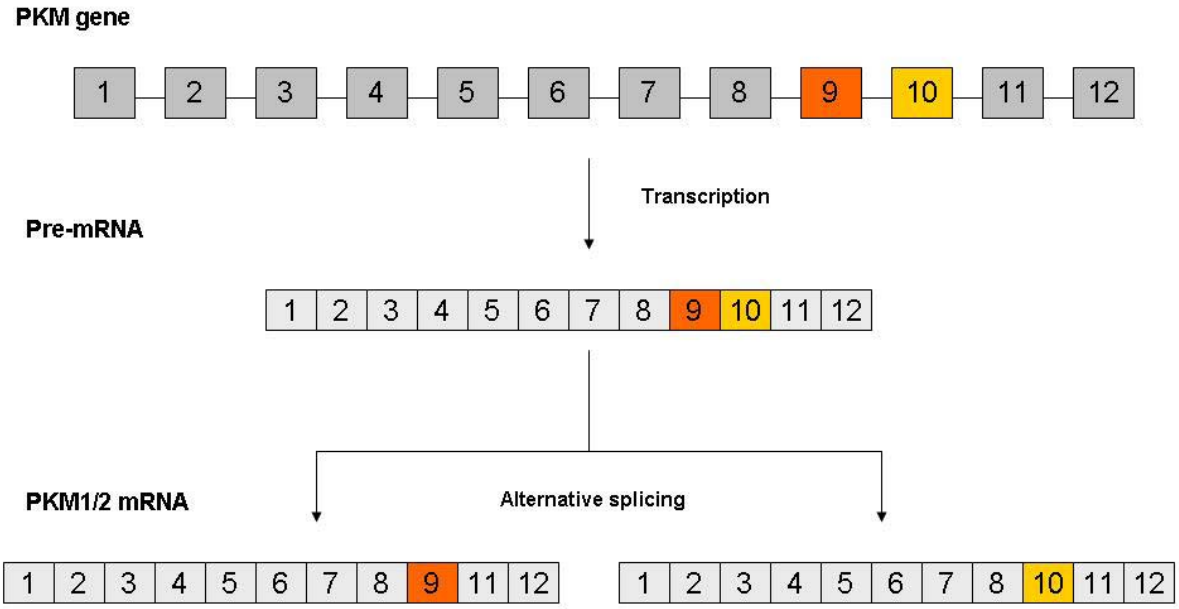


Figure 1: Schematic representation of the transcription of PKM1 and PKM2 mRNA from the PKM gene by mutually exclusive alternative splicing

The human PKM gene consists of 12 exons and 11 introns. PKM1 and PKM2 are different splicing products of the PKM gene. PKM1 contains exon 9, whereas PKM2 contains exon 10. Both of the mRNAs are 1593 base pairs long and differ from one another by 160 nucleotides from 1143-1303. At amino acid level, this results in a difference of 23 amino acid residues within this 56 amino acid stretch.

The PKM gene promoter consists of three *cis*-acting regions: box A, box B and box C downstream at -279 to -216 [12]. In addition, there are three GC boxes: GC Box 1, GC Box 2 and GC Box 3, which are located -133 bp from the transcription initiation site [13] (Figure 2). A mutation in either GC box 1 or 3, but not in GC box 2, resulted in a 50% decrease in promoter activity [8]. Five putative specificity protein (SP1) and (SP3) binding sites were found to be functional in PKM promoter [14]. SP1 and SP3 were functional as transcriptional activators at GC boxes 1 and 3 in the PKM promoter in SL2 cells [13]. In C2C12 myocytes, SP3 caused transcription repression, which was overcome under hypoxic conditions by down regulation of SP3 [9], [15]. Reactive oxygen species (ROS) decreased the SP1 mediated PKM promoter activity [16]. A carbohydrate-response element (ChoRE) was not precisely localized in the PKM promoter region, while putative consensus DNA-binding elements for upstream stimulating factor (USF) are present within the PKM promoter region [8]. Recently hypoxia response element (HRE) has been identified in intron 1 of PKM2 [17].

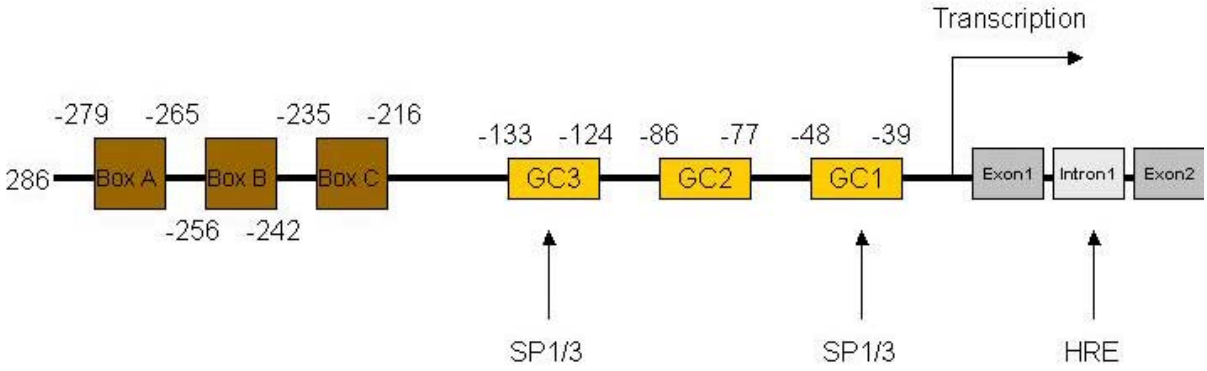


Figure 2: Schematic representation of the *cis*-acting elements and binding proteins of the PKM gene promoter

PKM gene promoter consists of three *cis*-acting regions downstream at -279 to –216: box A (-279 to -265), box B (-256 to -242) and box C (-235 to -216). Additionally, three GC boxes: GC Box 1 (-48 to -39), GC Box 2 (-86 to -77) and GC Box 3 (-133 to -124) are located 133 bp from the transcription initiation site. SP1 and SP3 had functional binding sites at GC boxes 1 and 3. Hypoxia response element HRE has been identified in intron 1 of PKM2.

1.1.3 Pyruvate kinase M2 (PKM2) expression

PKM2 expression is influenced by different factors, like glucose in rat hepatoma cells, which increased the amount of dephosphorylated transcription factor SP1 resulting a higher DNA binding activity [18]. In adipocytes, the PKM2 expression was increased by insulin independently of glucose and glucosamine, whereas in rat epididymal fat pads, the insulin required presence of either glucose and glutamine or glucosamine to increase PKM2 expression, indicating the involvement of the hexosamine biosynthesis pathway [9], [19], [20] [21], [22]. Thyroid hormone was found to increase PKM2 activity in the small intestine of hypothyroid rats [23]. Ras and the transcription factors SP1 and SP3 induced PKM2 [15], [22]. Stimulation with interleukin-2 increased PKM2 expression in proliferating thymocytes [24].

PKM2 expression was studied by investigating the PKM gene splicing mechanism of switching between PKM1 and PKM2. Heterogeneous nuclear ribonucleoprotein (hnRNP) protein family hnRNPA1, hnRNPA2 and polypyrimidine tract binding protein (PTB) were found to bind to intronic sequences, flanking exon 9 and resulting in exon 10 inclusion in Hela cells [25] (Figure 3). Depleting of hnRNPA1, hnRNPA2 and PTB using small

interfering RNA (si-RNA) showed an increase of PKM1 and a decrease of PKM2 in several different cell lines [25]. Changes in concentration of splicing factors in different tissues can be one mean of regulating tissue specific alternative splicing [26]. Highly differentiated mouse myoblast cell line C2C12 showed an increase of PKM1 and a decrease of PKM2, which was accompanied by decreases in hnRNPA1 and PTB. The expression of hnRNPA1, hnRNPA2 and PTB is controlled by the transcription factor c-Myc in gliomas and C2C12 [25] (Figure 3). Knockdown of c-Myc in NIH3T3 cells showed a decrease of hnRNPA1/A2 and PTB levels and an increase of the PKM1/PKM2 mRNA ratio. Depleting other proliferation transcription factors, such as E2F1 in HeLa cells and Rb in MCF-7 cells showed no decrease of hnRNP A1/A2 and PTB RNA levels [25], [26].

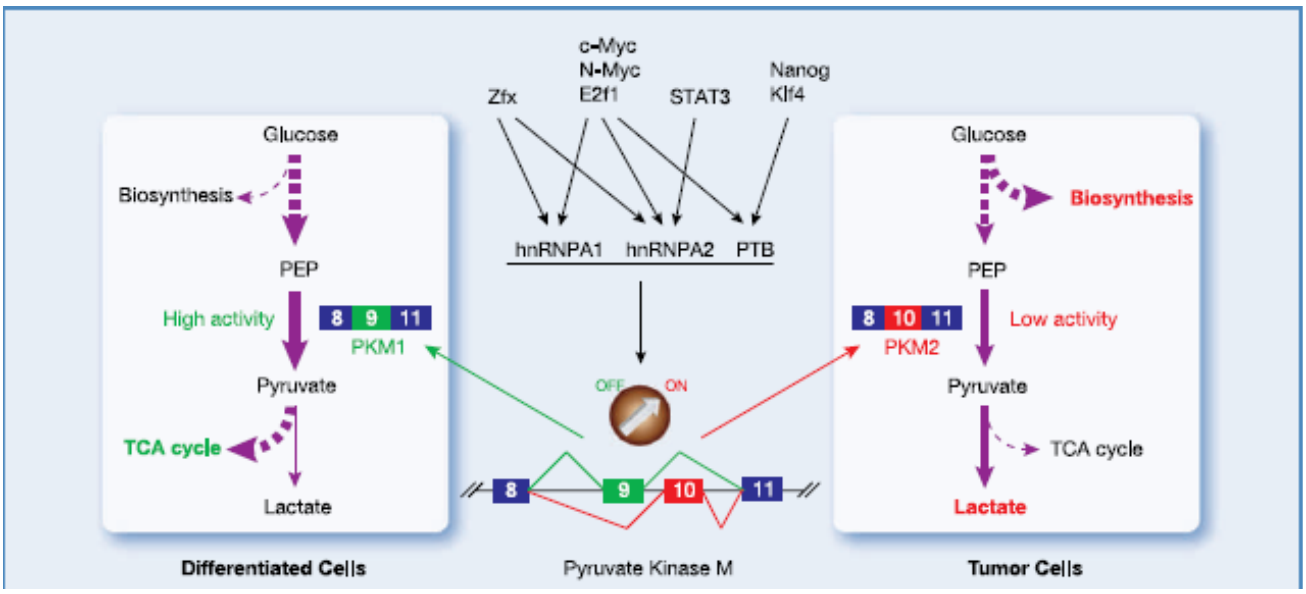


Figure 3: Differential splicing of PKM1 and PKM2

HnRNP proteins control the differential splicing of PKM1 and PKM2. In cancer cells, transcription of hnRNPA1, A2 and PTB genes is induced by c-Myc and likely one or more of the other factors indicated. Binding of the hnRNPs to the splice sites flanking exon 9 in PKM transcripts results in exon 9 exclusion and exon 10 inclusion, generating PKM2. PKM2 converts PEP to pyruvate less efficiently than PKM1, leading to the accumulation of glycolytic metabolites for anabolic metabolism. [26]

1.1.4 PKM2 dimerization: tetramer to dimer ratio of PKM2

The characterization of PKM2 in lung tissue and tumors showed no differences in the RNA and amino acid sequence, however gel permeation experiments had revealed a difference of

its structure [27], [28]. In lung it had a tetrameric structure and in tumors, it had a dimeric structure [22], [27], [29], [30], [31], [32], [33]. PKM2 protein consists of 531 amino acids and is subdivided into N-terminal domain from (aa 1-43), the A-domain, which is composed of (aa 44-116), as well as (aa 219-389), the B-domain from (aa 117-218) and the C-domain from (aa 390- 531) [6] (Figure 4). The A-domain is responsible for the intermolecular subunit contact to compose a dimeric form. The tetrameric form occurs by the association of the interface of the C-domains of two dimers. The C-domain comprises of an inducible nuclear translocation signal (NLS) (aa 393-531), which is not rich in arginine or lysine as classical NLS [34]. PKM1 and PKM2 differ in 23 amino acids located in the 56 amino acid stretch encoded by exon 9 or exon 10 respectively [35] (Figure 4). Its responsible for the different characteristics and regulation mechanisms between both isoforms.

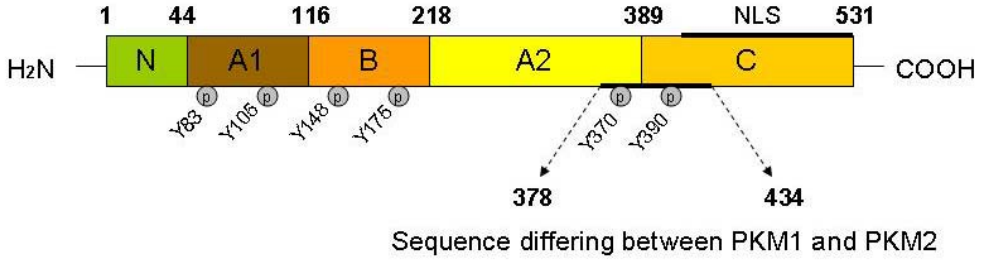


Figure 4: Schematic representation of PKM2 protein structure

PKM2 protein consists of 531 amino acids and can be subdivided into four domains: the N-domain (aa 1-43), the A-domain (aa 44-116 and 219-389), the B-domain (aa 117-218) and the C-domain (aa 390-531). The C-domain contains 23 amino acids of the 56 amino acid stretch (aa 378-434) which differs between M1 and M2 isoforms and inducible nuclear translocation signal NLS (aa 393-531). PKM2 can be dimerized by phosphorylation at the tyrosine residues Y83, Y105, Y148, Y175, Y370 and Y390.

In tumor cells, the dimerization of PKM2 is enhanced by its direct interaction with different oncoproteins such as (pp60v-src kinase and HPV-16 E7), which induces its tyrosine residues phosphorylation [27], [31], [32], [36], [37]. PKM2 was identified as a direct substrate of the oncogenic tyrosine kinase FGFR1, which phosphorylates PKM2 on the tyrosine residues Y83,

Y105, Y148, Y175, Y370 and Y390 (Figure 4). PKM2 was phosphorylated in various human solid tumor cell lines, including A549 and H1299 lung cancer cells, MDA-MB231 breast cancer cells, PC3 and DU145 prostate cancer cells, but not in 22Rv prostate cancer cells [38]. A-Raf and PKC delta have been identified as serine kinases, which bind to and phosphorylate PKM2 in serine. The physiological function of the interaction between PKM2 and HERC1, as well with PKC delta is unknown [39], [40].

1.1.5 Effects of pyruvate kinase M2 (PKM2) on glycolysis

Otto Warburg's has demonstrated that tumor cells rapidly use glucose and convert the majority of it from pyruvate to lactate, whereas in normal cells pyruvate enters the citrate cycle for oxidative phosphorylation, a phenomena known as aerobic glycolysis [41], [42]. This change with lactate production is accompanied by increased glucose uptake and high rate metabolism, which correlates with transformation and accounts for a significant percentage of ATP generated during cell proliferation [26], [43], [44], [45], [46], [47], [48]. Growing tumor cells have glycolytic rates that are 200 times higher than those of normal tissues, even if oxygen is present [49].One study showed that PKM2 expression and activity in cancer cells enables cells to use predominantly aerobic glycolysis instead of oxidative phosphorylation [50], [51]. Cells expressing PKM2 showed more lactate production and less oxygen consumption than cells expressing PKM1 combined with a stronger glycolytic phenotype, which is important for the cancer development *in vivo* [50].

Oxygen shortage, or the accumulation of glycolytic intermediates, such as Fructose-1,6-bisphosphate (FBP), induces the re-association of PKM2 dimeric form to the tetrameric form. PKM2 tetrameric form has a high affinity to its substrate PEP and is highly active at physiological PEP concentrations. PKM2 tetrameric form is associated with several other glycolytic enzymes: (hexokinase, glyceraldehydes 3-phosphoate dehydrogenase, phospho glycerate kinase, phospho glycerate mutase, enolase, lactate dehydrogenase), other enzymes (nucleotide diphosphate kinase, adenylate kinase, glucose 6-P dehydrogenase), components of the protein kinase cascade (RAF, MEK, ERK), as well as AU rich mRNA forming a glycolytic enzyme complex [22], [31], [32], [33], [39], [52], [53], [54] [55], [56], [57]. This association within the glycolytic enzyme complex leads to conversion of glucose to lactate, producing energy until the FBP levels drop below a certain value, which causes the dissociation of the PKM2 tetrameric form to the dimeric form. The cycle of oscillation repeats

again when the FBP levels reach a certain higher value and induces the tetramerization of PKM2. When PKM2 is in the less active dimeric form, energy is produced by the degradation of the amino acid glutamine to aspartate, pyruvate and lactate (glutaminolysis) [33] (Figure 5).

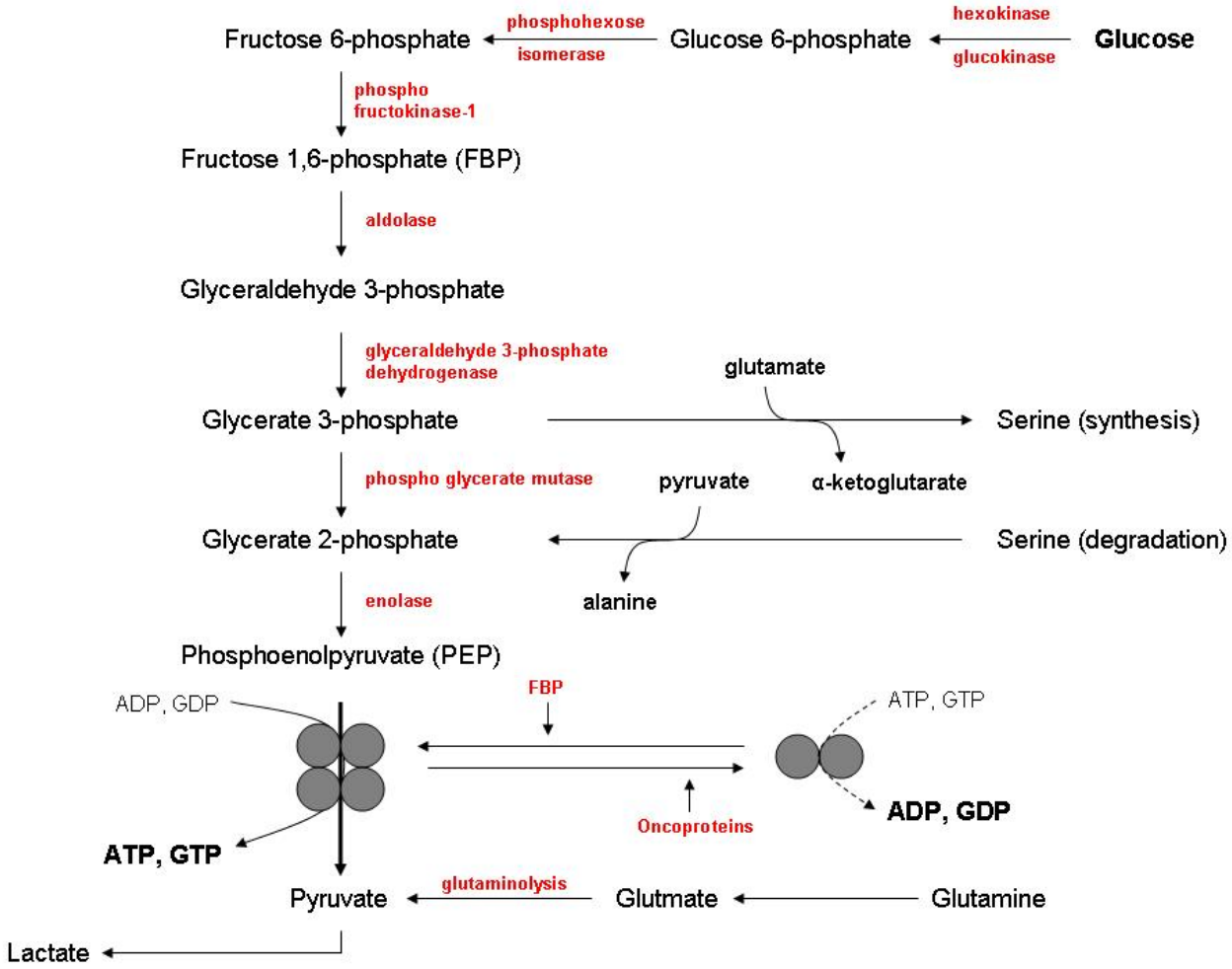


Figure 5: Schematic representation of PKM2 effect on glycolysis

Glycolysis starts by the phosphorylation of glucose by the hexokinase/glucokinase enzymes converting it to glucose 6-phosphate, which is in turn converted to fructose 6-phosphate by the phosphohexose isomerase enzyme. Fructose 6-phosphate is phosphorylated again and converted to fructose-1,6-diphosphate by the phosphofructokinase-1 enzyme. In the next step fructose-1,6-diphosphate is cleaved by the aldolase enzyme into two identical three-carbon compounds glyceraldehydes 3-phosphate. The enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) phosphorylates and oxidates glyceraldehydes 3-phosphate in two steps converting it into glycerate 3-phosphate, which is in turn is phosphorylated by the phosphoglycerate mutase enzyme by relocating the Phosphate from the third carbon to the second carbon to form glycerate 2-phosphate. The enolase enzyme removes a molecule of water from glycerate 2-phosphate acid to form phosphoenol pyruvate (PEP). Pyruvate kinase M2 (PKM2) phosphorylates PEP to ADP to form pyruvate and ATP. FBP favors PKM2

tetmerization and is correlated with low ADP/GDP and high ATP/GTP levels. Oncoproteins favors PKM2 dimerization and is correlated with high ADP/GDP and low ATP/GTP levels. Glutamine is degraded to pyruvate by glutaminolysis to provide energy. Serine is formed from glycerate 3-phosphate and the glutaminolytic intermediate glutamate. It increases the affinity of PKM2 to PEP and decreases the necessary amount of FBP for PKM2 tetramerization. Serine degradation produces alanine which in turn decreases the PEP affinity of PKM2 and increases the necessary FBP concentrations for PKM2 tetramerization.

Amino acid serine is formed from the glycolytic intermediate glycerate 3-phosphate and the glutaminolytic intermediate glutamate. Serine allosterically increases the affinity of PKM2 to PEP and reduces the amount of FBP necessary for tetramerization. The glutaminolytic intermediate alanine decreases the PEP affinity of PKM2 and increases FBP concentrations, which are necessary for tetramerization [33] (Figure 5). PKM2 dimeric form is correlated with high ADP/GDP levels and low ATP/GTP levels, which influence a high degree of malignancy [31], [32], [33], [58] (Figure 5).

1.1.6 Effects of pyruvate kinase M2 (PKM2) on tumor proliferation

PKM2 is expressed in some differentiated tissues and all cells with a high rate of nucleic acid synthesis, which include all proliferating cells, such as normal proliferating cells, embryonic cells, adult stem cells and tumor cells [27], [50], [59], [60], [61]. PKM2 can be negatively regulated in response to growth factor signalling by binding to tyrosine-phosphorylated proteins (pTyr) and decreasing its activity by releasing of the allosteric activator FBP [50]. PKM2 tetrameric form has a high affinity to PEP and converts it to pyruvate with production of energy, whereas PKM2 dimeric form has a low affinity to PEP [62] (Figure 6), resulting in the accumulation of all glycolytic intermediates and their availability as precursors for the synthetic processes, such as nucleic acid, amino acid and phospholipids synthesis [27], [33] [50], [62].

Cells expressing a mutant form of PKM2 that is unable to bind pTyr peptides had failed to increase lactate production [63]. Presence of the PKM2 Y105F mutant in cancer cells leads to decreased proliferation under hypoxic conditions, increased oxidative phosphorylation and reduced tumor growth [38]. These observations indicate a link between cell growth and/or proliferation signals, which are organized through tyrosine kinase signalling pathways [51].

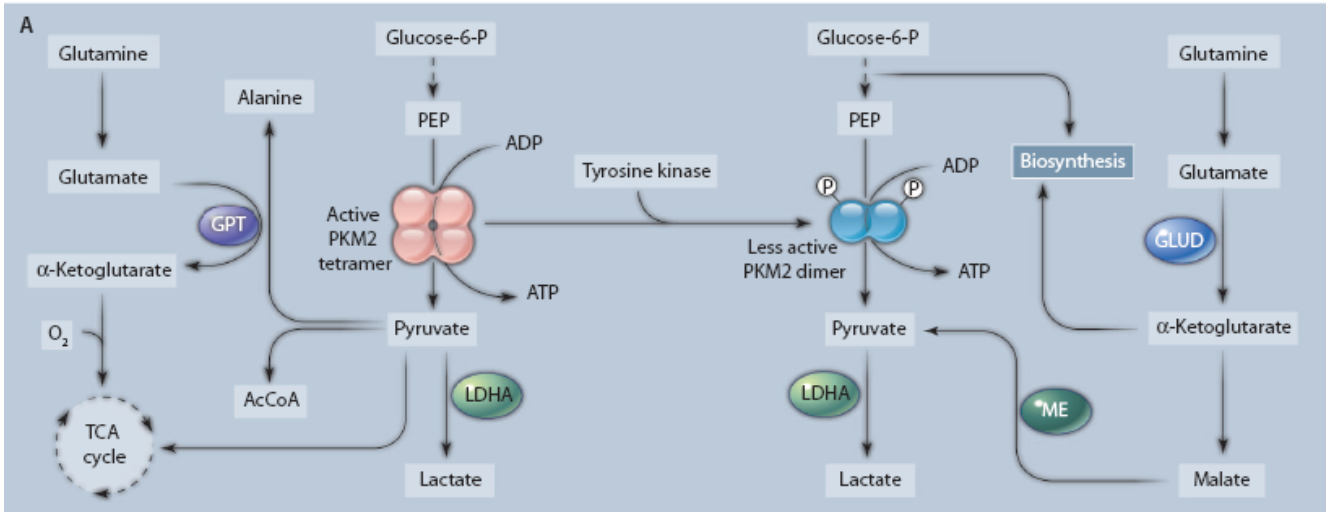


Figure 6: Effect of PKM2 tyrosine kinase phosphorylation on proliferation

The associated cellular changes with increased oxygen consumption by cells with the active PKM2 tetramer may be linked to glutamine or pyruvate oxidation (or both). The decreased pyruvate production by the less active tyrosine-phosphorylated PKM2 dimer may permit channeling of glucose carbons upstream of phosphoenolpyruvate (PEP) for biosynthetic purposes. Concurrent glutaminolysis provides a lactate source, as well as a carbon source for anapleurosis in the TCA cycle, which supports biosynthesis. [62]

PKM2 was also shown to be involved in: Hepatitis C HCV Ns5B genome synthesis and replication efficiency [61], [64], opacity associated proteins (Opa) in *Staphylococcus* pathogenesis [65], tumor suppressor protein promyelocytic leukemia (PML) translocation between nucleus and cytoplasm [66], Oct-4 increase target gene expression [67] and suppression of cytokines signaling 3 (SOCS3) resulting in disruption of antigen representing ability of dendritic cells [68], [1].

1.2 Tumor hypoxia and HIF

Hypoxia is a reduction in the normal level of tissue oxygen [69]. This reduction can occur when oxygen supply from the bloodstream does not meet demand of the cells in the tissue, as in the case of chaotic jumbles of blood vessels which are unable to perfuse blood to the tumor cells resulting in poor oxygen supply [70], [71]. The best studied example of chronic hypoxia is the hypoxia associated with the tumor microenvironment [72]. Hypoxia is a characteristic physiological abnormality in human solid tumors. While the oxygen tension (pO2) in normal

tissues ranges, depending on the tissue type is between 10 and 80 mmHg, tumors frequently contain regions that are deficient in oxygen (pO_2 < 5 mmHg).

Hypoxia influences tumor biology and physiology through a wide range of cellular and systemic adaptive responses, resulting in more aggressive tumor phenotypes. The adaptive hypoxia response program is coordinated by hypoxia-inducible factor 1 (HIF-1), which permits tumor cells to adapt by inducing hypoxia responsive genes [73], [74], [75]. The hypoxia-inducible transcription factors HIF-1α and HIF-2α are activated in hypoxic tumor regions [76], [77], [78], [79], [80], [81], [82], [83]. HIF-1α is not exclusively regulated by oxygen tension, but also by other factors, such as transition metals, nitric oxide, ROS, growth factors, mechanical stresses [84], as well as energy metabolism intermediates, like pyruvate, lactate and oxaloacetate [49], [85], [86].

1.2.1 Regulation of HIF

Upon activation, HIF-1α (120 kDa) dimerizes with HIF-1β (91-94 kDa) to form HIF-1 [87]. HIF-1 together with other coactivators triggers several transcriptional activities for different genes involved in the regulation of energy metabolism and angiogenesis. HIF-1α protein was found to accumulate in the cell nucleus within 2 minutes of hypoxic conditions and HIF-1α DNA-binding occurs earlier in this time range [88]. The basic helix loop helix (bHLH) domain participate in this process and the Per-ARNT-Sim (PAS) domain serves as sensor of oxygen and functions as a transducer of signals by protein–protein interactions [89]. The oxygen dependent degradation (ODD) domain is required for the stabilization and function of HIF-1α; it contains two prolyl residues (Pro402 and Pro564), asparaginyl N803 for hydroxylation and lysine 532 residues for acetylation, and is overlapping with the N-terminal tansactivation domain.

The N-terminal transactivation domain (NTAD) and C-terminal transactivation domain (CTAD) are important for HIF-1α transcriptional activity by providing docks for co-activators, such as p300/CBP (Figure 7). The inhibitory domain (ID) may repress CTAD activety under non-hypoxic conditions. Other ID is independent but hypoxia-dependent mechanisms may modulate NTAD and CTAD [90], [91], [92].

The regulation of HIF-1α activation occurs at the level of protein stabilization and transctivation. The prolyl hydroxylase domain (PHD) family contains three members: PHD1, PHD2 and PHD3 [93], [94], which hydroxylate specific prolyl residues (Pro402 / Pro564) in the ODD domain of HIF-1α with differential efficacy in the presence of O2. Hydroxylation of HIF-1α requires two subsets of 2-oxoglutarate dioxygenases from the iron (II) and 2-oxoglutarate-dependent oxygenases super family. PHD1 and PHD2 modify both of the prolyl residues, while PHD3 hydroxylates Pro564 [94]. Knockdown of PHD2, but not PHD1 or PHD3 had stabilized HIF-1α under normoxic conditions illustrating the importance of PHD2 in the process of HIF-1α hydroxylation [95], [96].

Hydroxylation of proline residues enhances the *von Hippel Lindau* (VHL) protein, binding to HIF-1α [94], [97], [98], [99] and targets it for proteasome degradation, mediated by ubiquitination by the E3 ubiquitin ligase (Figure 7). Cell renal carcinoma cells lacking functional VHL constitutively expressed HIF-1α and HIF-1 target genes under non-hypoxic conditions [100], [101], [102].

Factor inhibiting HIF-1 (FIH-1) hydroxylate asparagine residue 803 in the CTAD of HIF-1α in normoxia. This modification prevents the interaction of HIF-1α with CBP/p300 and stops the transcriptional activity of HIF-1α [103], [104], [105] (Figure 7).

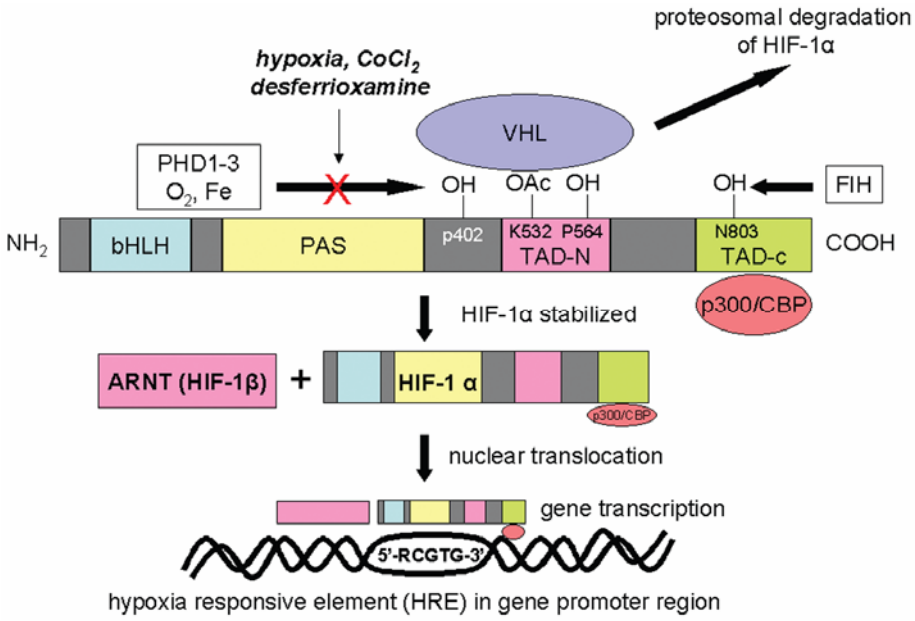


Figure 7: Regulation of HIF-1α in hypoxia

Hypoxic regulation of the hypoxia-inducible factor-1α (HIF-1α) transcription factor is primarily through inhibition of degradation. Under normoxic conditions, HIF-1α undergoes rapid proteosomal degradation once it forms a complex with *von Hippel–Lindau* tumor suppressor factor (VHL) and E3 ligase complex. This requires the hydroxylation of critical proline residues by a family of HIF-1α -specific prolyl hydroxylases (PHD-1, 2 and 3), which requires O_2 and several cofactors, including iron. Under hypoxic conditions, or when iron is chelated or competitively inhibited, proline hydroxylation does not occur, thus stabilizing HIF-1α and allowing it to interact with the constitutively expressed HIF-1α (aryl hydrocarbon nuclear translocator; ARNT). The HIF-1 complex then translocates to the nucleus and activates genes with hypoxia-responsive elements in their promoters. BHLH, basic helix-loop-helix; CBP, cAMP response element binding protein; FIH, factor inhibiting HIF-1α; PAS, PER-ARNT-SIM; TAD, transactivation domain. [106]

1.2.2 Effects of HIF-1 on glycolysis

Adaptation to the respiratory suppression owing to oxygen depletion causes tumor cells to switch glycolysis for ATP production and is accompanied by increased generation of lactate and acidification of tumor microenvironment [107], [108]. HIF-1α is a key molecule that mediates cellular response to hypoxia and can activate a set of genes involved in angiogenesis, glucose uptake and glycolysis [69]. There are some core mechanisms which are involved in the upregulation of glycolytic targets in cancer cells like: glucose uptake transporters GLUT1 and GLUT3, the lactate production MCT1 and MCT4, as well as other

metabolic targets, like PKM2, PDK1 and LDHA [109], [110], [111]. Most of the targets are controlled and induced in the hypoxic areas due to HIF-1α stabilization [86], [111], [112] (Figure 8).

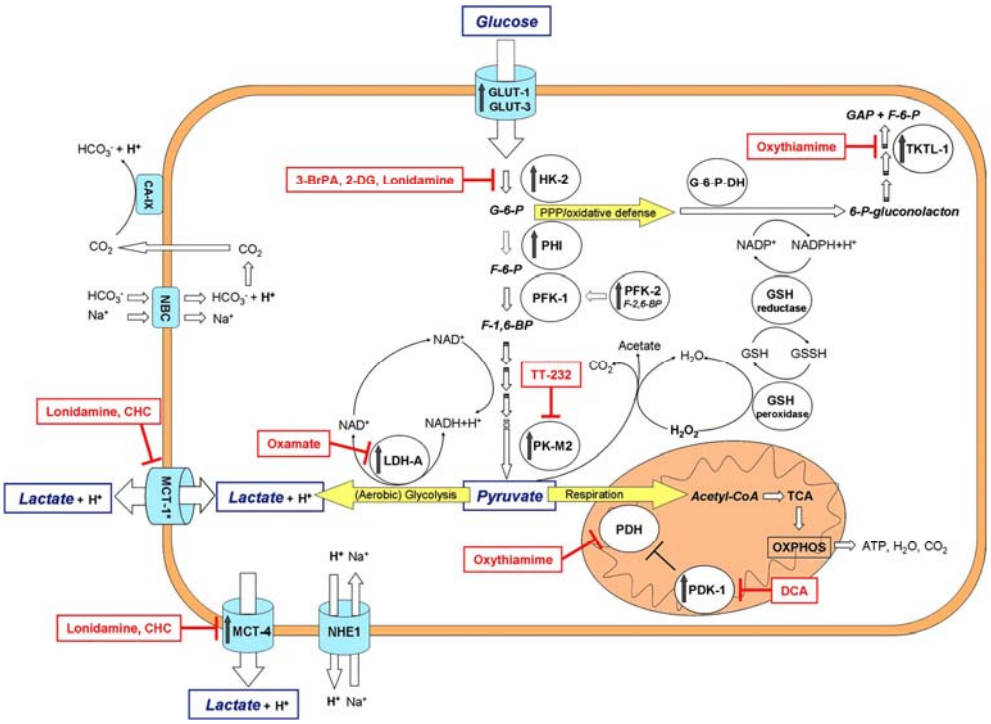


Figure 8: Molecular base of enhanced tumor glycolysis and manipulating targets

The diagram schematizes the regulation of glycolysis and energy metabolism in tumor cells. Glycolysis is enhanced mainly through increased expression of GLUT-1 and 3 leading to higher glucose uptake into the cell and glycolytic enzymes, such as HK-2, PHI, PKM2 and LDHA, which generates lactate and H+ from pyruvate. Higher expression of enzymes in malignant cells is depicted as gray upright arrows. Effective efflux of lactate is provided through MCTs, CA-IX and NHE1 to prevent intracellular acidosis. MCT-1 may function as a lactate importer especially in oxygenated tumor cells. Glycolysis is closely interrelated with the PPP and glutathione metabolism. Possible manipulation targets of tumor cell metabolism are demonstrated in red. [86]

2. Aims of the Study

The pyruvate kinase gene is expressed as two different splice variants of the PKM gene named PKM1 and PKM2. Originally for no evident reason, the products of these two splice variants have strong opposing effects on cell metabolism. Whereas PKM1 favours oxidative phosphorylation, PKM2 favours glycolysis and conversion of pyruvate to lactate. In particular, this situation is observed under hypoxic conditions or in tumor cells that display enhanced glycolytic activity even under normoxic conditions, a phenomenon known as the Warburg effect. Hypoxia is a condition that strongly contributes to tumor progression by initiating complex adaptive processes, such as tumor angiogenesis and metabolic changes through hypoxia-inducible factor (HIF). These HIF dependent processes are relevant in solid tumors including lung and prostate cancer that are the sources of the cancer cell lines (A549, PC3 and LNCaP) investigated here.

At the beginning of this work, the expression and regulation of PKM1 and PKM2 in hypoxia and the dependence of PKM1 and PKM2 on hypoxia-inducible transcription factors were not explored.

- Thus, the first aim of this study was to characterize PKM1 and PKM2 regulation under hypoxic conditions and to analyze the dependence of PKM1 and PKM2 on HIF-1α and HIF-2α.
- Since HIF is a factor that strongly shifts the activity from oxidative phosphorylation towards glycolysis and may contribute to metabolic changes dependent on PKM2 favoring this direction, possible effects of PKM1 and PKM2 on HIF-1α were analyzed as well.
- As metabolism is important for cell proliferation and tumorigenesis, studies about the effects of PKM1 and PKM2 on cell clonogenic survival were included.

To meet these aims the following principal approaches were undertaken in A549, PC3 and LNCaP cancer cell lines:

- Gene expression analysis of pyruvate kinase isoforms (PKM1/ PKM2) under normoxic and hypoxic conditions employing silencing of HIF-1α and HIF-2α in order to analyze consequences for PKM1 and PKM2 expression.

- Establishment of selective silencing of PKM1 and PKM2 by siRNA for testing the role of these splice variants for HIF dependent gene regulation and for cell proliferation and clonogenic survival.

Foot note:

During the progress of this work Semenza's group published an important article in May 2011 that is relevant for this thesis. It was identified that hypoxia dependent regulation of PKM2 is dependent on HIF-1α and that PKM2 favors transactivation of HIF-1α. Thus, this article covers similar aspects as analyzed in this thesis [17].

3. Materials and methods

3.1 Materials

3.1.1 Cells

Human pulmonary epithelial cell line A549 adenocarcinoma non-small cell lung cancer, advanced androgen independent bone metastasis prostate cancer PC3 and androgen sensitive human prostate adenocarcinoma LNCaP, which used in the experiment were purchased from American type culture collection (ATCC).

3.1.2 Oligonucleotides for quantitative RT-PCR

The oligonucleotides were synthesized by Biomers (Biomers, Ulm, Germany)

PBGD

Forward	5′-TGT CTG GTA ACG GCA ATG CG-3′
Reverse	5′-CCC ACG CGA ATC ACT CTC AT-3′

PKM1

Forward	5′-GAA GGC AGT GAT GTG GCC AAT G-3′
Reverse	5′-ACT CCG TCA GAA CTA TCA AAG CTG C-3′

PKM2

Forward	5′-CTT GCA ATT ATT TGA GGA ACT CCG C-3′
Reverse	5′-CAC GGT ACA GGT GGG CCT GAC-3′

HIF-1α

Forward	5′- TAA AGG AAT TTC AAT ATT TGA TGG G -3′
Reverse	5′- AAA GGG TAA AGA ACA AAA CAC ACA G -3′

HIF-2α

Forward	5′-GAT CTT TCT GTC AGA AAA CAT CAG C-3′
Reverse	5′-GTT GAC AGT ACG GCC TCT GTT G-3′

3.1.3 Small interfering RNA sequences

The oligonucleotides were synthesized by Biomers (Biomers, Ulm, Germany)

si-PKM

Forward	5′- GCUGUGGCUCUAGACACUA dTdT-3′
Reverse	5′- UAGUGUCUAGAGCCACAGC dTdT-3′

si-PKM1

Forward	5′- GCGUGGAGGCUUCUUAUAA dTdT-3′
Reverse	5′- UUAUAAGAAGCCUCCACGC dTdT-3′

si-PKM2

Forward	5′- CCAUAAUCGUCCUCACCAA dTdT -3
Reverse	5′- UUGGUGAGGACGAUUAUGG dTdT -3′

si-HIF-1α

Forward	5′- CUGAUGACCAGCAACUUGA dTdT -3′
Reverse	5′- UCAAGUUGCUGGUCAUCAG dTdT -3′

si-HIF-2α

Forward	5′- CAGCAUCUUUGAUAGCAGU dTdT -3′
Reverse	5′- ACUGCUAUCAAAGAUGCUG dTdT -3′

si-random

Forward	5′- UAGCGACUAAACACAUCAA dTdT -3′
Reverse	5′- UUGAUGUGUUUAGUCGCUA dTdT -3′

3.1.4 Enzymes

DNase I

DNase I (RNase-free) was purchased from Ferments Company. DNase I is an endonuclease that none specifically cleaves DNA to release di-, tri- and oligonucleotide products with 5′-phosphorylated and 3′-hydroxylated ends. DNase I act on single- and double-stranded DNA, chromatin and RNA: DNA hybrids. Its frequently used to remove contaminating genomic DNA from RNA samples.

MMuLV Reverse Transcriptase

MMulV was purchased from Ferments Company. Moloney murine leukemia virus reverse transcriptase (M-MLV RT) is an RNA-dependent DNA polymerase that can be used in cDNA synthesis with long messenger RNA templates (>5kb). The enzyme is a product of the *pol* gene of M-MLV and consists of a single subunit with a molecular weight of 71kDa. The RNase H activity of M-MLV RT is weaker than the commonly used Avian Myeloblastosis virus (AMV) reverse transcriptase.

RiboLock RNase Inhibitor inhibits

RiboLock RNase inhibitor was purchased from Ferments Company. It inhibits the activity of RNases by binding them in a noncompetitive mode at a 1:1 ratio. It does not inhibit eukaryotic RNases T1, T2, U1, U2, CL3, as well as prokaryotic RNases I and H. Its source is *E.coli* cells with a cloned gene encoding mammalian ribonuclease inhibitor with molecular wight 49.6 kDa monomer.

3.1.5 Antibodies

Antibodies used in the experiments are all commercially available. Their parameters were described as follows:

Primary antibodies	**Company**
Anti-human HIF-1α (rabbit)	BD Biosciences / San Diego, CA, USA
Anti-human HIF-2α (rabbit)	Lifespan Biosciences/ WA, USA
Anti-human PKM2 (rabbit)	Cell Signaling / USA
Anti-human PKM2 (Tyr105) (rabbit)	Cell Signaling / USA
Anti-human β-actin (mouse)	Abcam / Cambridge, UK
HRP secondary antibodies	
Anti-mouse IgG goat	Thermo Scientific / Rockford, IL, USA
Anti-rabbit IgG goat	Thermo Scientific / Rockford, IL, USA

3.2 Methods

3.2.1 Culture of A549, PC3 and LNCaP cells

The cell culture was performed according to the protocol given by the American type culture collection (ATCC). The cells frozen in 10% DMSO in liquid nitrogen (approx. 5 x 10^6 cells/ml) were thawed rapidly at 37°C and then added drop wise to 100 mm dish containing 10 ml of pre-warmed DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA,USA) (1:1) culture medium (supplemented with 10% FCS (v/v) (Greiner BioOne, Frickenhausen, Germany), 1% (v/v) penicillin and streptomycin, 1% vitamins, 1% glutamine and 1% non essential amino acids) or RPMI (PAA, Germany) (supplemented with 10% FCS (v/v), 1% (v/v) penicillin and

streptomycin (all supplements were from Gibco, Invitrogen, Carlsbad, CA,USA). When the cells became confluent, they were trypsinized with 2 ml 1 x trypsin per 100 mm plate for approximate 5 min at 37°C. The reaction was stopped by adding 5 ml of medium with 10% FCS which containing trypsin inhibitors. For continuous culture, about 1/4 of the medium containing the cells were transferred to a fresh plate and cultured in a gas controlled ProOx incubator (Innova CO-48, New Brunswick Scientific, Edison, NJ, USA) with saturated gas mixture of 0.1% O_2, 5% CO_2, and 94% N_2 or water-saturated atmospheric air with 5% CO_2 (BioSpherix, Ltd., Redfield NY, USA) under hypoxic and normoxic conditions respectively.

1 x Trypsin	Volume
10 x Trypsin	10 ml
HEPES (200 mM)	10 ml
Isotonic NaCl (0.9%)	80 ml

3.2.2 Small interfering RNA preparation and transfection

Selective inhibition of target gene was performed using specific si-RNAs. As a control, si-RNA sequence (si-random) was employed that does not target any gene in the human genome and has been tested by micro-array analysis (Dharmacon Inc., Chicago, IL, USA). The forward and reverse RNA strands with two 5′ deoxy-thymidine overhangs were commercially synthesized and annealed at a final concentration of each 20 µM at 37°C for 1h in annealing buffer.

RNA duplex annealing buffer	Final concentration
potassium acetate	20 mM
HEPES-KOH	6 mM
magnesium acetate	0.4 mM
pH	7.4

The liposome mediated transfection method was employed for transfection of A549, PC3 and LNCaP cells. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent was used for transfection (si-RNA) in cells. One day before transfection, appropriate number of A549, PC3 and LNCaP cells was plated on respective culture dishes with growth medium; so

that they will become 60% confluent for si-RNA transfection at the time of transfection. Si-RNA was diluted in Opti-MEM reduced serum medium and mixed gently. Lipofectamine 2000 was mixed gently before use and then diluted in the appropriate amount of Opti-MEM medium. Mixtures were mixed gently and incubated for 5 min at room temperature. After 5 min incubation, the diluted si-RNA and the diluted lipofectamine 2000 were mixed and incubated for 20 min at room temperature to allow the DNA-lipofectamine 2000 complexes to form. DNA-lipofectamine complexes were added to each well containing cells and medium and mixed gently by rocking the plate back and forth. Cells were incubated in CO_2 incubator at 37°C for 5 h and the medium was replaced. Cells were further incubated either in normoxia or hypoxia incubator according to the experiment need.

3.2.3 RNA and protein isolation from cultured cells

Cells were washed with PBS and scraped in extraction buffer (peqGOLD TriFast, Peqlab Biotechnology GmbH, Erlangen, Germany), for dissociation of the nucleoprotein complexes the samples were kept for 5 min at RT, then were shaken by hand vigorously after adding 0.1 ml of chloroform and incubated for 10 min at RT, then were centrifuged for 10 min at 12.000g (4°C). The mixture separates into the lower red (phenol-chloroform phase), the interphase and the colorless upper aqueous phase. RNA is forced exclusively into the aqueous phase, whereas DNA and the proteins partition into the interphase and lower phenol phase. The aqueous phase was transferred to a fresh appropriate tube. To precipitate the RNA, 0.3 ml of isopropanol was added to the aqueous phase, after that the samples were shaken and incubated on ice for 15 min and centrifuged for 10 min at 12.000g (4°C). The RNA pellet should form a gel like precipitate on the bottom side of the tube. The supernatant was removed carefully and the RNA pellet was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 8 min at 7,500g (4°C). The excess 75% ethanol was removed from the RNA pellet by air-drying; later the RNA pellet was resuspended in RNAse-free water. RNA was quantified by spectrophotometer (Nanodrop ND-100).

For protein precipitation, 1.3 ml of isopropanol was added to the ethanol/phenol supernatant, samples were shaken and incubated at RT for 10 min, then centrifuged for 10 min at 12.000g (4°C). The supernatant was removed and samples protein pellet was washed three times with 2 ml solution of 0.3 M guanidinium hydrochloride 95% ethanol and for once with 2 ml 100% ethanol and incubated for 20 min at RT each time, then centrifuged for 5 min at 7.500g (4°C).

The samples protein pellet was dried for 10 minutes at RT and it was solubilized by adding 110 µl laemmli buffer to each sample followed by sonication.

3.2.4 Preparation of cDNA from RNA probes

For the preparation of cDNA, 2 µg RNA per sample was used. RNA was copied to cDNA using reverse transcriptase (MMuLV-RT) (Invitrogen, Carlsbad, CA, USA) and random-hexamer primer ($p(dN)_6$). For the negative control, MMuLV-RT was omitted.

2 µg RNA in DEPC H_2O (total volume 20 µl) including DNaseI was denatured at 65°C for 5 min followed by rapid cooling and addition of 20 µl of the following master mixture:

5 x first strand buffer	8 µl
40 mM dNTP mix	4 µl
H2O	2 µl
$p(dN)_6$ primer	2 µl
RNase inhibitor	2 µl
MMuLV-RT (200 U/µl)	2 µl

The denatured RNA mixed with master solution was then subjected for cDNA synthesis by incubating at 39°C for 1 h followed by inactivation of enzymes at 96°C for 2 min.

3.2.5 Real-time quantitative PCR (qPCR)

The transcriptional regulation of selected genes was analyzed using real-time quantitative PCR (Applied biosystem 7300). Real-time PCR is a method based on the detection and quantification of a fluorescent reporter signal that increases in direct proportion to the amount of the PCR product in reaction [113], [114], using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). CDNA was detected and quantified with the fluorescent dye SYBR Green, which offers a linear dose response over a wide range of target concentrations. As cDNA accumulates, the dye generates a signal that is proportional to the cDNA concentration. ROX reference dye was used to normalize the fluorescent signal between reactions. PCR reactions were performed in 25 µl volume by using the qPCR mix.

qPCR mix	Stock solution	Quantities per reaction
SYBR®Green mix	2x (Taq DNA Polymerase, SYBR Green dyeI, Tris-HCl, KCl, 6 mM MgCl2, 400 µM dGTP,dATP,dCTP, 800 µM dUTP,uracil DNA glycosylase, stabilizers)	12.5 µl
$MgCl_2$	50 mM	1 µl
Forward primer	10 pmol/µl	1 µl
Reverse primer	10 pmol/µl	1 µl
Rox Reference dye		0.5 µl
H2O		8 µl
cDNA	2 µg	1 µl

Cycling conditions were as follows: 95°C for 5 min, 45 cycles of 95°C for 10 s, 55°C for 15 s and 72° C for 30 s. Formation of a single specific PCR product was confirmed by melting curve analysis. Porphibilinogen deaminase (PBGD) served as a reference gene for all real-time PCR reactions. Relative changes in gene expression were determined with the ΔCt method. Differential gene expression between conditions is expressed as ΔΔCt which corresponds to the log2 fold-difference in mRNA levels between the conditions compared [115].

3.2.6 Protein analysis

3.2.6.1 BCA protein concentration assay

The Pierce BCA protein assay (Pierce, Rockford, USA) is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using a unique reagent containing bicinchoninic acid [116]. The purple-colored reaction product of this assay is formed by the chelating of two molecules of BCA with one cuprous ion. The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are responsible for color formation with BCA [117]. Protein concentrations were determined with reference to standards of bovine serum albumin (BSA).

In this method, a series of dilutions of known concentration of BSA and protein samples with 1:10 dilution by 0.9% NaCl were prepared. Working solution was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (reagent A:B, 50:1). Then 200 µl working solution was added to each well after pipetting 25 µl of each standard or unknown sample replicate into a 96 well microplate shown as follows. After incubation the plate at 37°C for 30 min, the absorbance of samples at 492 nm was measured by spectrofluorometer (FL-600) (BioTek Instruments GmbH, Bad Friedrichshall, Germany) and concentration of protein was determined based on the standard curve.

3.2.6.2 Western-blot

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In SDS-PAGE the denatured polypeptides bind SDS and become negatively charged. The amount of bound SDS is always proportional to the molecular weight of the polypeptide and is independent of its size and charge, therefore the SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the molecular weight of the polypeptides. By using protein molecular weight standard, it is therefore possible to estimate the molecular weight of the polypeptide chains. Protein samples from cell extracts were mixed with (2% of bromophenol blue and β-mercaptoethanol) then incubated for 30 min at 37°C then temperature was raised up to 85°C for 10 min then samples cooled on ice immediately. The samples were loaded on self-made 10% polyacrylamide gel. Glasses for gels, combs, electrophoresis chambers, electro blotting chambers, power supplies (all from Bio-Rad, Hercules, CA, USA), The electrophoresis was performed with 120 V constant and the gel was run till the bromophenol blue reaches the bottom of the separating gel (for about 2 h).

Electro blotting of immobilized proteins

The separated proteins on the SDS-polyacrylamide gel were electrically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) by semi-dry electro blotting. The PVDF membrane was activated by methanol before use. The transfer equipment was prepared in the following way: two layers of whatmann 3 mm filter paper soaked with transfer buffer followed by activated PVDF membrane washed with transfer buffer were placed onto the electro-blotting chamber. On the PVDF membrane, the gel and the other two layers of filter paper soaked with transfer buffer were placed. The cathode and anode from the

power supply were connected with the electro-blotting chamber. Electro blotting was performed at constant current (2 mA/cm^2) for 90 min.

Immunological detection of immobilized proteins

The membrane was blocked with milk TBS blocking buffer at RT on shaker for 1 h followed by incubation with primary antibody overnight at 4°C. After washing with 1 x TBS-T three times for 10 min each, the membrane was incubated with the respective HRP-labeled secondary antibody at RT for 2 h followed by three times washing with 1 x TBS-T buffer for 10 min each. The protein bands were detected by ECL (Enhanced Chemi-luminescence, Amersham, Germany) treatment, followed by exposure of the membrane in fluorChem 8900 chemi-luminescence imager.

All the buffers used in western-blot as follows:

- **4x Laemmli sample buffer**: 0.25 M Tris (pH adjusted with HCl to 6.8), 8% of SDS, 40% of glycerol, adjusted with dest. H_2O
- **Collecting buffer**: 0.625 M Tris (pH adjusted with HCl to 6.8)
- **Separating buffer**: 1.125 M Tris, 30% of sacharose, pH adjusted with HCl to 8.8
- **Transfer buffer**: 25 mM Tris, 192 mM glycine, 20% of methanol, 0.01% of SDS
- **10x TBS buffer**: 24.2g Trsi base, 80g NaCl, 1L aqua dest
- **1x TBS-T**: 100ml 10x TBS, 1ml Tween-20 (0.1%)
- **Blocking buffer**: 1x TBS, 0.1% Tween-20, 5% W/V SMP

3.2.7 Reporter gene assay

The pGL3-TK plasmid (Promega) with the thymidine kinase minimal promoter was used to construct the HRE-plasmid employing the NheI and XhoI restriction sites. For cloning, forward and reverse oligonucleotides corresponding to the hypoxia response element (HRE) from the phosphoglycerate kinase (PGK) gene was used after annealing and restriction digest with NheI and XhoI: HRE-PGK: CTA GCG CGT CGT GCA GGA CGT GAC AAA TAG CGC GTC GTG CAG GAC GTG ACA AAT AGC GCG TCG TGC AGG ACG TGA CA AAT. Finally, a construct with five repeats of HRE-PGK ligated to the 5`end of the TK-mp promoter were isolated and verified by sequencing.

For the reporter gene assay 30000 A549 cells/well, 40000 PC3 cells/well and 50000 LNCaP cells/well were plated in 48-wells plates (Greiner Bio-One, Germany). On the second day, cells were transfected with HRE luciferase reporter plasmid and si-RNA according to the liposome mediated transfection method described above. On the third day of transfection, cells were incubated under normoxic and hypoxic conditions for 24h. The detection of luciferase activity of transfected cells with reporter plasmid was performed with the luciferase reporter assay kit (Promega, Mannheim, Germany). The luciferase assay is based on the enzyme-catalyzed chemiluminescence. Luciferin present in the luciferase assay reagent is oxidized by luciferase in the presence of ATP, air oxygen and magnesium ions. This reaction produces light with a wavelength of 562 nm that can be measured by a luminometer. On the fourth day media was discarded and wells were washed once with 1 x PBS, then 100 µl of 1 x lysis buffer was added to each well and plates were shaken for 15 min at RT and then frozen at -80 °C over night. Next day plates were thawed on shaker at RT. For measurement of firefly luciferase activity, 20 µl of the lysate was mixed in white and flat bottom 96 well plates with 100 µl luciferase assay reagent, which was freshly prepared by mixing substrate and the luciferase assay buffer. The activity of luciferase in cells transfected with HRE-reporter plasmids was measured as relative light units (RLU) employing spectrofluorometer (FL-600 BioTek Instruments GmbH).

3.2.8 Colony survival assay

A clonogenic assay is a microbiology technique for studying the effectiveness of specific agents on the survival and proliferation of cells. Its frequently used in cancer research laboratories to determine the effect of drugs or radiation on proliferating tumor cells, describing the relationship between the cell treatment and the proportion of cells that survive in the sense that they are able to grow into a colony, thereby demonstrating retention of their reproductive integrity. If these cells are seeded as single ones and exposed to radiation or to some other cytotoxic agent, its possible to count the proportion of cells that are able to form macroscopic colonies after graded doses of the cytotoxic agent.

For this assay three cell lines were characterized: A549, PC3 and LNCaP. Four Petri dishes were seeded from each cell line according to the cell culture protocol given by the ATCC, as one Petri dish for each si-RNA (si-PKM, si-PKM1, si-PKM2 and si-random). On the second day the four Petri dishes from each cell line were transfected according to the liposome

mediated transfection method. On the third day when cells became confluent, they were trypsinized with 2 ml 1 x trypsin per 100 mm plate for approximate 5 min at 37°C. The reaction was stopped by adding 5 ml of medium with 10% FCS containing trypsin inhibitors. For continuous culture three Petri dishes were established from each of the four Petri dishes of the three cell lines, as medium containing (100 cells A549, 400 cells LNCaP and 250 cells PC3). On the fourth day Petri dishes were incubated under normoxic and hypoxic conditions for 48 h. After that they were moved from hypoxia chamber to culture incubator for 8 days. On day 13, media of the Petri dishes was discarded and Petri dishes were washed by 1x PBS, then 2 ml of crystal violet stain (0.1% crystal violet, 10% formaldehyde, PBS) was added to each Petri dish and incubated for 15 min at RT. The crystal violet stain was discarded and Petri dishes were washed by normal water.

3.3 Statistical analysis

All the data in the figures and text are expressed as means ± SEM of n independent observations unless indicated otherwise. Statistical evaluation was performed by unpaired t-test and one way Anova test.

4. Results

4.1 Expression of PKM1 and PKM2 under normoxic and hypoxic conditions

In this study we analyzed the expression of PKM isoforms (PKM1 and PKM2) in different cancer cell lines under normoxic and hypoxic conditions. We analyzed PKM1 and PKM2 mRNA levels from A549, PC3 and LNCaP cells by real-time RT-PCR. PKM1 and PKM2 were expressed at a higher level in A549 and PC3 cells than in LNCaP cells under normoxic conditions. PKM2 mRNA expression level was increased significantly under hypoxic conditions in all cell lines studied and this increase was most strongly pronounced in LNCaP cells. PKM1 mRNA expression level showed a significant increase under hypoxic conditions in LNCaP cells, but this increase did not reach a significant level in A549 and PC3 cells (Figure 9).

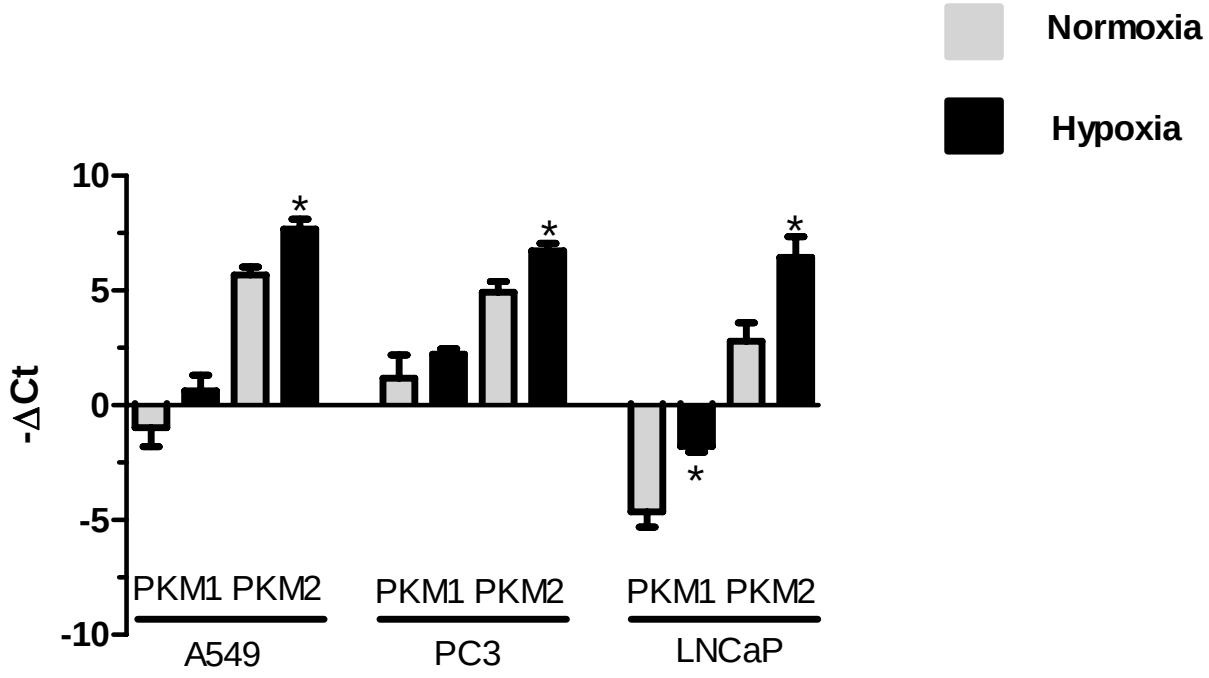


Figure 9: PKM1 and PKM2 mRNA levels in normoxia and hypoxia in different cell lines

Quantification of PKM1 and PKM2 mRNA levels by real time RT-PCR of RNA extracts from A549, PC3 and LNCaP cells cultured for 24 h in normoxia or hypoxia. PBGD gene was used as a reference gene. (* Significant differences under normoxic and hypoxic conditions, n=4, SEM, $p<0.05$, unpaired t-test).

In order to analyze the expression of PKM2 in hypoxia, we analyzed PKM2 protein levels from A549, PC3 and LNCaP cells by western blot. Densitometric protein quantification showed a significant upregulation of PKM2 protein expression level under hypoxic conditions as compared with normoxic conditions in LNCaP cells, but this increase did not reach a significant level in A549 cells. No change was obsereved in PC3 cells (Figure 10).

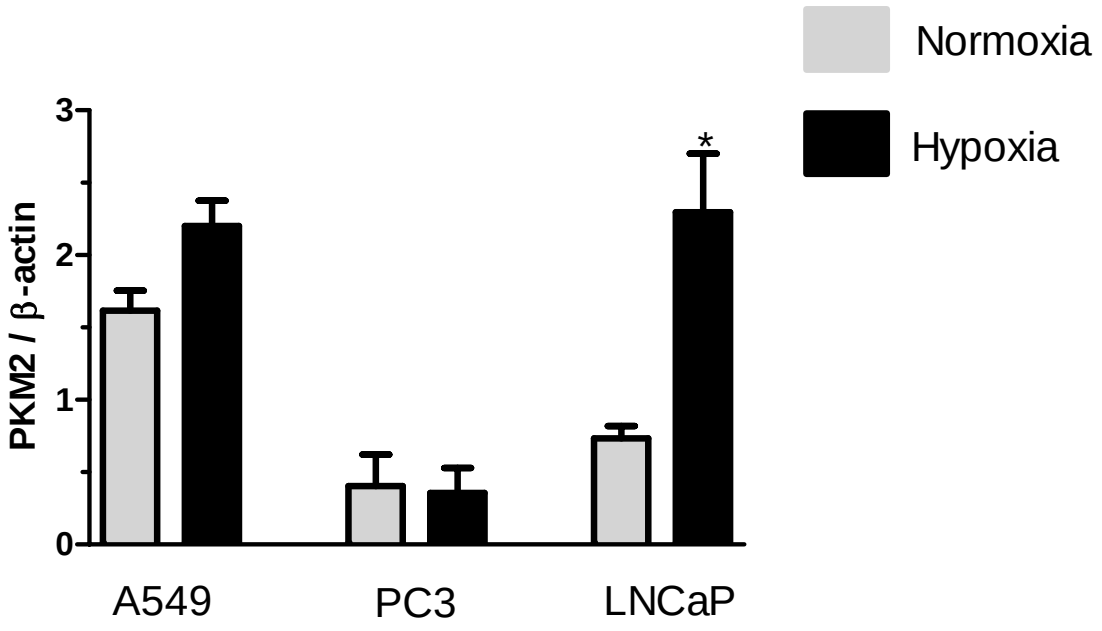


Figure 10: PKM2 protein level in normoxia and hypoxia in different cell lines

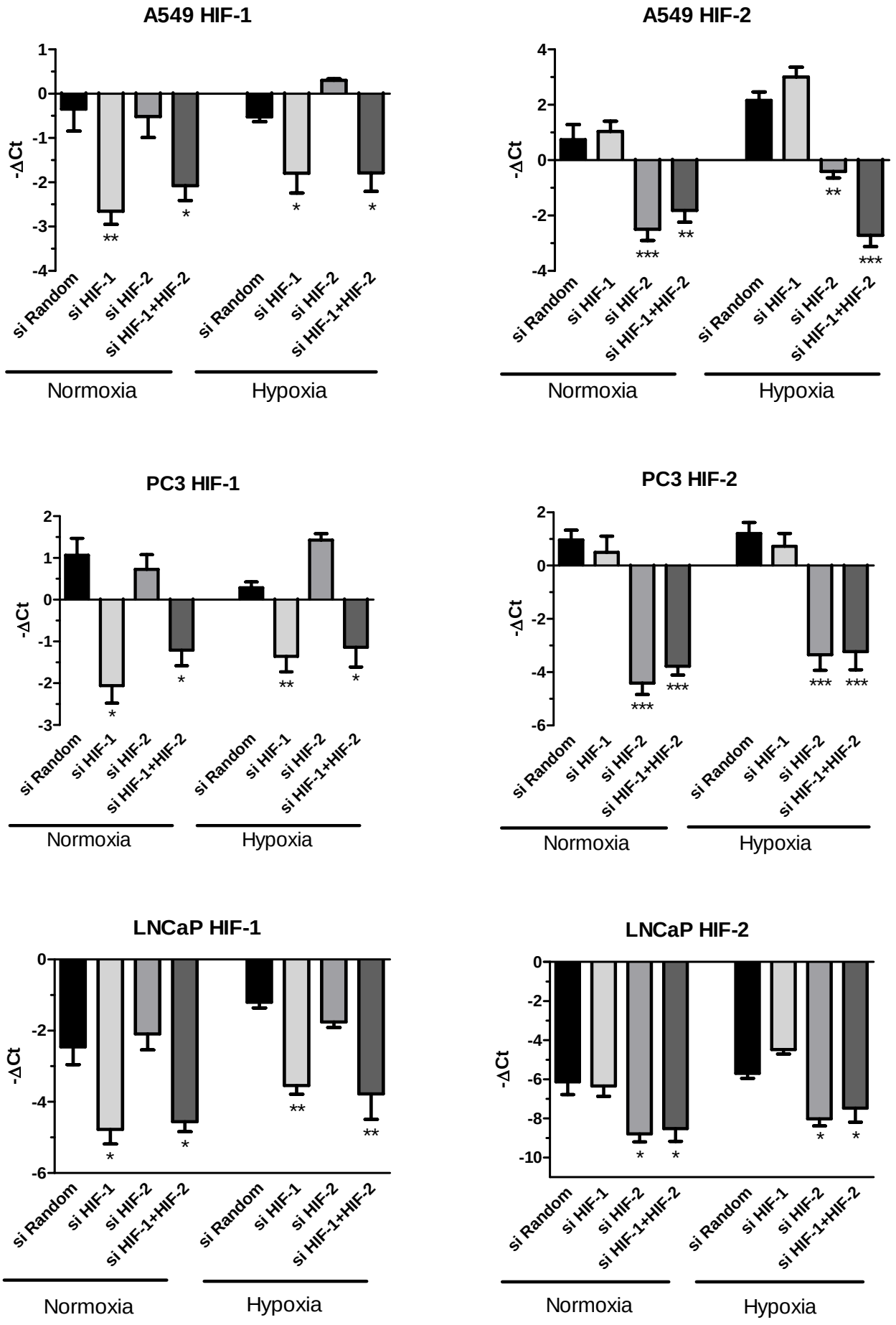
Densitometric analysis of the western blot for PKM2 normalized to β-actin. (* Significant difference under normoxic and hypoxic conditions, n=3, SEM, $p<0.05$, unpaired t-test).

4.2 Inhibition of HIF-1α and HIF-2α expression by small interfering RNA and its effect on PKM2 expression

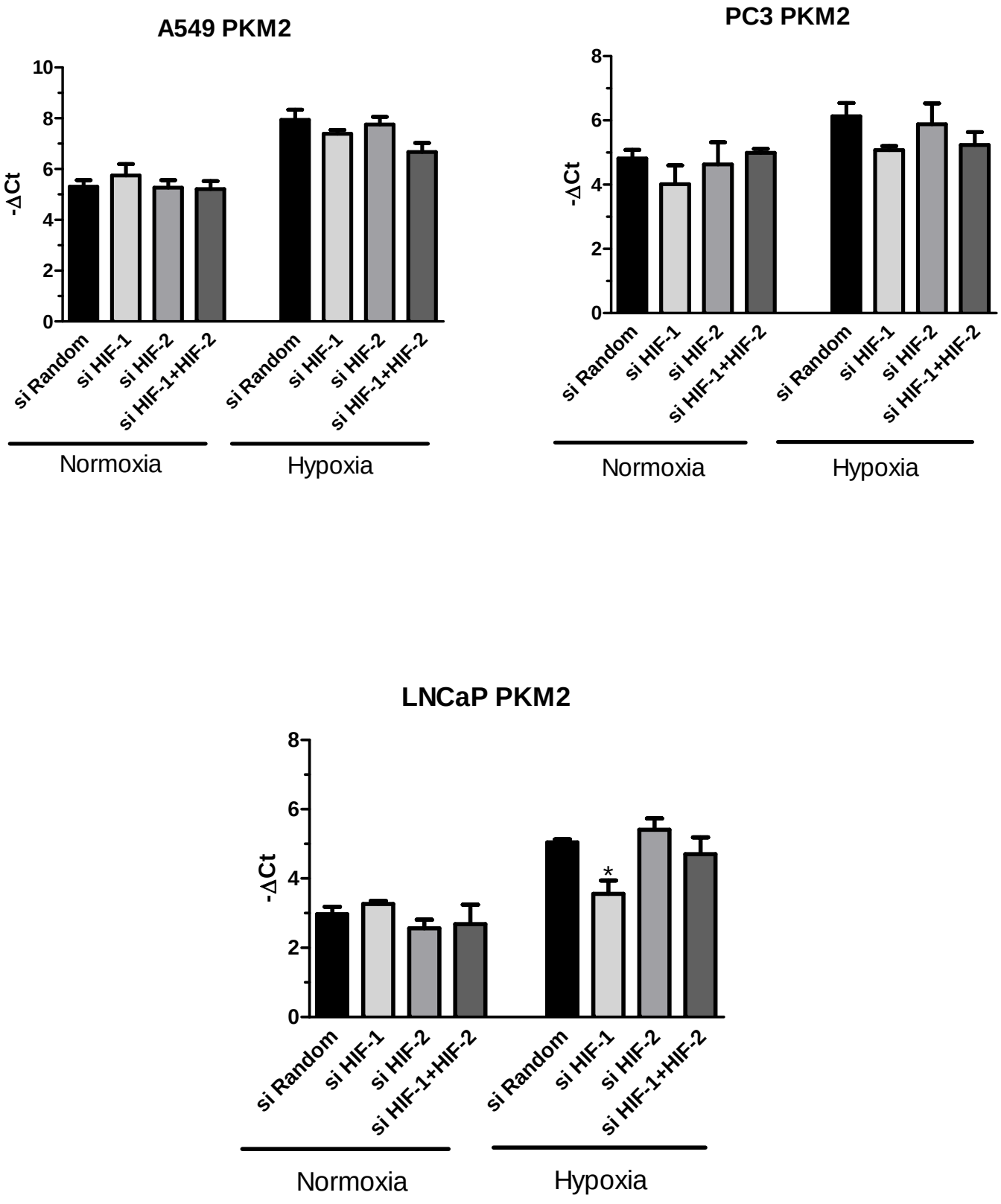
In order to analyze the role of HIF for PKM1 and PKM2 upregulation in hypoxia, we silenced HIF-1α and HIF-2α expression by small interfering RNA. Silencing was validated by real time RT-PCR analysis of mRNA extracts from A549, PC3 and LNCaP cells transfected with non-specific si-RNA (si-random), si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) under normoxic and hypoxic conditions. A significant downregulation of HIF-1α and HIF-2α mRNA expression levels were observed under si-HIF-1α and si-HIF-2α respectively, or both together (si-HIF-1α + si-HIF-2α) as compared with si-random treated cell lines in hypoxia and normoxia (Figure 11A). The si-RNA validation was further confirmed by HIF-1α and HIF-2α western-blot analysis. A remarkable downregulation of HIF-1α and HIF-2α protein expression levels were observed under si-HIF-1α and si-HIF-2α respectively, or both together (si-HIF-1α + si-HIF-2α) as compared with si-random treated cell lines in hypoxia (Figure 11C).

We analyzed whether hypoxia inducible factor (HIF) regulates PKM2 and phospho PKM2 (Tyr105) expression by silencing of HIF-1α or HIF-2α by a small interfering RNA. RT-PCR analysis of mRNA extracts from A549 and PC3 cells showed some downregulation of PKM2 mRNA expression level under si-HIF-1α and (si-HIF-1α + si-HIF-2α) as compared with si-ranodm that did not reach significance, whereas no effect was obvious under si-HIF-2α in hypoxia. However, in LNCaP cells we observed a significant downregulation of PKM2 mRNA expression level under si-HIF-1α, but not under si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) as compared with si-random in hypoxia (Figure 11B). Also, Western-blot analysis showed no change of PKM2 or phospho PKM2 (Tyr105) protein expression levels in A549 and PC3 cells neither under si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) as compared with si-random in hypoxia and normoxia. However, in LNCaP cells we observed a downregulation of PKM2 and phospho PKM2 (Tyr105) protein expression levels under si-HIF-1α and both together (si-HIF-1α + si-HIF-2α), but not under si-HIF-2α as compared with si-random in hypoxia (Figure 11C).

A)


A549 HIF-1
A549 HIF-2
PC3 HIF-1
PC3 HIF-2
LNCaP HIF-1
LNCaP HIF-2
-ΔCt
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
Normoxia
Hypoxia

B)


A549 PKM2
-ΔCt
10
8
6
4
2
0
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
Normoxia
Hypoxia
PC3 PKM2
-ΔCt
8
6
4
2
0
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
Normoxia
Hypoxia
LNCaP PKM2
-ΔCt
8
6
4
2
0
*
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
si Random
si HIF-1
si HIF-2
si HIF-1+HIF-2
Normoxia
Hypoxia

C)

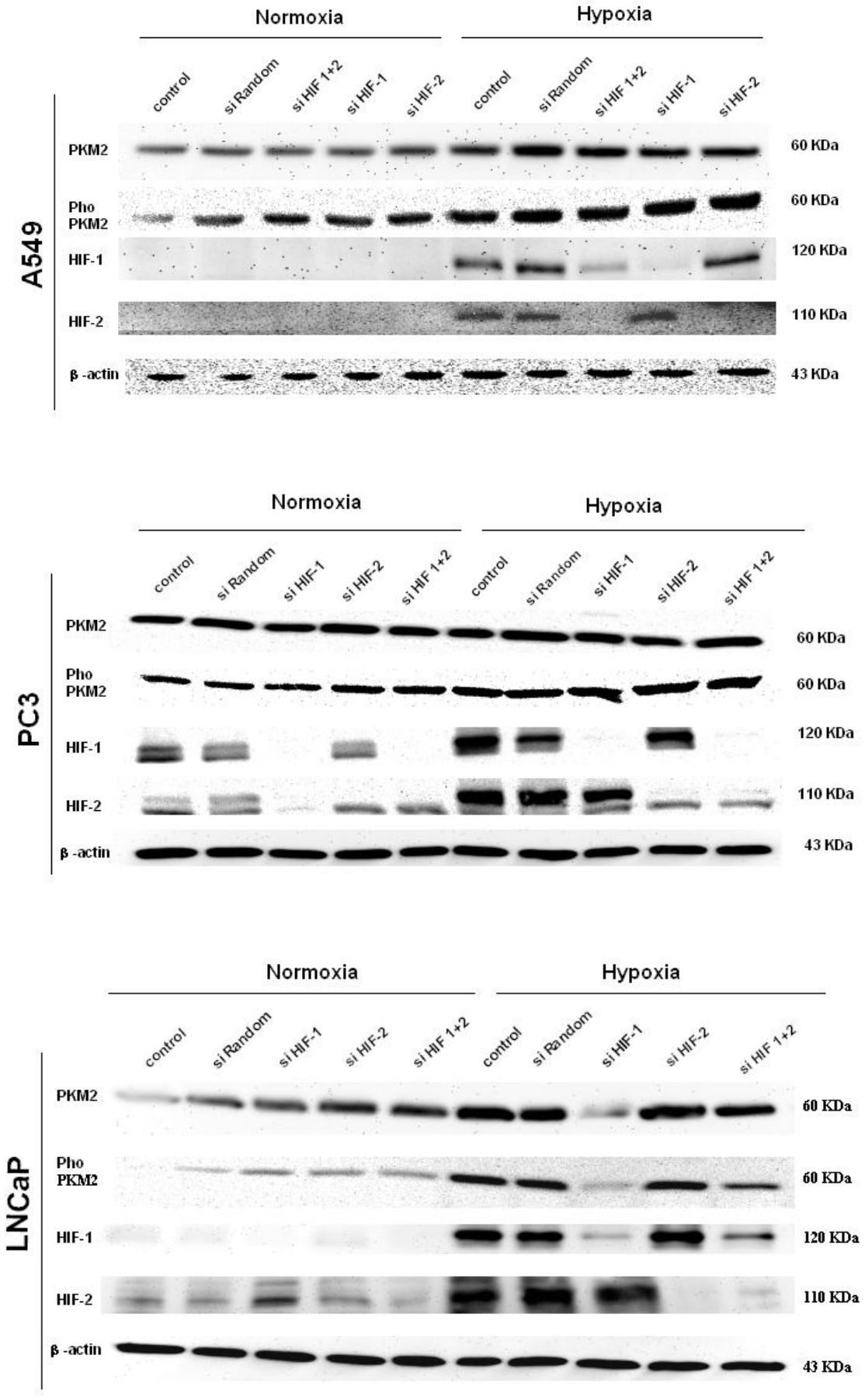
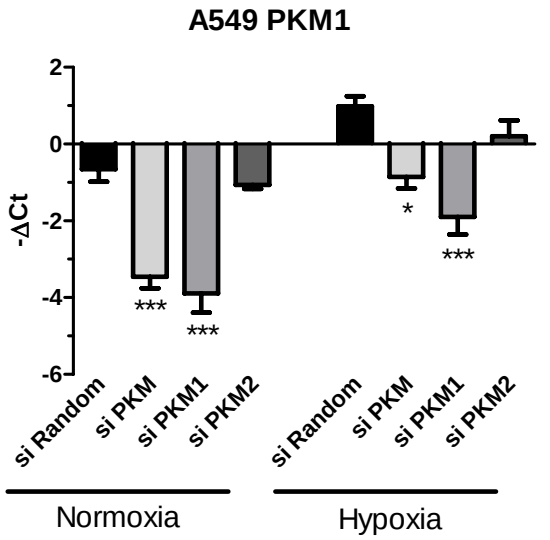

Normoxia
Hypoxia
control
si Random
si HIF 1+2
si HIF-1
si HIF-2
A549
PKM2
Pho PKM2
HIF-1
HIF-2
β -actin
60 KDa
60 KDa
120 KDa
110 KDa
43 KDa
PC3
LNCaP

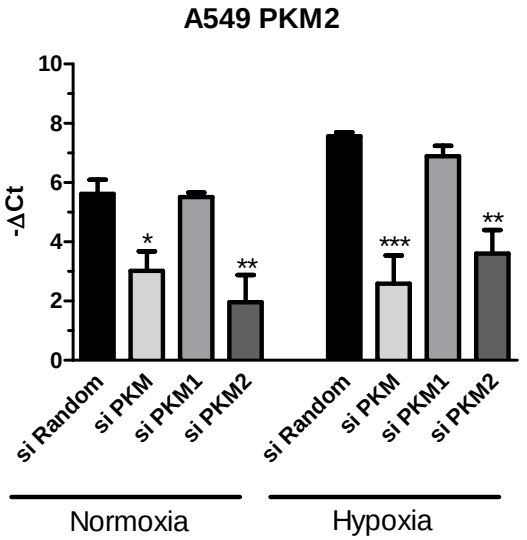
Figure 11: Effect of silencing of HIF-1α and HIF-2α by si-RNA on PKM2 expression

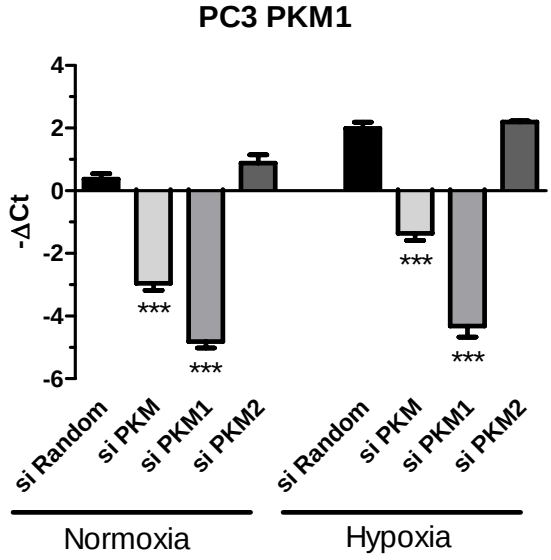
A) HIF-1α and HIF-2α mRNA analysis by real time RT-PCR of RNA extracts from A549, PC3 and LNCaP cells transfected with si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) and si-random, cultured for 24 h in normoxia or hypoxia. PBGD gene was used as a reference gene. (* Significant difference for si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) compared with si-random under normoxic or hypoxic conditions, n=4, bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was performed for statistical analysis SEM, * $p<0.05$) **B)** PKM2 mRNA analysis by real time RT-PCR of RNA extracts from A549, PC3 and LNCaP cells transfected with si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) and si-random, cultured for 24 h in normoxia or hypoxia. PBGD gene was used as a reference gene. (* Significant difference for si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) compared with si-random under normoxic or hypoxic conditions, n=4, bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was performed for statistical analysis SEM, * $p<0.05$) **C)** Western blot analysis of PKM2 and pho PKM2 expression under si-HIF-1α, si-HIF-2α or both together (si-HIF-1α + si-HIF-2α) compared with si-random and the validation of the suppressive effects of si-HIF-1α, si-HIF-2α and (si-HIF-1α + si-HIF-2α) on HIF-1α and HIF-2α expression compared with si-random. Cellular protein extracts (60 μg) from A549, PC3 and LNCaP cells, cultured for 24 in normoxia or hypoxia were used. β-actin was used as loading control

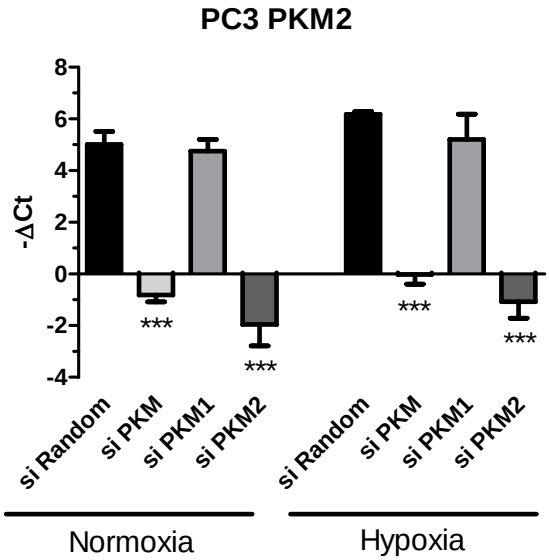
4.3 Inhibition of PKM1 and PKM2 expression by small interfering RNA and its effect on HIF-1α or HIF-2α

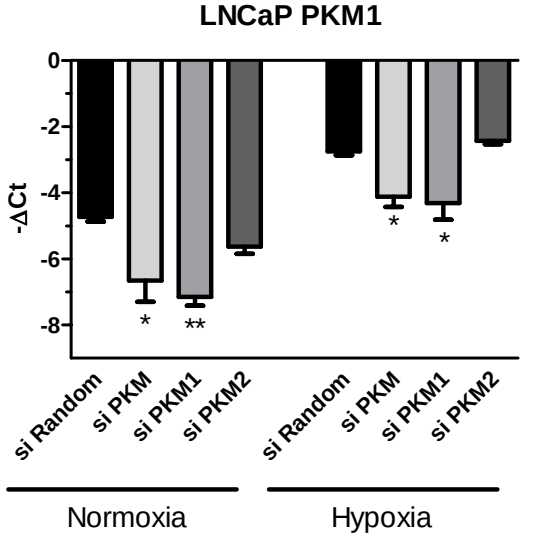
In order to analyze the role of PKM1 and PKM2 for HIF-1α and HIF-2α expression, we silenced PKM1 and PKM2 expression by small interfering RNA. For silencing of PKM1 a siRNA targeting exon 9 of the PKM gene and for PKM2 a siRNA targeting exon 10 of the PKM gene were selected. Silencing was validated by real time RT-PCR analysis of mRNA extracts from A549, PC3 and LNCaP cells transfected with non-specific si-RNA (si-random), si-PKM, si-PKM1 and si-PKM2 under normoxic and hypoxic conditions. A significant downregualtion of PKM1 and PKM2 mRNA expression levels were observed under si-PKM1 and si-PKM2 respectively, and si-PKM as compared with si-random treated cell lines in normoxia and hypoxia (Figure 12).


A549 PKM1
-ΔCt
2
0
-2
-4
-6
***
***
*
***
si Random
si PKM
si PKM1
si PKM2
si Random
si PKM
si PKM1
si PKM2
Normoxia
Hypoxia


A549 PKM2
-ΔCt
10
8
6
4
2
0
*
**
***
**
si Random
si PKM
si PKM1
si PKM2
si Random
si PKM
si PKM1
si PKM2
Normoxia
Hypoxia


PC3 PKM1
-ΔCt
4
2
0
-2
-4
-6
***
***
***
***
si Random
si PKM
si PKM1
si PKM2
si Random
si PKM
si PKM1
si PKM2
Normoxia
Hypoxia


PC3 PKM2
-ΔCt
8
6
4
2
0
-2
-4
***
***
***
***
si Random
si PKM
si PKM1
si PKM2
si Random
si PKM
si PKM1
si PKM2
Normoxia
Hypoxia


LNCaP PKM1
-ΔCt
0
-2
-4
-6
-8
*
**
*
*
si Random
si PKM
si PKM1
si PKM2
si Random
si PKM
si PKM1
si PKM2
Normoxia
Hypoxia

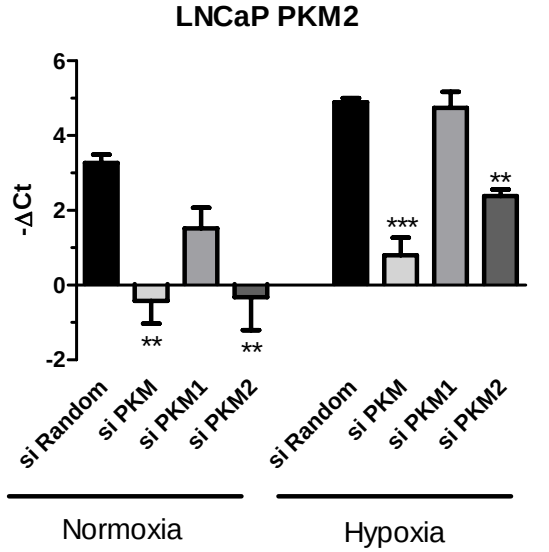
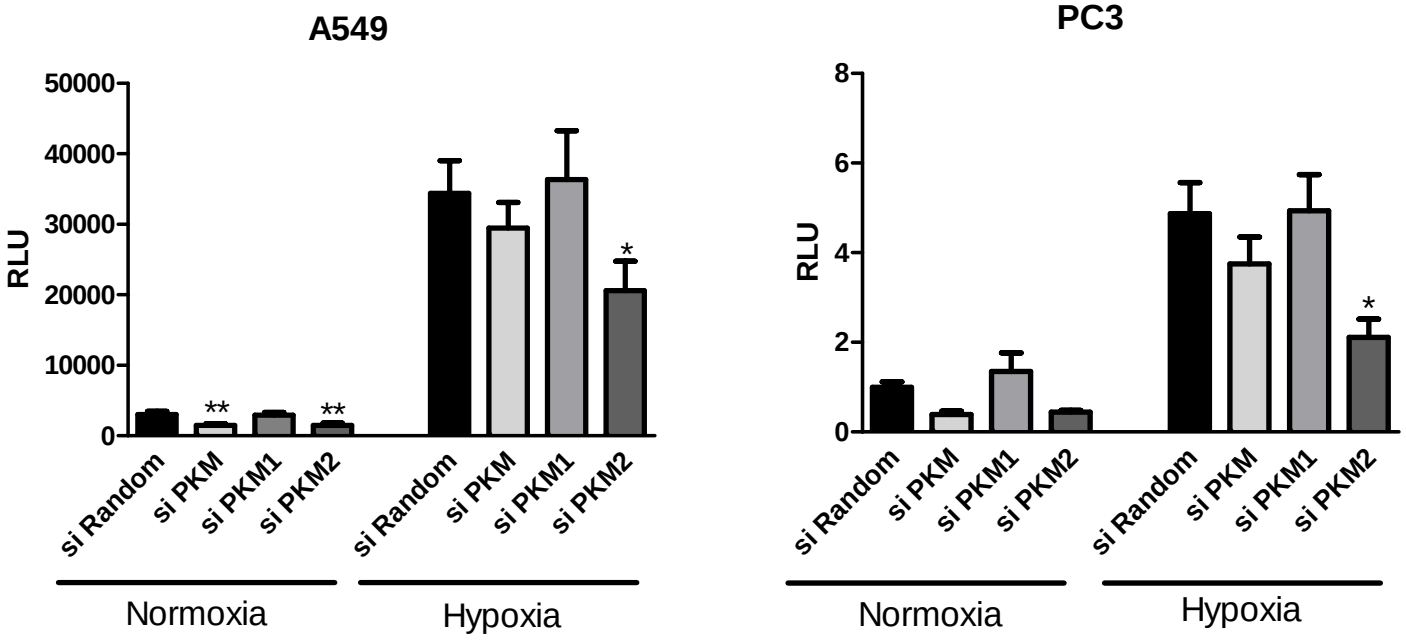

LNCaP PKM2
-ΔCt
6
4
2
0
-2
**
**
***
**
si Random
si PKM
si PKM1
si PKM2
si Random
si PKM
si PKM1
si PKM2
Normoxia
Hypoxia

Figure 12: Inhibition of PKM1 and PKM2 expression by small interfering RNA

PKM1 and PKM2 mRNA analysis by real time RT-PCR of RNA extracts from A549, PC3 and LNCaP cells transfected with si-PKM, si-PKM1, si-PKM2 and si-random, cultured for 24 h in normoxia or hypoxia. PBGD gene was used as a reference gene. (*Significant differences for si-PKM, si-PKM1 and si-PKM2 compared with si-random under normoxic and hypoxic conditions, n=4, bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was performed for statistical analysis SEM, $p<0.05$).

4.3.1 Effect of silencing of PKM1 and PKM2 on HRE activation

Next we analyzed the interaction between PKM2 and HIF-1α. We employed hypoxia responsive element (HRE) luciferase reporter gene assay for the measurement of HIF-1α dependent target gene expression. A549, PC3 and LNCaP cells were co-transfected by non-specific si-RNA (si-random), si-PKM, si-PKM1 and si-PKM2 with the phosphoglycerate kinase reporter plasmid (PGK-HRE) under normoxic and hypoxic conditions. We observed a significant downregulation of the luciferase activity in A549 and PC3 cells under si-PKM2, but not under si-PKM or si-PKM1 as compared with si-random under hypoxic conditions. No change of luciferase activity was observed under si-PKM or si-PKM2 in LNCaP cells as compared with si-random under hypoxic conditions, but we observed a significant increase of luciferase activity under si-PKM1. These effects were also notable at lower levels under conditions of normoxia (Figure 13).



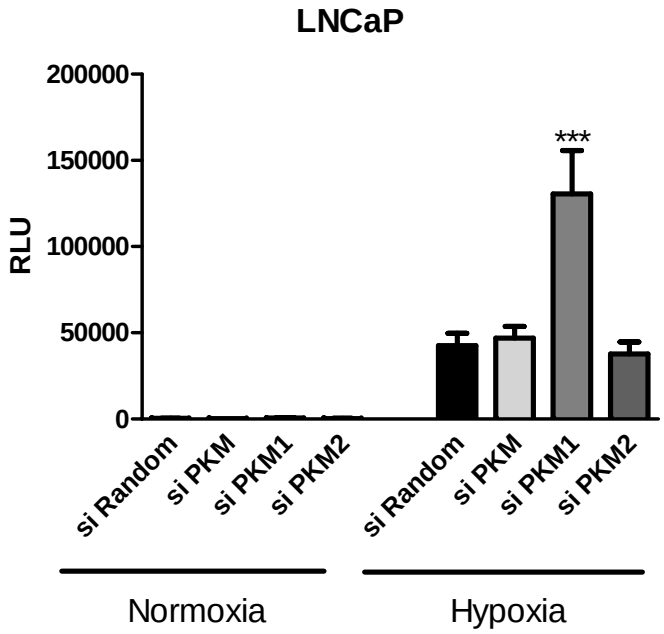


Figure 13: Effect of silencing of PKM1 and PKM2 by si-RNA on HRE activation

HRE reporter gene assay in A549, PC3 and LNCaP cells transfected with si-PKM, si-PKM1, si-PKM2 and si-random, cultured for 24h in normoxia or hypoxia. Luciferase activity was measured after 48h of transfection. (* Significant differences for si-PKM, si-PKM1, si-PKM2 compared with si-random under normoxic and hypoxic conditions, n=4, bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was performed for statistical analysis SEM, $p<0.05$).

In the next step, we analyzed the effects of the inhibition of PKM1 and PKM2 on HIF-1α by densitometric analysis of HIF-1α western blot. The validation of si-RNA was confirmed by PKM2 and phospho PKM2 (Tyr105) western blot in relation to the loading control β-actin. A remarkeble downregulation of PKM2 and phospho PKM2 (Tyr105) protein expression levels were observed under si-PKM and si-PKM2, but not under si-PKM1 as compared with si-random treated cell lines in hypoxia (Figure 14).

The densitometric scanning of blots showed no significant effect on HIF-1α protein expression levels in A549, PC3 and LNCaP cells under si-PKM, si-PKM1 and si-PKM2 as compared with si-random in hypoxia (Figure 14).

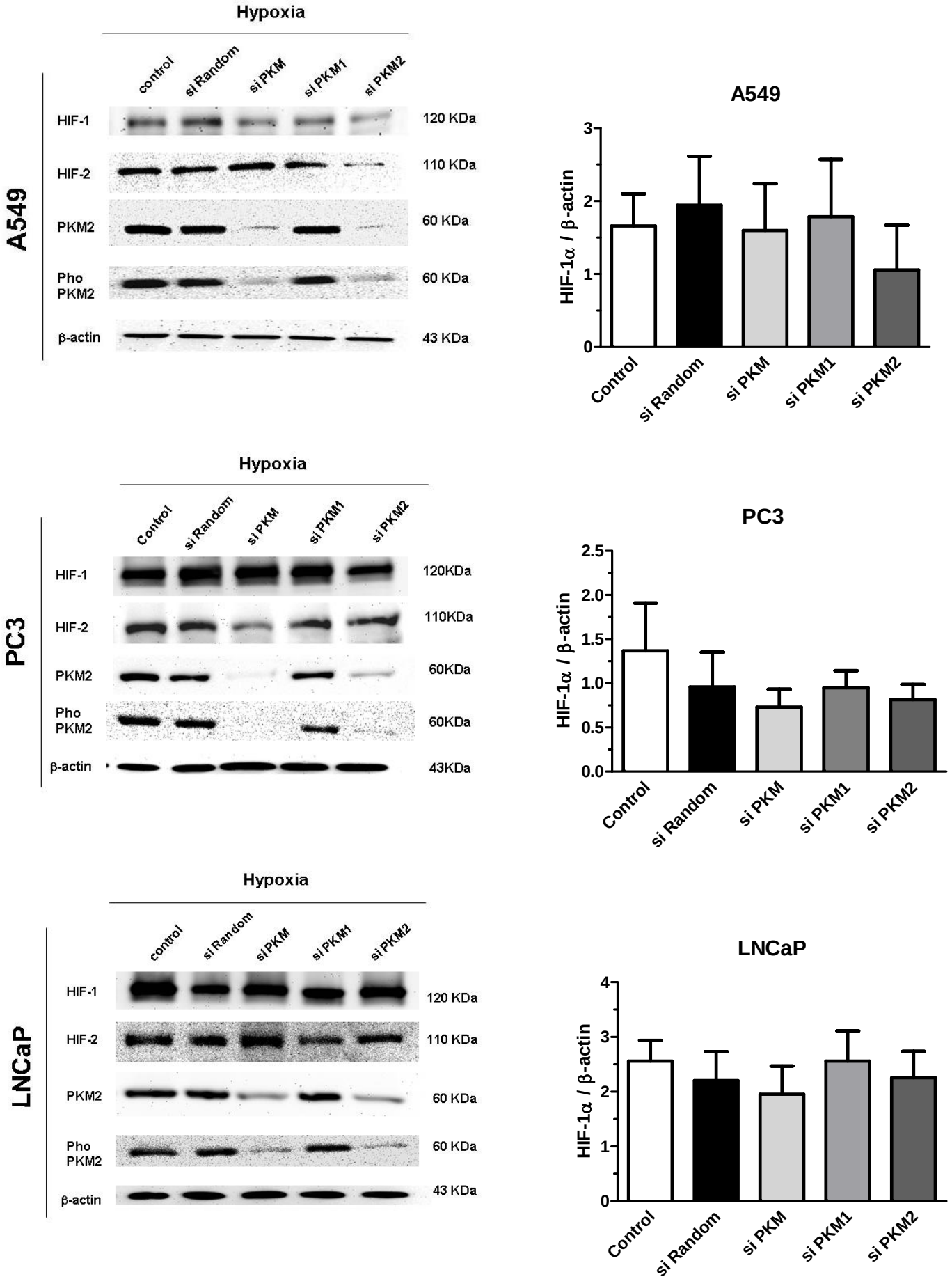

A549
Hypoxia
control
si Random
si PKM
si PKM1
si PKM2
HIF-1
HIF-2
PKM2
Pho PKM2
β-actin
120 KDa
110 KDa
60 KDa
60 KDa
43 KDa
HIF-1α / β-actin
Control
si Random
si PKM
si PKM1
si PKM2
PC3
Hypoxia
Control
si Random
si PKM
si PKM1
si PKM2
HIF-1
HIF-2
PKM2
Pho PKM2
β-actin
120KDa
110KDa
60KDa
60KDa
43KDa
HIF-1α / β-actin
Control
si Random
si PKM
si PKM1
si PKM2
LNCaP
Hypoxia
control
si Random
si PKM
si PKM1
si PKM2
HIF-1
HIF-2
PKM2
Pho PKM2
β-actin
120 KDa
110 KDa
60 KDa
60 KDa
43 KDa
HIF-1α / β-actin
Control
si Random
si PKM
si PKM1
si PKM2

Figure 14: Effect of silencing of PKM1 and PKM2 by si-RNA on HIF-1α expression

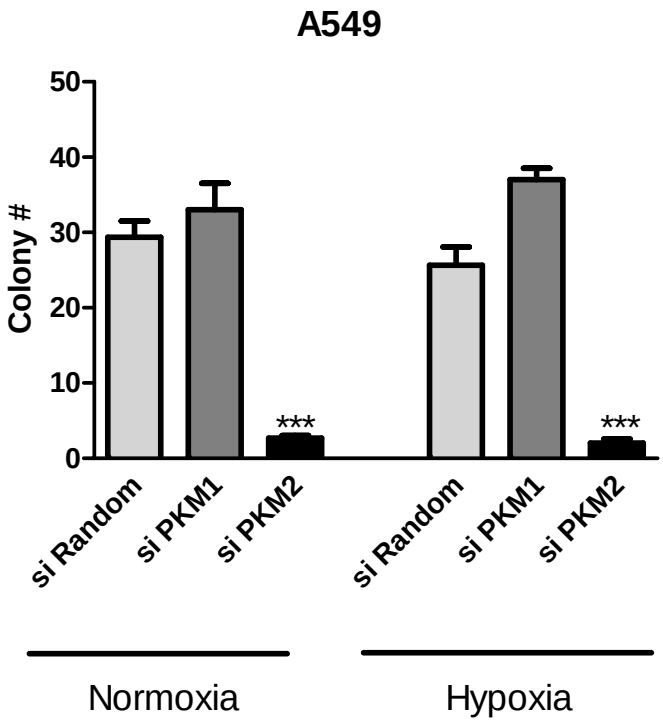
Western blot analysis of HIF-1α and HIF-2α expression under si-PKM, si-PKM1 and si-PKM2 compared with si-random and the validation of the suppressive effects of si-PKM, si-PKM1 and si-PKM2 on PKM2 and pho PKM2 expression compared with si-random. Cellular protein extracts (60 µg) from A549, PC3 and LNCaP cells, cultured for 24 in hypoxia were used. β-actin was used as loading control. Densitometric analysis of HIF-1α western-blot normalized to β-actin from A549, PC3 and LNCaP cells transfected with si-PKM, si-PKM1, si-PKM2 and si-random, cultured for 24 h in hypoxia. (* Significant differences for si-PKM, si-PKM1, si-PKM2 compared with si-random under hypoxic conditions, n=4, bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was performed for statistical analysis SEM, $p<0.05$).

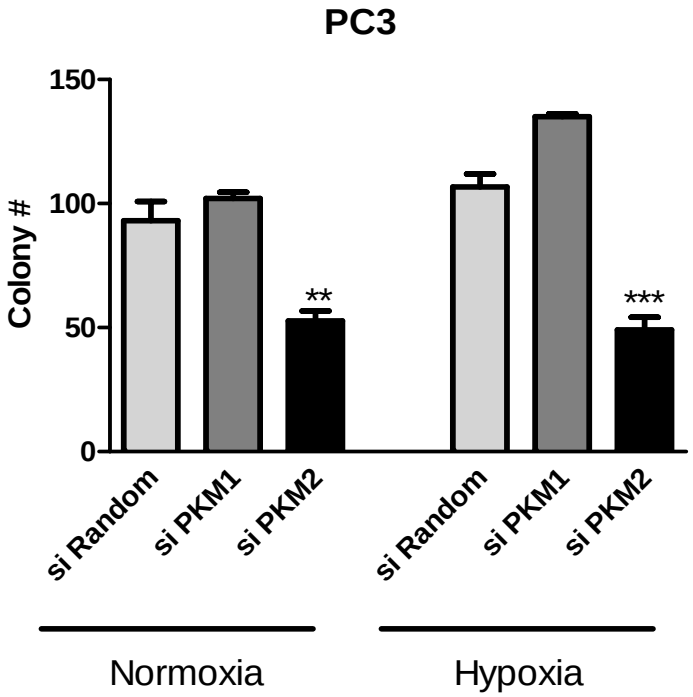
4.4 Effect of silencing of PKM1 and PKM2 on proliferation

We next studied the effect of PKM1 and PKM2 on cell proliferation by colony survival (clonogenic assay), reflecting the cell growth under difficult conditions and the oncogenic characteristic of the cells

4.4.1 Effect of silencing of PKM1 and PKM2 by si-RNA on colony survival

A549, PC3 and LNCaP cells were transfected by non-specific si-RNA (si-random), si-PKM1 and si-PKM2 under normoxic and hypoxic conditions. The clonogenic survival was analyzed after 14 days of transfection by counting colonies. Inhibition of PKM2 expression by si-PKM2 resulted in a significant decrease of colony formation in A549, PC3 and LNCaP cells as compared with si-random in normoxia and hypoxia, while no decrease was observed under si-PKM1 (Figure 15).


A549
Colony #
50
40
30
20
10
0
***
***
si Random
si PKM1
si PKM2
si Random
si PKM1
si PKM2
Normoxia
Hypoxia


PC3
Colony #
150
100
50
0
**
***
si Random
si PKM1
si PKM2
si Random
si PKM1
si PKM2
Normoxia
Hypoxia

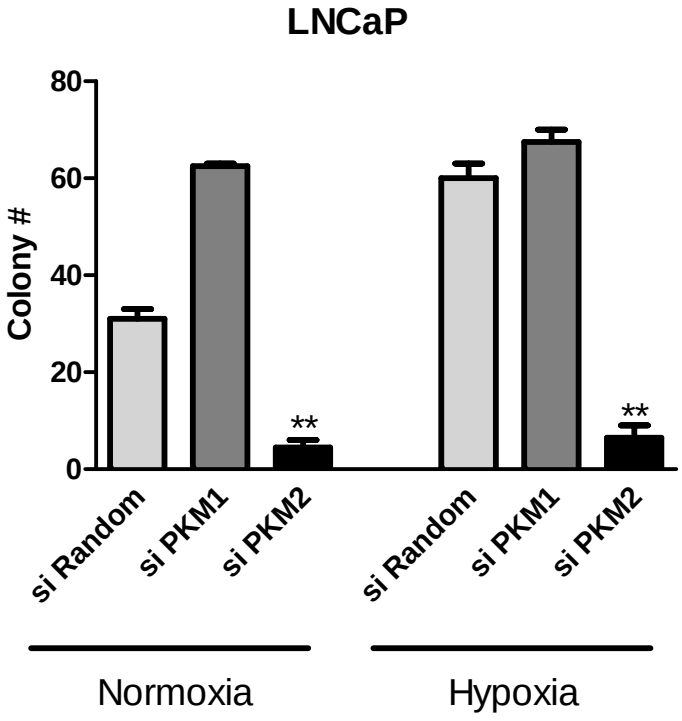

LNCaP
Colony #
80
60
40
20
0
**
**
si Random
si PKM1
si PKM2
si Random
si PKM1
si PKM2
Normoxia
Hypoxia

Figure 15: Effect of silencing of PKM1 and PKM2 by si-RNA on colony survival

Colony survival assay in A549, PC3 and LNCaP cells transfected with si-PKM1, si-PKM2 and si-random, cultured in normoxia and hypoxia. (* Significant differences for si-PKM1 and si-PKM2 compared with si-random on colony formation under normoxic and hypoxic conditions, n=3, bars represent mean ± SEM. One-way ANOVA with Dunnett's multiple comparison test was performed for statistical analysis SEM, $p<0.05$).

5. Discussion

In this study we investigated the regulation of PKM1 and PKM2 in hypoxia and their dependence on HIF-1α and HIF-2α. For this purpose we analyzed the gene expression of PKM1 and PKM2 in A549, PC3 and LNCaP cell lines under normoxic and hypoxic conditions. Silencing of HIF-1α, HIF-2α or both was used for the analysis of the dependence of PKM on HIF. Vice verse, we investigated the effects of PKM1 and PKM2 on HIF-1α and HIF-2α expression. Furthermore, the role of PKM1 and PKM2 for cancer cell survival was analyzed. For these experiments selective silencing of PKM1 and PKM2 employing synthetic siRNA was established. Vice verse, we investigated the effects of PKM1 and PKM2 on

5.1 Regulation of PKM1 and PKM2 in hypoxia and dependence on HIF

We observed that PKM2 was induced at the mRNA level in hypoxia as compared to normoxia, in all cell lines studied. PKM1 mRNA level was significantly induced in hypoxia only in LNCaP cells, whereas this hypoxia induced increase did not reach significance in A549 and PC3 cells (Figure 9). At the protein level, PKM2 was induced in hypoxia as compared to normoxia, significantly only in LNCaP cells, but not in PC3 and A549 cells. Thus, an increase at the mRNA level of PKM2 was followed by an increase at the protein level only in LNCaP cells (Figure 10). In other studies PKM2 induction in hypoxia was seen at the mRNA level in hepatoblastoma cells (HepG2) [118] and mouse embryonic fibroblast (MEFs) [17]. PKM2 induction in hypoxia was seen also at the protein level in hepatoblastoma cells (HepG2) [118] and Hela cells [17].

Silencing of HIF-1α and HIF-2α revealed no significant change of PKM2 mRNA expression level in hypoxia in A549 and PC3 cells. However, we observed a significant downregulation of PKM2 mRNA expression level in LNCaP cells (Figure 11B). Also, western blot analysis showed no change of PKM2 or phospho PKM2 (Tyr105) protein expression levels in A549 and PC3 cells. However, we observed a strong downregulation of PKM2 and phospho PKM2 (Tyr105) protein expression levels in LNCaP cells (Figure 11C). Thus, HIF-1α dependent induction of PKM2 is not obvious in all cells. The dependence of PKM2 on HIF-1α was recently demonstrated and linked to a functional HRE site within intron 1 of the PKM gene, which allows HIF-1α dependent regulation of PKM2 and PKM1 [17]. Silencing of HIF-1α

significantly reduced PKM2 HRE-dependent firefly luciferase (FLuc) activity in hypoxic HeLa cells [17]. Thus, the effect of HIF-1α on PKM2 regulation may differ between cell lines or the detailed protocol of hypoxic incubation. In two other publications, an effect of HIF-1α on PKM2 was observed to be mediated by c-Myc, which binds and activates the hnRNPA1, hnRNPA2 and polypyrimidine tract binding protein (PTB) promoters. These proteins regulate differential splicing of PKM1 and PKM2. Depletion of c-Myc in NIH-3T3 fibroblasts reduced the accumulation of the hnRNP proteins and increased the ratio of PKM1 to PKM2. However, this effect was not observed in HeLa cells, suggesting cell type specific mechanisms in this regard [25], [119].

5.2 Regulation of hypoxia inducible factor by pyruvate kinase

Using HRE reporter gene assay, we observed that silencing of PKM2 showed a significant downregulation of HIF-1α in A549 and PC3 cells, but not in LNCaP cells in hypoxia (figure 13). Western blot quantification results showed no significant effect of HIF-1α protein expression level in A549 and PC3 cells (Figure 14). This result suggests that HIF-1α expression may be controlled at the transactivation level as observed and mechanistically explored by another group [17]. In this study, it was demonstrated that PKM2 is hydroxylated at a prolin residue by PHD3. Phosphorylated PKM2 then participates in a transcription factor complex with HIF-1α and enhances transactivation of HIF-1α dependent target genes. This effect is likely dependent on the critical amino acid motif LXXLAP found in exon 10 of PKM2, which is identical to the prolyl hydroxylation domain of HIF-1α. Its suggested that PKM2 is hydoxlyated by PHD3 at this domain under mild hypoxic conditions (3-0.1% O_2), however an effect that should not be observed under anoxic conditions [17].

Of note is that PKM2 hydroxylation still occurs at 1% O_2, a condition where hydroxylation of HIF-1α by PHD2 does not occurs. This can be explained by a lower oxygen related Km value of the hydroxylation of PKM2 by PHD3 than the Km value for hydroxylation of HIF-1α by PHD2 [120], [17]. Overall, studies revealed that the Km values of purified PHDs for oxygen are close to the oxygen partial pressure (pO_2) in air, making these enzymes suitable as oxygen sensors [121]. Differences may occur between different enzymatic isoforms and substrates [121], [122], [123]. Hydroxylation of PKM2 by PHD3 in hypoxia is favored by enhanced PHD3 expression in hypoxia since PHD3 is a member of the HIF-1α target genes

[124], but the degree of induction apparently varies between cell type and pO_2 [95], [125], [94], [126]. Furthermore, enzymatic activity of PHD3 is induced in hypoxia and may further favor hydroxylation of PKM2 in hypoxia, in spite of lowered oxygen concentration [127].

Our observation that silencing of PKM1 significantly enhances HIF-1α target gene expression only in LNCaP cells is descriptive and may involve cell specific characteristics that are obscure.

HIF dependence on PKM2 could also be affected by the metabolites lactate and pyruvate. PKM2 is crucial for metabolic and glycolytic flux, which is reflected to a several fold increase in glucose consumption and lactate production [33], [128], [129]. It was suggested that the end products of glycolytic metabolism can promote HIF-1α protein stability and activate HIF-1α inducible gene expression. Pyruvate can have an inducible role by preventing HIF-1α degredation involving the steps of HIF-1α proline hydroxylation, *VHL* tumor suppressor protein binding or ubiquitin conjugation [85].

Figure 16 represents a schematic diagram that summarizes the regulation and interaction of PKM2 with HIF-1α in hypoxia. This diagram is mainly based on the literature [17], [25], [119] for details see figure legend.

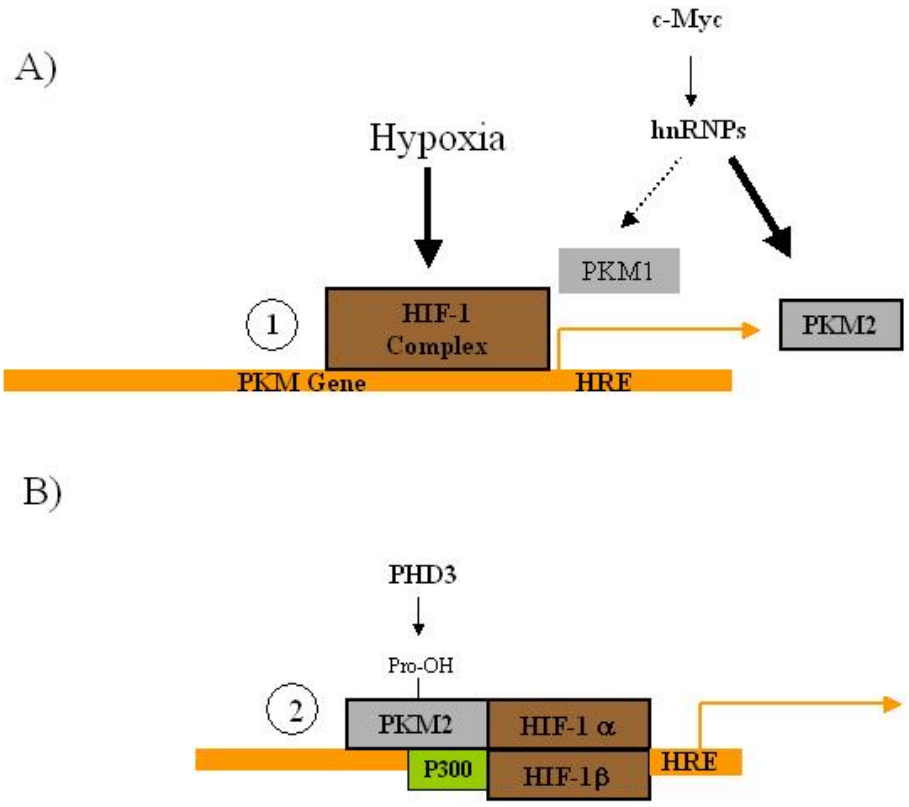


Figure 16: Schematic representation of the regulation of PKM2 and its interaction with HIF-1α

The principle mechanisms of PKM2 regulation involve HIF and c-Myc proteins. **A)** Hypoxia activates HIF-1α that can induce PKM2 at the transcriptional level mediated by the HRE element. C-Myc is relevant for PKM2 since it regulates critical hnRNP proteins that affect differential splicing of PKM1 and PKM2 favoring the latter one. Overall, these pathways determine the absolute and relative levels of PKM1 and PKM2. **B)** The interaction of PKM2 with HIF-1α involving PHD3 hydroxylation of PKM2 is displayed. This modification results in HIF-1α transactivation by interaction with the transcription factor complex [17], [25], [119]. Pathways affecting PKM2 and HIF that were observed in our study are labeled by circled numbers. The first is induction of PKM2 at the mRNA and protein levels in hypoxia, which was dependent on HIF-1α but not HIF-2α in LNCaP cells. The second is PKM2 dependent transactivation of HIF-1α that was observed in A549 and PC3 cells in our study.

5.3 Role of pyruvate kinase for cell proliferation

Silencing of PKM2, but not PKM1 expression resulted in a significant decrease of colony formation in studied cell lines under normoxic and hypoxic conditions (Figure 15). This may fit functionally with the observation that PKM2 activity shifts glycolytic metabolites away from energy production towards anabolic processes of cellular compounds required for

proliferation. PKM2 expression would better sustain cell growth by providing cells with a growth advantage from enabling them greater flexibility in dividing glucose metabolites between energy production and anabolic processes, such as lipid, nucleotide and amino acid synthesis [50], [62], [130].

In conclusion, we have presented data on the gene expression and function of PKM2. We demonstrated that PKM2 is induced in hypoxia at the mRNA level in all cell lines studied. However, this induction of PKM2 mRNA only resulted in higher abundance of PKM2 protein in one cell line. According to our study, HIF-1α, but not HIF-2α, was relevant for PKM2 induction in one cell line reflecting cell specific differences in hypoxic PKM2 regulation. We showed that PKM2, but not PKM1, has an effect on HIF-1α stabilization at the transactivition level, but not in all studied cell lines. At the functional level, we observed that PKM2 induces cell proliferation both in normoxia and hypoxia in all cell lines studied.

6. Summary

PKM1 and PKM2 represent two splice variants of the glycolytic enzyme pyruvate kinase that differ in the inclusion of exon 9 or exon 10, respectively. PKM2 appears to be highly expressed in tumor cells and is suggested to favor the Warburg effect that is characterized by high glycolytic activity of tumor cells.

In the first part of the thesis, we analyzed the regulation of PKM1 and PKM2 under normoxic and hypoxic conditions in different tumor cell lines from lung (A549) and prostate (PC3 and LNCaP). Furthermore, we investigated whether HIF-1α and HIF-2α are relevant for hypoxic regulation of PKM1 and PKM2.

At the mRNA level, PKM1 and PKM2 were expressed in all cell lines studied. PKM2 displayed significant upregulation under hypoxic conditions in all cell lines studied, whereas PKM1 mRNA was only increased significantly under hypoxic conditions in LNCaP cells. At the protein level, PKM2 expression was upregulated significantly in LNCaP cells in hypoxia and to some extent in A549 cells that did not reach significance. No change of the PKM2 protein level was observed in PC3 cells in hypoxia. Silencing of HIF-1α and HIF-2α did not show any effect on PKM2 mRNA and protein expression levels in A549 and PC3 cells. However, HIF-1α dependent upregulation of PKM2 was observed at the mRNA and protein level in LNCaP cells in hypoxia.

Since HIF-1α is a mediator of the Warburg effect, we next analyzed a possible role of PKM2 on the HIF-1α pathway. Interestingly, silencing of PKM2 decreased HRE activation in A549 and PC3 cells, whereas HIF-1α protein levels were not significantly reduced after silencing of PKM2, suggesting that HIF-1α dependence on PKM2 is mediated at the transactivation level. LNCaP cells responded differently in this regard, whereas no effect was observed after silencing of PKM2. Silencing of PKM1 increased HRE activation in these cells, an effect that also was observable only at the transactivation but not at the protein level of HIF-1α.

PKM1 and PKM2 were further functionally characterized with respect to clonogenic survival. Silencing of PKM2, but not PKM1, significantly reduced clonogenic survival in all cell lines studied, confirming the role of PKM2 in tumorigenesis in contrast to PKM1.

In conclusion, we revealed that PKM2 was upregulated at the mRNA level in hypoxia in all cell lines studied. At the protein level, this effect became obvious only in LNCaP cells and was dependent on HIF-1α in these cells. Vice verse, PKM2 appears to have an effect on HIF-1α transactivation in two from three cell lines studied. Thus, PKM2 may contribute to the Warburg effect by induction of HIF dependent signal transduction in at least some cancer cell lines. PKM2 has strong effects on tumorigenesis to a similar extent in all cell lines studied.

7. Zusammenfassung

PKM1 und PKM2 repräsentieren zwei Spleißvarianten des glykolytischen Enzyms Pyruvatkinase, die sich jeweils durch die Inklusion von Exon 9 oder Exon 10 unterscheiden. PKM2 scheint in Tumorzellen hoch exprimiert zu werden und begünstigt den Warburg-Effekt, der durch eine hohe glykolytische Aktivität in Tumorzellen charakterisiert ist.

Im ersten Teil der Arbeit, analysierten wir die Regulation von PKM1 und PKM2 unter normoxischen und hypoxischen Bedingungen in verschiedenen Tumor-Zelllinien aus Lunge (A549) und Prostata (PC3 und LNCaP). Darüber hinaus wurde untersucht, ob HIF-1 α und HIF-2α relevant für eine Induktion von PKM1 und PKM2 in Hypoxie sind.

Auf mRNA Ebene wurden PKM1 und PKM2 in allen untersuchten Zelllinien exprimiert. PKM2 zeigte eine signifikante Hochregulation in Hypoxie in allen untersuchten Zelllinien während PKM1 mRNA nur in LNCaP-Zellen signifikant erhöht war. Auf Protein-Ebene war die PKM2 Expression in LNCaP-Zellen in Hypoxie signifikant erhöht und in geringerem Maße Grad in A549 Zellen ohne signifikant zu sein. In PC3-Zellen war keine Änderung von PKM2 Protein in Hypoxie zu beobachten. *Silencing* von HIF-1α und HIF-2α zeigte keine Auswirkungen auf PKM2 mRNA- und Protein-Expression in A549 und PC3 Zellen. Allerdings wurde eine HIF-1α abhängige Induktion von PKM2 auf mRNA-und Protein-Ebene in LNCaP-Zellen in Hypoxie beobachtet.

Da HIF-1 α ein Vermittler des Warburg-Effekt ist, analysierten wir als nächstes eine mögliche Rolle von PKM2 auf HIF-1 abhängige Signaltransduktion. Interessanterweise verringerte *Silencing* von PKM2 die HRE-Aktivierung in A549 und PC3 Zellen, während die HIF-1α-Protein-Spiegel nicht signifikant reduziert waren, was darauf hindeutet, dass PKM2 die Transaktivierung von HIF-1 α begünstigt. LNCaP-Zellen reagierten in dieser Hinsicht unterschiedlich. Während kein Effekt nach *Silencing* von PKM2 auf die die HRE-Aktivierung gefunden wurde, erhöhte *Silencing* von PKM1 die HRE-Aktivierung in diesen Zellen ohne, dass die HIF-1α Protein-Spiegel geändert waren.

PKM1 und PKM2 wurden weiter funktionell in Bezug auf ihr klonogenes Überleben charakerisiert. *Silencing* von PKM2 aber nicht PKM1 reduzierte das klonogene Überleben in

allen untersuchten Zelllinien deutlich und bestätigte so die Rolle von PKM2 im Gegensatz zu PKM1 in der Tumorgenese.

Zusammenfassend lässt sich sagen, dass wir eine PKM2 Hochregulation auf mRNA-Ebene in Hypoxie in allen untersuchten Zelllinien nachweisen konnten. Auf Protein-Ebene wurde dieser Effekt nur in LNCaP-Zellen offenbar und war abhängig von HIF-1 α in diesen Zellen. Umgekehrt hatte PKM2 eine positive Wirkung auf die Transaktivierung von HIF-1 α in zwei von drei Zelllinien. So könnte PKM2 durch die Induktion HIF abhängiger Signaltransduktion zumindest in einigen Tumorzellen zum Warburg-Effekt beitragen. PKM2 zeigte eine starke Auswirkung auf die Tumorgenese in den untersuchten Tumorzelllinien.

8. References

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Declaration

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Place and Date

Signature

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

9. Appendix

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Research and practical experince

Oct 2007 – Oct 2011 Giessen Hospital Marburg Hospital	(A549, H23, H226, LLC, PC3, LNCaP and DU145) cell lines: cell culture, treatment by hypoxia and radiation conditions, siRNA and plasmid transfection. Tumor mouse model (radiation and hypoxia) molecular characterization including: HIF, angiogenesis, cell cycle, autophagy and radiation markers. Protein analysis (protein purification, western blot), genomic analysis (RNA isolation, mRNA expression by Real time), immunocytochemistry staining, FACS, gene reporter assay, proliferation assay (BrdU, clonogenic survival). Patient's prostate tumor analysis, Marburg Hospital, Urology Department. Development of new approaches for drug delivery system including treatment, analysis and molecular characterization of cancer cell lines by new peptide system (iRGD) and tyrosine kinase inhibitor (TKI) Novartis company.
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2) Florentine Kamlah, Jörg Hänze, Andrea Arenz, Ulrike Seay, Diya Hasan Janko Juricko, Birgit Bischoff, Oana R. Gottschald, Claudia Fournier, Gisela Taucher-Scholz, Michael Scholz, Werner Seeger, Rita Engenhart-Cabillic, Frank Rose; Comparison of the effects of carbon ion and photon irradiation on the angiogenic response in human lung adenocarcinoma cells; International Journal of Radiation Oncology • Biology • Physics, 2011

3) Diya Hasan, Viktor Malec, Werner Seeger, Jörg Hänze; Gene expression and functional characterization of pyruvate kinase isoforms M1 and M2 and their inhibition by siRNA in cancer (in process)

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The 15th AEK International Cancer Congress 2009, (Berlin) Germany

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