/Regulation of pyruvate kinase M2 (PKM2) by hypoxia/

By

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A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
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Masters of Science

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The undersigned hereby recommend to
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1. ABSTRACT

Sequence analysis of the M2 isoform (PKM2) promoter revealed that it contained a Hypoxia Response Element (HRE) which may bind the Hypoxia Inducible Factor (HIF) and induce hypoxic regulation. This is also the only PK isoform that contains the same LXXLAP sequence that is present as two copies in HIF- α which, when hydroxylated under normoxic conditions, is ubiquitinated and targeted for degradation by the proteasome. PKM2 protein expression and activity was significantly enhanced after cobalt and long-term exposure to hypoxia. Real-time RT-PCR analysis, however, showed that gene levels were not affected in L6E9 cells. Cell viability of HEK29A cells, under knockdown of PKM2 with siRNA and exposure to hypoxia, was examined using the MTT assay. When treated with hypoxia, there were no significant decreases in cell viability when comparing between cells that did not contain the knockdown to those that did.

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5. LIST OF ABBREVIATIONS

ADP: Adenosine-Diphosphate

APS: Ammonia Persulfate

ARNT: Aryl Hydrocarbon Nuclear Receptor Translocator

ATP: Adenosine-Triphosphate

bHLH: Basic Helix Loop Helix

BSA: Bovine Serum Albumin

CBP: CREB-Binding Protein

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

dsRNA: Double Stranded RNA

EDTA: Ethylenediaminetetraacetic Acid

FBP: Fructose-1,6-Bisphosphate

FBS: Fetal Bovine Serum

FIH: Factor Inhibiting HIF

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

HEK: Human Embryonic Kidney

HEPES: 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HK: Hexokinase

HIF: Hypoxia Inducible Factor

HRE: Hypoxic Response Element

HS: Horse Serum

IEF: Isoelectric Focusing

IPG: Immobilized pH Gradient

LDH: Lactate Dehydrogenase

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NAD⁺: Nicotinamide Adenine Dinucleotide (oxidized form)

NADH: Nicotinamide Adenine Dinucleotide (reduced form)

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NLS: Nuclear Localization Signals

ODD: Oxygen-Dependent Degradation Domain

PAS: Period/ARNT/Similar

PBS: Phosphate Buffered Saline

PDB: Protein Data Bank

PEP: Phosphoenolpyruvate

Pfu: *Pseudomonas fluorescens*

PHD: Prolyl Hydroxylase

PGK: Phosphoglycerate Kinase

PK: Pyruvate Kinase

PK-L: Pyruvate Kinase Isoform L

PKM1: Pyruvate Kinase Isoform M1

PKM2: Pyruvate Kinase Isoform M2

PK-R: Pyruvate Kinase Isoform R

pI: Isoelectric Point

PI: Proteasome Inhibitor

PMSF: Phenylmethylsulfonyl Fluoride

P/S/A: Penicillin/Streptomycin/Amphotercin

PVDF: Polyvinylidene Fluoride

RISC: RNA-Induced Silencing Complex

RNAi: RNA Interference

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

siRNA: Small Interfering RNA

TAD: Transactivation Domain

TBST: Tris-Buffered Saline Tween-20

TBE: Tris/Borate/EDTA

TEMED: Tetramethylethylenediamine

VEGF: Vascular Endothelial Growth Factor

vHL: von Hippel Lindau

6. INTRODUCTION

6.1 Pyruvate Kinase (PK)

Pyruvate Kinase (PK, E.C. 2.7.1.40) is an enzyme located in the cytosol that catalyzes the final step of glycolysis. The enzyme catalyzes the conversion of phosphoenolpyruvate (PEP) and adenosine-diphosphate (ADP) to pyruvate and adenosine-triphosphate (ATP) (**Figure 1**). The first step of the reaction involves a phosphoryl transfer from PEP to ADP forming an enolate intermediate and ATP. The second step requires the protonation of the enolate intermediate forming pyruvate (Dombrauckas *et al.*, 2005). This reaction is strongly irreversible since the dephosphorylation of PEP to form ATP releases a significant amount of free energy (Dombrauckas *et al.*, 2005). The regulation of this reaction is very important because pyruvate is used in many biochemical processes including aerobic respiration, gluconeogenesis, anaerobic respiration and amino acid synthesis (Wang *et al.*, 2001).

The enzyme is ubiquitously expressed in all tissues of all eukaryotic organisms. PK is a tetramer of four identical subunits of approximately 60 kDa each unit (**Figure 2**) (Takenaka *et al.*, 1989). Four forms exist in order to satisfy the needs of different metabolic and growth patterns in specific tissues (Jurica *et al.*, 1998). They also differ in their enzymatic properties and regulation of their gene expression (Noguchi *et al.*, 1986). In humans, there are two genes that encode for different PK isoforms: the L gene produces the L (in liver) and R (in red blood cells) isozymes by the use of different

Figure 1: The pyruvate kinase reaction. The first step of the reaction involves a phosphoryl transfer from PEP to ADP forming an enolate intermediate and ATP. The second step requires the protonation of the enolate intermediate forming pyruvate. This reaction is strongly irreversible since the dephosphorylation of PEP to form ATP releases a significant amount of free energy (modified from Dombrauckas *et al.*, 2005).

Figure 2: Ribbon diagram of the structure of human PKM2 tetramer. PK is a tetramer of four identical subunits of approximately 60 kDa each unit (each unit is shown in a different colour) (Dombrauckas *et al.*, 2005). PDB ID 1T5A is shown here drawn with PyMOL.

tissue-specific promoters (Dombrauckas *et al.*, 2005) whereas the M gene produces the M1 (in skeletal muscle and brain) and M2 (in rapidlyproliferating fetal tissues) isoforms by alternative splicing of the same gene (Jurica *et al.*, 1998). There is a high degree of structural identity among the different isoforms (**Figure 3**) where PKM2 is gradually replaced by the other 3 isoforms as cells differentiate (Noguchi *et al.*, 1986). Isoforms of PK (specifically the L, R and M2) are allosterically regulated; they are either activated by fructose-1, 6-bisphosphate (FBP, produced earlier from glycolysis) or inactivated by ATP (Kiyoto *et al.*, 1991).

Deficiency of the human erythrocyte PK-R is the most common cause of the hereditary non-spherocytic haemolytic anaemia, a hereditary disease with severe clinical manifestations that can lead to death in the neonatal period (Mattevi *et al.*, 1996). It occurs when there are mutations in the main PK-R isoform that then interferes with allosteric regulation. This leads to an increase in concentration of 2,3-diphosphoglycerate (hemoglobin regulator and isomer of the glycolytic intermediate 1,3-bisphosphoglycerate) which results in a decrease in the affinity of hemoglobin for oxygen (Jurica *et al.*, 1998).

The tetrameric form of PK is associated with several other glycolytic enzymes in a huge structure known as the glycolytic enzyme complex, within which PK is active. The other enzymes associated with the glycolytic complex include hexokinase (HK), glyceraldehyde-3-P dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglyceromutase, enolase and lactate dehydrogenase (LDH) (Mazurek *et al.*, 2005).

Figure 3: Multiple sequence alignment of the complete sequence of all four human PK isoforms (R, L, M1 and M2) with ClustalW. The amino acid residues that are 100% identical among the human isoforms are starred.

hPK-M1 -----MSKPHSEAGTAFIQTQQ 17
hPK-M2 -----MSKPHSEAGTAFIQTQQ 17
hPK-R MSIQENISSQLRSWVSKSQRD LAKS ILIGAPGGPAGYLRRASVAQLTQELGTAFQQQQ 60
hPK-L -----MEGPAGYLRRASVAQLTQELGTAFQQQQ 29
* * * * *

hPK-M1 LHAAMADTFLEHMCRLDIDSPITARN TGIICTIGPASRSVETLKEMIKSGMNVARLNFS 77
hPK-M2 LHAAMADTFLEHMCRLDIDSPITARN TGIICTIGPASRSVETLKEMIKSGMNVARLNFS 77
hPK-R LPAAMADTFLEHLCLLDIDSEPVAARSTSI IATIGPASRSVERLKEMIKAGMNIARLNFS 120
hPK-L LPAAMADTFLEHLCLLDIDSEPVAARSTSI IATIGPASRSVERLKEMIKAGMNIARLNFS 89
* * * * *

hPK-M1 HGTHEYHAETIKNVRTATESFASDPILYRPVAVALDTKGPEIRTGLIKSGTAEVELKKG 137
hPK-M2 HGTHEYHAETIKNVRTATESFASDPILYRPVAVALDTKGPEIRTGLIKSGTAEVELKKG 137
hPK-R HGSHEYHAESI ANVREAVESFAGSPLSYRPVAIALDTKGPEIRTGILQGGPESEVELVKG 180
hPK-L HGSHEYHAESI ANVREAVESFAGSPLSYRPVAIALDTKGPEIRTGILQGGPESEVELVKG 149
* * * * *

hPK-M1 ATLKITLDNAYMEKCDENILWLDYKNICKVVEVGSKIYVDDGLISLQVKQKGADFLVTEV 197
hPK-M2 ATLKITLDNAYMEKCDENILWLDYKNICKVVEVGSKIYVDDGLISLQVKQKGADFLVTEV 197
hPK-R SQVLVTVDPAFRTRGNANTVWVDYPNIVRVVPVGGRIYIDDGLISLVVQKIGPEGLVTQV 240
hPK-L SQVLVTVDPAFRTRGNANTVWVDYPNIVRVVPVGGRIYIDDGLISLVVQKIGPEGLVTQV 209
* * * * *

hPK-M1 ENGGSLGSKKGVNLPGAAVDLPVASEKDIQDLKFGVEQDVMVFASFIRKASDVHEVRKV 257
hPK-M2 ENGGSLGSKKGVNLPGAAVDLPVASEKDIQDLKFGVEQDVMVFASFIRKASDVHEVRKV 257
hPK-R ENGGVLGSRKGVNLPGAQVDLPGLSEQDVRDLRFGEHGVDIVFASFVRKASDVAAVRAA 300
hPK-L ENGGVLGSRKGVNLPGAQVDLPGLSEQDVRDLRFGEHGVDIVFASFVRKASDVAAVRAA 269
* * * * *

hPK-M1 LGKEGKNIKIISKIENHEGVRRFDEILEASDGIMVARGDLGIEIPA EKVF LAQKMMIGRC 317
hPK-M2 LGKEGKNIKIISKIENHEGVRRFDEILEASDGIMVARGDLGIEIPA EKVF LAQKMMIGRC 317
hPK-R LGPEGHGIKII SKIENHEGVKRFDEILEVSDGIMVARGDLGIEIPA EKVF LAQKMMIGRC 360
hPK-L LGPEGHGIKII SKIENHEGVKRFDEILEVSDGIMVARGDLGIEIPA EKVF LAQKMMIGRC 329
* * * * *

hPK-M1 NRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLSGETAKGDYPLEAVRM 377
hPK-M2 NRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLSGETAKGDYPLEAVRM 377
hPK-R NLAGKPVVCATQMLESMITKPRPTRAETSDVANAVLDGADCIMLSGETAKGNFPVEAVKM 420
hPK-L NLAGKPVVCATQMLESMITKPRPTRAETSDVANAVLDGADCIMLSGETAKGNFPVEAVKM 389
* * * * *

A domain → | ← C domain

hPK-M1 QHLIAREAEAMFHRKLFEELVRASSHSTD LMEAMAMGSVEASYKCLAAALIVLTESGRS 437
hPK-M2 QHLIAREAEAAIYHLQLFEELRRLAPITSDPTEATAVGAVEASFKCCSGAIIVLT KSGRS 437
hPK-R QHAIAREAEAAVYHRQLFEELRRAAPLSRDPTEVTAIGAVEAAFKCCAAAIIVLT TTTGRS 480
hPK-L QHAIAREAEAAVYHRQLFEELRRAAPLSRDPTEVTAIGAVEAAFKCCAAAIIVLT TTTGRS 449
* * * * *

hPK-M1 AHQVARYRPRAPI IAVTRNPQTARQAHLYRGIFPVLCKDPVQEAWAEDVDLRVNFAMNVG 497
hPK-M2 AHQVARYRPRAPI IAVTRNPQTARQAHLYRGIFPVLCKDPVQEAWAEDVDLRVNFAMNVG 497
hPK-R AQLLSRYRPRAAVIAVTRSAQAARQVHLCRGVFPLLYREPPEAIWADDVDRRVQFGIESG 540
hPK-L AQLLSRYRPRAAVIAVTRSAQAARQVHLCRGVFPLLYREPPEAIWADDVDRRVQFGIESG 509
* * * * *

hPK-M1 KARGFFKKGDVVIVLTGWRPGSGFTNTMRVVPVP 531
hPK-M2 KARGFFKKGDVVIVLTGWRPGSGFTNTMRVVPVP 531
hPK-R KLRGFLRVGDLVIVVTGWRPGSGYT NIMRVLSIS 574
hPK-L KLRGFLRVGDLVIVVTGWRPGSGYT NIMRVLSIS 543
* * * * *

6.2 Regulation of PKM2

The message RNAs of PKM1 and PKM2 have the same length of 5' and 3' untranslated regions where the entire noncoding sequences of the two isozymes are identical (Noguchi *et al.*, 1986). Excluding the poly (A) tail, the mRNAs of the two isozymes have the same sequence except for a 160 nucleotide stretch within the coding region (Noguchi *et al.*, 1986). It is also unlikely that PKM1 and PKM2 have different sites of initiation and termination of transcription because the mRNAs of the two have identical noncoding 5' and 3' sequences (Noguchi *et al.*, 1986).

The two isozymes consist of 530 amino acids each and they are almost structurally identical except for a region of 45 residues (amino acids 388-432). It has been discovered that the two forms are translated by two different mRNAs transcribed from the same gene (Noguchi *et al.*, 1986). PKM1 and PKM2 are encoded by 12 exons and they differ by the alternate splicing of exons 9 and 10, where other exons upstream and downstream from these 2 unique exons code amino acid sequences common to the two isozymes (**Figure 4**) (Stall *et al.*, 1988). Human PKM1 and PKM2 are 96% identical at the amino acid level (Spoden *et al.*, 2008). This unique region in PKM1 corresponds to the $\alpha 1$ and $\alpha 2$ strands of the C-domain, which is the major region responsible for intersubunit contact. This unique stretch of 45 amino acids in PKM2 may be important for mediating the allosteric properties of this isoform since, unlike PKM1, PKM2 is allosterically regulated. This is confirmed by the sequence identity of PKM2 and PK-L types (which can also be allosterically regulated) are highest in this region compared to PKM1 (**Figure 5**). Allosteric regulation of enzyme activity is an extremely important

Figure 4: Schematic representation of the expression of the PK-M gene. The M1 exon is indicated by the hatched boxes and the M2 exon by the black boxes. M₁PK and M₂PK represent PKM1 and PKM2 respectively. PKM1 and PKM2 are encoded by 12 exons which differ by the alternate splicing of exons 9 and 10, whereas other exons upstream and downstream from these 2 unique exons code amino acid sequences common to the two isozymes. TATA represents TATA box and AATAA represents the polyadenylation signal (modified from Noguchi *et al.*, 1986)

Figure 5: Comparison of amino acid sequences specific to cat PKM1, PKM2 and PKL. Sequence homology of PKM2 and PK-L types (which can also be allosterically regulated) are highest in the region of 45 residues where PKM1 and PKM2 are not identical. These differences in amino acids seen in PKM2 and PK-L may be important for mediating the allosteric properties of these isoforms since, unlike PKM1, they can be allosterically regulated. Identical residues are boxed in and the positions of the elements of the secondary structure ($C\alpha 1$, $C\alpha 2$ and $C\beta 1$) are shown above the sequences (modified from Noguchi *et al.*, 1986).

biological mechanism to maintain an appropriate balance of intracellular substrate and product concentrations and to also coordinate with other cellular reactions and signalling pathways (Jurica *et al.*, 1998).

The molecular architecture of PK is a tetramer composed of a dimer of dimers where each identical subunit is comprised of four domains: **1)** the N-terminal N-domain (residues 1-43), composed of a short α -helical stretch, **2)** the A-domain (residues 44-116 and 219-389), responsible for the catalytic activity of the enzyme and is formed by two separate stretches of amino acids that fold together into the classic $(\beta/\alpha)_8$ barrel topology with two additional α -helices, **3)** the B-domain (residues 117-218), made up of a small, nine-stranded irregular β barrel motif that forms a cap over the active site and **4)** the C-terminal C domain (residues 390-531) which has an α/β open-sheet motif responsible for regulating the enzyme (**Figures 6 and 7**) (Jurica *et al.*, 1998; Wang *et al.*, 2001). Intermolecular subunit contacts for the first dimer occur between the A-domains of each monomer (Dombrackas *et al.*, 2005). The cleft between the A- and B-domains is where PEP binds. FBP binding is entirely located on the C-domain in a pocket formed from a loop between the C β 1 strand and the Ca3 helix (Wooll *et al.*, 2001). The residues involved in FBP binding are highly conserved and the binding of FBP allows for conformational changes in PK that lead to an increase in the affinity of the enzyme for PEP (Jurica *et al.*, 1998). The activation of PK involves a combination of domain and subunit rotations in which all of the domains of each subunit are capable of undergoing highly concerted motions (Mattevi *et al.*, 1996). Large domain and subunit rotations represent a common example of a protein architecture developed by nature for the purpose of intricate enzyme regulation (Mattevi *et al.*, 1996).

Figure 6: Molecular structure of the human PK-M protein. The 4 domains are: **1)** N: N-terminal N-domain, **2)** A1 and A2: A-domain, **3)** B-domain and **4)** C-terminal C domain (modified from Mazurek *et al.*, 2008).

The image shows a three-dimensional crystal structure of a single PKM2 subunit. The structure is composed of several domains: the N-terminal domain is colored orange, the A domain is red, the B domain is cyan, and the C domain is green. FBP is shown in magenta. The structure is rendered in a stick representation, showing the atoms and bonds. The background is a light gray gradient.

Figure 7: Three-dimensional crystal structure of a single PKM2 subunit. The N-terminal domain is shown in orange, the A domain in red, the B domain in cyan and the C domain in green. FBP is shown in magenta (modified from Zanella *et al.*, 2007). Figure created in PyMOL.

6.3 PKM2 and Tumourigenesis

Regulation of PK is not only critical in normal cells but also in rapidly proliferating tissues, because PK controls both consumption of metabolic carbon for biosynthesis and utilization of pyruvate for energy production (Jurica *et al.*, 1998). This places special demands on the metabolism of tumour cells since they have to survive in environments with varying oxygen and it has been shown that tumour cells increase the rate of lactate production, even in the presence of oxygen (aerobic glycolysis). PK has consistently been shown to be altered during tumourigenesis, since glycolytic intermediates are necessary as precursors for the synthesis of cell components. Observations show that, during tumour formation, there is a shift in the PK isoform composition, such that the particular tissue-specific PK isoform disappear and PKM2 is then expressed (Mazurek *et al.*, 2005). Studies reveal that it is the dimeric form, and not the tetrameric form of PKM2 found in the glycolytic complex, that is predominant in tumours and has therefore been designated as tumour-specific PKM2 (Dombrackas *et al.*, 2005). The switch between the tetrameric and dimeric forms of PK enables tumour cells to grow in environments with varying oxygen and nutrient supplies (Jurica *et al.*, 1998). Dissociation of the tetramer to the dimer in tumour cells is induced by direct interaction of PKM2 with different oncoproteins (Mazurek *et al.*, 2005). Characterization of the kinetic properties of the tetrameric and dimeric forms of PKM2 revealed that the tetrameric form has a high affinity for PEP (active form) whereas the dimeric form has low affinity (inactive form), due to the fact that the dimer is not associated with the glycolytic enzyme complex (**Figure 8 and Table 1**) (Jurica *et al.*, 1998). The glycolytic

complex is a multienzyme complex containing all the enzymes necessary to metabolize glucose to pyruvate (Brooks and Storey, 1993). Association of the glycolytic enzymes and other compounds within the glycolytic complex leads to a compartmentalization of glycolysis in the cytosol, resulting in very effective glycolytic enzyme activity (or substrate tunnelling; Mazurek *et al.*, 2005). According to Brooks and Storey (1993), three criteria must be met before considering the glycolytic enzyme complex to be functional: **1)** the enzymes must easily associate with each other at physiological ionic strength and protein concentrations, **2)** the enzymes must be kinetically active when bound in the complex and **3)** formation of the complex must demonstrate a kinetic advantage to the bound enzymes. When in the inactive dimeric form, phosphometabolites above PKM2 accumulate and are then available as precursors for synthetic processes such as nucleic acid, amino acid and phospholipids synthesis as well as NADPH production through the Pentose Phosphate Pathway (a requirement for tumourigenesis, since cell proliferation is only possible if high energetic phosphometabolites are available). The switch between the tetrameric and dimeric forms is not constant, where conversion between the two forms is regulated by the levels of cellular FBP (Mazurek *et al.*, 2005). When the cell senses that the FBP has reached a certain level, the inactive dimer will re-associate to form the active tetrameric form. From there, glucose can then be converted to lactate and energy until the level of FBP drops and the tetrameric form dissociates to the inactive dimeric form and the cycle starts again (Mazurek *et al.*, 2005).

Figure 8: Glycolysis and its debranching synthetic pathways. When PKM2 is in the inactive dimer form, phosphometabolites above PKM2 accumulate and are then available as precursors for other synthetic processes such as nucleic acid, amino acid, phospholipids and NADPH synthesis (Mazurek *et al.*, 2005).

Table 1: A summary of the kinetic properties and metabolic consequences of the tetrameric and dimeric forms of PKM2 (modified from Mazurek *et al.*, 2005).

<u>PKM2</u>	<u>Tetramer</u>	<u>Dimer</u>
PEP affinity	High	Low
Activity at physiological PEP concentrations	High	Inactive
Association within the glycolytic enzyme complex	Associated	Not associated
ADP and GDP levels	Low	High
ATP and GTP levels	High	Low
ATP:ADP ratio	High	Low
GTP:GDP ratio	High	Low
Phosphometabolite levels	Low	High
Glucose carbons	Used for energy production	Channelled into synthetic processes
(AtP + GTP):(UTP+CTP)	High	Low

More recent studies have shown that it is possible that PKM2 has functions other than its role in glycolysis, having the unique ability to interact with tyrosine phosphorylated proteins. A study by Christofk *et al.*, (2008) has demonstrated that PKM2 binds directly and selectively to phosphotyrosine-containing peptides which resulted in the release of FBP, leading to the inhibition of PKM2 enzymatic activity. Phosphotyrosine-containing protein binding is a transient event that results in a conformation change in the PKM2 structure that releases the tightly bound FBP molecule (Christofk *et al.*, 2008). Regulation of PKM2 in this manner diverts glucose metabolites for anabolic processes instead of energy production which is used for rapid cell proliferation in cancer cells (Christofk *et al.*, 2008).

6.4 Oxygen and Hypoxia

Oxygen is one of the most abundant chemical elements in the universe and in its gaseous form comprises 21% of the Earth's atmosphere (Park *et al.*, 2004). To maintain the stability of the Earth's biosphere, there must be precise balance between oxygen production and utilization (Bunn and Poyton, 1996). The main source of oxygen comes from a process called photosynthesis where carbon dioxide (CO_2) and water (H_2O) is converted to oxygen (O_2) and glucose, with the help of solar energy (Campbell and Reece, 2002). Oxygen is required in so many biological reactions and it is essential in humans for aerobic metabolism where it acts as the terminal electron acceptor in the mitochondrial electron transport chain, producing energy in the form of adenosine triphosphate (ATP) in a process called oxidative phosphorylation (Campbell and Reece,

2002). The body is able to obtain oxygen from the environment and distribute it to tissues and cells so that the required biochemical reactions can occur in order to survive (Semenza 1999). Therefore the regulation of O₂ homeostasis is essential in the development and physiology of living (aerobic) organisms (Park *et al.*, 2004).

In everyday life, humans experience many different environmental stressors that may result in a disease state. Such stressors may limit the availability of an essential component of normal cellular metabolism. Humans can experience low oxygen conditions during development *in utero* and at high altitudes; a condition known as hypoxia where the oxygen conditions are below normal atmospheric concentrations (or 21% O₂) (Safran and Kaelin, 2003). Hypoxia is also associated with disease states such as cardiac arrest and stroke where the blood supply to a particular tissue or organ is restricted (also known as ischemia) (Huang and Bunn, 2003).

To survive under oxygen-deficient conditions, most organisms have numerous regulatory mechanisms that aid in the adaptation to hypoxia, including alterations in gene and protein expression (Semenza, 1999). Genes and proteins expressed may be involved in the shift from aerobic (O₂-dependent) to anaerobic (O₂-independent) metabolism (known as the Pasteur Effect) and in ways to increase oxygen transport to particular tissues which typically involve increasing blood flow to tissues such as erythropoiesis (production of red blood cells), angiogenesis (growth of pre-existing blood vessels) and vasodilation (expansion of pre-existing blood vessels) (Kress *et al.*, 1998). The regulation of many proteins involved in hypoxia adaptation occurs at the level of the gene. A specific factor that controls most hypoxic gene expression is the Hypoxia Inducible

Figure 9: Hypoxia inducible genes that have been found to be directly regulated by HIF. HRE represents the hypoxic response element (5'-ACGTG-3') and ARNT is the aryl hydrocarbon nuclear receptor translocator, the binding partner protein for HIF- α (Chun *et al.*, 2002).

Factor (HIF) (Semenza, 2003), which has been identified to have more than 60 target genes (**Figure 9**), including the PK-M gene (Park *et al.*, 2004).

6.5 Hypoxia Inducible Factor (HIF)

HIF is the most important transcription factor mediating cellular responses to physiological decreases in oxygen tension. HIF is a ubiquitously expressed transcriptional regulator of hypoxia-inducible gene expression (Hagele *et al.*, 2006). HIF is an 826 amino acid protein encoded by 15 exons (Park *et al.*, 2004). The transcription factor is a heterodimer made up of two different subunits (an alpha (α) and a beta (β) subunit) that have basic helix-loop-helix (bHLH) motifs at the N-terminus (Ratcliffe, 2002). The β subunit is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Park *et al.*, 2004). The C-terminus of both proteins contain transactivation domains (NAD and CAD for N- and C-terminal transactivation domains respectively; TAD for simply transactivation domain) (**Figure 10**). The mRNA of both the α and the β subunits is constitutively expressed; whereas only the α -subunit protein responds to hypoxia. Both subunits, however, are required for HIF-1 dimerization, DNA-binding and transactivation (Huang and Bunn, 2003). The HLH domain allows for subunit dimerization and the basic domain mediates DNA-binding (Safran and Kaelin, 2003). Also, both subunits contain the Period/ARNT/Similar (PAS) domain that assists the dimerization of the two subunits (Huang and Bunn, 2003). PAS domains are known to be involved in the direct detection in changes of diatomic gases, light and redox potential in

Figure 10: Schematic representation of HIF-1 α and β subunits indicating the location of the bHLH and PAS domains, N-terminal (NAD) and C-terminal (CAD) transcriptional activation domains (in red) and the unique oxygen-dependent degradation domain (ODD; in yellow) present only in the α subunit (modified from Huang and Bunn, 2003).

more primitive organisms, but function as a dimerization domain in higher organisms (Gilles-Gonzalez and Gonzalez, 2003).

Under normoxic conditions, HIF- α undergoes oxygen-dependent posttranslational modifications and then is degraded by the ubiquitin-proteasome pathway (**Figure 11**) (Ratcliffe, 2002). The α subunit contains an oxygen-dependent degradation domain (ODD) which is responsible for the oxygen-dependent degradation of HIF- α (Park *et al.*, 2004). The initiation of this mechanism occurs when oxygen is present and there is oxygen-dependent hydroxylation of Pro-402 or Pro-564 within the ODD by enzymes known as prolyl hydroxylases (Safran and Kaelin, 2003; Huang and Bunn, 2003). These prolines are present within a particular conserved sequence of amino acids (LXXLAP; where L = leucine, X = any amino acid, A = alanine and P = proline) found twice within the ODD (Huang and Bunn, 2004). Once hydroxylated, the LXXLAP sequence is recognized and bound by the von Hippel Lindau (vHL) tumour suppressor protein, which is the substrate recognition subunit of a much larger complex of proteins that act as an E3 ubiquitin ligase (**Figure 12**). With the assistance of ubiquitin activating and conjugating proteins, HIF- α is rapidly polyubiquitinated and consequently targeted for proteolytic degradation by the 26S proteasome (**Figure 11**) (Semenza, 2003).

Hydroxylation of the HIF-1 α prolines is carried out by a family of proline hydroxylases known as the prolyl hydroxylase domain-containing (PHD) proteins (Boeckman *et al.*, 2003). These enzymes require oxygen, α -ketoglutarate, iron (Fe^{2+}) and ascorbic acid as cofactors to convert proline to hydroxyproline where ascorbic acid is used to keep the iron in its ferrous state and iron coordinates α -ketoglutarate at the active site of the enzyme (Boeckman *et al.*, 2003). PHDs transfer one oxygen atom to the

Figure 11: The mechanism of HIF- α regulation by oxygen. Under normoxic conditions, HIF- α is hydroxylated by prolyl hydroxylases at two prolines contained within two LXXLAP sequences present in the HIF- α ODD, bound by the vHL protein, ubiquitinated by vHL-containing E3 ubiquitin ligases and subsequently degraded by the proteasome. vHL does not recognize or bind to non-hydroxylated HIF- α . Under hypoxic conditions, HIF- α is not hydroxylated, ubiquitinated or degraded and is stabilized, travelling to the nucleus where it dimerizes with HIF- β , binds to HREs present upstream of hypoxia-inducible genes and transactivates hypoxia-inducible gene expression. Transactivation occurs through a bridging protein such as p300 or CBP (modified from Willmore, 2004).

Figure 12: The mechanism of the degradation of HIF- α under normoxic conditions. The vHL subunit of E3 ubiquitin ligase complex (which includes the proteins Cul-2, Elongins B and C, Rbx-1, vHL, E1 and E2 enzymes) recognizes hydroxylated HIF-1 α and binds to it. HIF- α is then subsequently polyubiquitinated for proteolytic degradation by the proteasome (modified from Willmore, 2003).

Figure 13: The proposed binding order of co-substrates and prime substrate by the HIF hydroxylases. Following binding of dioxygen, one atom of oxygen is incorporated into the hydroxylated HIF residue and the other oxidizes 2-oxoglutarate forming succinate and CO₂ (Masson and Ratcliffe, 2003).

proline substrate, producing hydroxyproline, while the second oxygen atom reacts with α -ketoglutarate, producing succinate and CO₂ (**Figure 13**).

In the absence of oxygen (i.e. under hypoxic conditions), HIF- α does not get hydroxylated and therefore cannot be degraded (stabilized). Instead, it accumulates in the cytoplasm and eventually travels into the nucleus where it dimerizes with the β subunit forming the active HIF- (**Figure 11**) (Willmore, 2004). HIF- α contains two nuclear localization signals (NLS) which mediate HIF- α 's translocation to the nucleus (**Figure 14**) (Chun *et al.*, 2002). HIF then binds to the hypoxic-responsive element (HRE) (**Figure 14**), a sequence of DNA (5'-(G/C/T)ACGTG-3') found in the promoters and enhancers of most hypoxia-inducible genes, activating their transcription (Willmore 2004). CREB-binding protein (CBP or p300) is a co-activator protein that bridges the gap between HIF and the basal transcription machinery and increases the rate of transcription of downstream genes involved in the response to hypoxia. HIF interaction with either CBP or p300 is primarily at the transcriptional activation domains (TAD) found on both HIF- α and HIF- β (**Figure 14**) (Safran and Kaelin, 2003). HIF- α is also hydroxylated on an asparagine in its C-terminal transactivation domain by Factor Inhibiting HIF (FIH; an asparagine/aspartate hydroxylase). This disrupts its interaction with CBP or p300 (Safran and Kaelin, 2003). FIHs are transcription factors that possess asparaginyl hydroxylase activity and are dependent on oxygen in a similar manner to PHDs (Safran and Kaelin, 2003).

Figure 14: Structure of the heterodimeric transcription factor HIF. The transcription factor is a heterodimer composed of an α and a β subunit. Each subunit has a DNA-binding (basic) domain, a helix-loop-helix (HLH) dimerization domain and a Period/ARNT/Single-minded (PAS) dimerization domain. While HIF- β contains only a C-terminal transactivation domain (CAD), HIF- α contains both an N-terminal (NAD) and a C-terminal (CAD) transactivation domain as well as the oxygen dependent degradation (ODD) domain (that contains the two LXXLAP amino acid sequences) and two PEST-like motifs (rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues). The HIF heterodimer recognizes and binds to an E-box hypoxic response element (HRE) (modified from Willmore, 2004).

6.6 Hypoxic Mimetics

It is critical to understand how proteins are regulated by oxygen, by both O₂-dependent and O₂-independent mechanisms. Hypoxia mimetics can help study O₂-independent mechanisms by mimicking hypoxia. They do so by inhibiting the function of the HIF prolyl hydroxylases, thus preventing HIF's hydroxylation and subsequent degradation. Such mimetics allow for the study of hypoxia-like conditions without the reduction in oxygen.

Oxygen is an essential substrate for PHDs, but their catalytic activity also depends upon iron. It is well known that various transition metals promote a response similar to hypoxia, stabilizing HIF- α under normoxic conditions and resulting in the expression of HIF-controlled genes (Vengellur and LaPres, 2004). Metals such as cobalt can mimic hypoxia by competing with iron for the iron-binding site on the PHDs, locking it in its deoxygenated state and therefore inactivating it since hydroxylation can no longer occur (Chun *et al.*, 2002). Reports suggest that cobalt also acts to decrease the available levels of ascorbate in the cell (Bergmeyer *et al.*, 1974). Ascorbate is necessary for the transition of iron between oxidation states (the recycling of iron in the active sites) of PHDs and decreasing the levels of ascorbate would cause the bound iron in PHDs to oxidize, inactivating these enzymes (Vengellur and LaPres, 2004; Bergmeyer *et al.*, 1974). Examples of this have been shown many times; for example, the expression of erythropoietin in Hep3B cells is induced by hypoxia as well as by cobalt (Ho and Bunn, 1996). Also, cobalt has been shown to mimic hypoxia in stimulating the expression of VEGF in a number of different cell types (Bunn and Poyton, 1996). Therefore using

Figure 15: The peptide aldehyde proteasome inhibitor MG132 (Cbz-Leu-Leu-Leu-al). The aldehyde group forms a transition state hemiacetyl complex with the threonine active site of the proteasome which interferes with its ability to degrade proteins.

cobalt as a hypoxia mimetic can help in the investigation of oxygen-dependent posttranslational modifications, even in the presence of oxygen.

Another hypoxic mimetic is the use of proteasome inhibitors. Inhibition of the proteasome results in the accumulation of HIF- α , even if it is hydroxylated and ubiquitinated. If such inhibitors cause changes in the level of the protein, then it is a good indication that the protein may be ubiquitinated and specifically degraded by the proteasome (Chun *et al.*, 2004). The proteasome inhibitor MG132 (Cbz-Leu-Leu-Leu-al) (**Figure 15**) is from the peptide aldehyde family that blocks the proteolytic activity of the 26S proteasome. The aldehyde group forms a transition state hemiacetyl complex with the threonine active site of the proteasome, interfering with its ability to degrade proteins (Bush *et al.*, 1996).

6.7 LXXLAP Database Search

A database search on the LXXLAP peptide sequence revealed over 5,000 different proteins which contained the conserved sequence. PKM2, but not PKM1, contained the sequence in many mammalian species (amino acid residues 398 to 403 in humans). Posttranslational modification of this sequence could be critical for targeting PKM2 to the proteasome under normoxic conditions. Hypoxia could suppress the hydroxylation modification of PKM2 by removing one of the key substrates of the reaction; oxygen. It is believed that oxygen-dependent regulation of this protein could be an essential adaptation to hypoxia and can have a role in initiating the expression of hypoxia-inducible genes.

6.8 Hypothesis

The regulation of most genes induced by hypoxia is mediated by HIF. HIF-independent pathways of hypoxic protein regulation may exist and other proteins may be degraded under normoxic conditions in a manner similar to that of HIF- α . Oxygen-dependent posttranslational modifications of the protein under normoxic conditions may a) lead to its ubiquitination and subsequent degradation by the proteasome or b) may change the protein's function (enzymatic activity, protein-protein interactions, nucleic acid-binding, transactivation, etc.) without leading to its degradation by the proteasome. Under hypoxic conditions, proteins involved in the adaptation to low oxygen may be stabilized and therefore more optimal under lowered oxygen conditions. These proteins would play a key role in the regulation and survival of the cell under hypoxic conditions.

The hypothesis is that PKM2 is regulated in a manner similar to that of HIF- α . This thesis examines PKM2 protein stability and function under hypoxic and hypoxic mimetic conditions. PKM2 is an excellent candidate protein for HIF-independent hypoxic regulation, not only because it plays a crucial role in the last step of glycolysis, but also because it contains the same LXXLAP sequence that is present as two copies in HIF- α . The PKM2 LXXLAP sequence is present in the unique exon for this isoform and thus may be the only PK isoform potentially regulated at the level of the protein by oxygen. PKM2 was investigated to determine if it is post-translationally regulated in an oxygen-dependent manner, similar to HIF- α , and if it plays a significant role in the cellular adaptation to hypoxia and hypoxic signalling, independent of HIF- α regulation.

7. MATERIALS AND METHODS

7.1 LXXLAP Database Search

The SWISS-PROT and TrEMBL databases (Boeckmann *et al.*, 2003) were searched for proteins containing the conserved LXXLAP sequence of amino acids. The results were sorted using a PERL-based algorithm and the proteins were organized and ranked according to: **a)** their identity, **b)** the number of species in which the protein appeared and **c)** their possible involvement in oxygen-sensing pathways.

7.2 Chemical and Biological Products

All biological and chemical products were obtained from Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO) respectively unless otherwise specified. Tris-HCl, glycine and Tween 20 were purchased from BioShop Canada Inc. (Burlington, ON). All gases were purchased from Praxair Inc. (Ottawa, ON). All tissue culture plates and flasks were obtained from Corning (Lowell, MA). Products for SDS-PAGE were purchased from National Diagnostics (Atlanta, GA). All primers were purchased from Integrated DNA Technologies (Coralville, IA). Rat muscle L6E9 cells were a gift from Cathy Merritt (Biology, Carleton University, Ottawa, ON) and maintained in 87% Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 3% penicillin/streptomycin/amphotercin B (P/S/A). Human Embryonic Kidney (HEK293A) cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in 87% DMEM, 10% horse serum (HS) and 3% P/S/A.

Cell cultures were kept at 37°C and 5% CO₂ in a humidified tissue culture incubator (Thermo Forma, Marietta, OH).

7.3 Cell Culture

7.3.1 Cell Maintenance

Cells were split approximately every 48 hours (doubling time was approximately 18 to 24 hours) in a 75 cm² tissue culture flask. Media was discarded and cells were rinsed with 5 mL of phosphate buffered saline (PBS (pH 7.4), 138 mM NaCl, 2.7 mM KCl). One mL of 1X trypsin was added on top of the adhered cells and left to incubate for approximately 1 minute. Five mL of fresh media was added to deactivate the trypsin and cells were pipetted up and down until all clumps were broken. Approximately 1 mL of the cells solution was kept in the flask and topped up with 19 mL of fresh media. The remainder of the cells was discarded or used for experimental purposes.

7.3.2 Cell Lysis

Frozen cell pellets were placed on ice and a few crystals of phenylmethylsulfonyl fluoride (PMSF) was added as a serine protease inhibitor. Ice-cold whole cell lysis buffer (20 mM HEPES (pH 7.9), 20 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 25% glycerol) was added to the pellet and centrifuged at 13,000 g in a microcentrifuge (Heraeus, Hanau, Germany) for 10 minutes at 4°C. The supernatant (“soluble protein”) was then collected into another Eppendorf tube and immediately stored at -80°C or on ice

until further use. The pellet (“insoluble protein”) was further extracted using a urea extraction buffer as described below.

7.3.3 Urea Extraction

Urea lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.1 mM PMSF and 8 M urea) was added to the pellet collected after whole cell lysis and incubated for 1 hour at room temperature. A second buffer (pH 8.0, 50 mM KH_2PO_4 (pH 10.7), 1 mM EDTA, 50 mM NaCl) was added to the lysed pellet and incubated for another 30 minutes at room temperature. The entire sample was then centrifuged at 12,000 g in a microcentrifuge (Heraeus, Hanau, Germany) for 15 minutes at 4°C. The supernatant was then collected and stored at -80 °C or on ice for until further use.

7.3.4 Protein Determination

Protein masses of cell lysates were determined according to the Bio-Rad Protein Assay kit protocol using the Bio-Rad Protein Assay Dye Reagent Concentrate (Hercules, CA) diluted (1:4) with Milli-Q water. After approximately 10 minutes incubation with cell lysates, the absorbance was read at 595 nm on a microplate reader (SpectroMax 340^{PC}, Molecular Devices, Sunnyvale, CA). Bovine Serum Albumin (BSA, 1 mg/mL) was used as standard, providing a relative measurement of protein concentration.

7.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Each 20 μ L of homogenized whole cell lysate was diluted with Milli-Q water, 5 % β -Mercaptoethanol and Laemmli sample buffer (Bio-Rad, Hercules, CA) to the desired protein concentration and heated at 95°C for 3 minutes, to denature the proteins, on a dry heat block (Fisher Scientific, Ottawa, ON). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10 % resolving gel. 4% stacking gel) in running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) was performed on the samples using Precision Plus Protein Kaleidoscope Standards (Bio-Rad, Hercules, CA) for molecular weight determination at 120 V.

7.5 Non-Denaturing Polyacrylamide Gel Electrophoresis (Native Gel)

Each 20 μ L sample was diluted with Milli-Q water to desired protein concentration in addition to 2 μ L of 50% glycerol. Non-denaturing polyacrylamide gel electrophoresis (10X TBE, 30% acrylamide/bisacrylamide (Bio-Rad, Hercules, CA), 80% glycerol, 10% ammonium persulfate (APS) and TEMED) was performed on the samples using Precision Plus Protein Kaleidoscope Standards (Bio-Rad, Hercules, CA) for molecular weight determination at 120 V.

7.6 Western Blotting

After electrophoresis, proteins on polyacrylamide gels were transferred overnight onto Immobilon-P PVDF transfer membrane (Millipore, Billerica, MA) at 180 mA and

4°C (Towbin et al., 1979) in transfer buffer (20 mM Tris-HCl (pH 8.0), 150 mM glycine and 20% methanol). Following transfer, the membrane(s) were then blocked for 1 hour with gentle shaking in a blocking solution of non-fat dry milk (5% w/v) dissolved in TBST buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween 20). The membrane(s) were then incubated with primary antibodies diluted in blocking solution for detection of the desired protein at room temperature for 1 hour. The unbound antibodies were removed by three 30 minute washes of TBST buffer at room temperature. If needed, secondary antibodies (anti-mouse IgG-HRP (Cat. No. P0447), Dako Canada Inc., Mississauga, ON and goat anti-rabbit IgG-HRP (Cat. No. sc-2004), SantaCruz Biotechnology Inc., Santa Cruz, CA) were incubated as described similarly to primary antibodies and washed again with TBST. The membrane(s) were subjected to chemiluminescent substrate (Millipore, Billerica, MA) for 1 minute, exposed to film (Kodak XB-1, Kodak, Toronto, ON), developed (Multigrade Developer, 1:10 dilution, Ilford, Concord, ON) and fixed (Multigrade Fixer, 1:5 dilution, Ilford, Concord ON).

7.7 Ponceau Red Staining

To stain PVDF membranes with Ponceau Red (used as a control for loading and normalization), PVDF membranes were placed in a solution of 0.1% (w/v) Ponceau Red and 5% acetic acid for 30 to 60 minutes and washed repeatedly with Milli-Q water until bands were seen.

7.8 Pyruvate Kinase Enzyme Assay

The enzyme activity of PK and PKM2 was measured in L6E9 cells using a continuous spectrophotometric kinetic assay modified and described by Bergmeyer *et al.* (1974). The reaction occurs in two steps, where the reaction velocity is determined in a lactate dehydrogenase (LDH)-coupled assay system by measuring the decrease in the absorbance of the oxidation of NADH at 340 nm. The production of pyruvate by PK (reaction 1) to the production of lactate by lactate dehydrogenase (reaction 2) is shown by the following reactions:

where PEP = phosphoenolpyruvate, ADP = adenosine 5'-diphosphate, ATP = adenosine 5'-triphosphate, NADH = nicotinamide adenine dinucleotide (reduced form) and NAD = nicotinamide adenine dinucleotide (oxidized form).

The enzymatic reaction was followed continuously with a spectrophotometric microplate reader (SpectroMax 340^{PC}, Molecular Devices, Sunnyvale, CA) over a period of 5 minutes at room temperature. The reaction mixture contained 1.5 mM NADH, 0.7 mM ADP, 10 Units of LDH and 0.25 mM PEP diluted in assay buffer (50 mM Imidazole (pH 7.0), 50 mM KCl and 5mM MgCl₂). The reaction was initiated with the addition of PEP. One unit of PK activity was defined as the conversion of 1.0 μmole of PEP to pyruvate per minute. To distinguish between PKM2 and PKM1 activities, cell lysates

were pre-incubated with 2 mM fructose-1,6-bisphosphate (FBP) an allosteric activator of PKM2 (Dombrauckas *et al.*, 2005). The difference between the activity of the unactivated and activated samples is the activity of the PKM2 isoform only.

7.9 Hypoxia and Hypoxic Mimetic Treatments

To assess the effect of hypoxia and hypoxic mimetics on the expression of endogenous and recombinant PKM2, L6E9 and HEK293A cells were treated with 6 to 48 hours of normoxia (21% O₂) versus hypoxia (1% O₂) and hypoxic mimetics (10 µM proteasome inhibitor (PI, Cbz-Leu-Leu-Leu-al) for 6 hours and 100 µM cobalt chloride (CoCl₂) for 24 hours as separate treatments maintained under normoxic conditions). Cells treated under hypoxic conditions (1% O₂, 5% CO₂, 37°C) were placed in a triple-gas incubator (Thermo Forma, Marietta, OH) for the duration of the treatment.

7.10 Real-Time Reverse-Transcriptase Polymerase Chain Reaction

(Real-Time RT-PCR)

Real-time RT-PCR was used to quantify the relative changes in PKM2 gene expression between normoxia and hypoxia or hypoxic mimetic treatments. Total RNA was isolated using QIAGEN's RNeasy Mini Kit (Valencia, CA) according to the manufacturer's instructions. RNA integrity was verified by OD₂₆₀/OD₂₈₀ nm absorption ratio > 1.7. Gene expression was quantified using Bio-Rad's iScriptTM One-Step RT-PCR Kit with SYBR[®] Green. After RNA isolation and quantification, a 50 µL reaction (2X SYBR[®] Green RT-PCR Reaction Mix, 10 µM primers, nuclease-free water, 100 ng RNA

sample and iScript Reverse Transcriptase for One-Step RT-PCR) was prepared for each sample. Gene-specific primers were used for the amplification of the target gene *PKM2* (213 bp amplicon):

Forward primer: 5'- CCCATTACCAGCGACCCCACAGAAGCCACC -3'

Reverse primer: 5'- CAGCACAGGGAAGATGCCACGGTACAG -3'

Also, primers for the amplification of the control housekeeping gene *β -actin* (Sigma Genosys, Oakville, ON) (279 bp amplicon):

Forward primer: 5'- GCGGGAAATCGTGCGTGCATT -3'

Reverse primer: 5'- GATGGAGTTGAAGGTAGTTTCGTG-3'

The reaction protocol was set up on the real-time RT-PCR machine (Rotor-GeneTM 6200-HRM, Corbett Research, Montreal Biotech Inc., Montreal, QC) as follows:

- 1) cDNA synthesis: 50°C for 10 minutes
- 2) iScript Reverse transcriptase inactivation: 95°C for 5 minutes
- 3) PCR cycling and detection (40 cycles): 95°C for 10 seconds
60°C for 30 seconds (data collection step)
72°C for 20 seconds (extension step)
- 4) Melt curve analysis: 55-95°C increasing by 0.5°C each cycle

The critical threshold (CT; the point at which the fluorescence increased above the background fluorescence of the sample) was obtained manually for each sample. The

expression of PKM2 (target gene) was quantified according to the Pfaffl method (Pfaffl, 2001). In this mathematical model, the relative expression ratio (R) of a gene is calculated based on amplification efficiencies of the housekeeping gene (E) and CT deviation of a treated sample versus an untreated (control) sample and expressed in comparison to a reference gene. Normoxic and hypoxic samples were designated as untreated and treated respectively and E was set to 2 for 100% efficiency (assuming that there were no changes between the treatments).

$$\text{Relative Expression Ratio (R)} = \frac{(\text{E target})^{\Delta\text{CT target (normoxia-treated)}}}{(\text{E target})^{\Delta\text{CT reference (normoxia-treated)}}$$

The RT-PCR products were confirmed by running a 2% DNA agarose gel electrophoresis at 140 V for approximately 45 minutes. The gel was stained with ethidium bromide and viewed under UV light.

7.11 RNA Interference (RNAi)

The knockdown of *PKM2* was carried out using synthesized siRNA molecules (SantaCruz Biotechnology, Santa Cruz, CA, Cat. No. sc-62820), which includes a pool of 3 target-specific, 20 to 25 nucleotide siRNAs designed to knockdown specifically the *PKM2* gene in human cells. This siRNA protocol was modified from Santa Cruz's siRNA and Invitrogen's LipofectamineTM 2000 Reagent protocols. Modifications were: **1)** Replacement of Santa Cruz's Transfection medium with Opti-MEM, **2)** Replacement of Santa Cruz's Transfection Reagent with Invitrogen's LipofectamineTM 2000 Reagent, **3)** siRNA was diluted in Opti-MEM instead of Santa Cruz's Dilution Buffer, **4)** siRNA and

transfection reagent were incubated for 20 minutes and **5)** Cells were incubated for 24 hours after transfection.

7.11.1 PKM2-siRNA Concentration and Cell Line Optimization

PKM2-specific siRNA knockdown was studied from whole cell homogenates of HEK293A and L6E9 cells transfected with 0, 0.25, 0.5 and 1.0 µg of siRNA for 24 hours by Western blotting with anti-PKM2 (1:2,500 dilution, Cell Signaling Technology, Danvers, MA, Cat. No. 3198S).

7.11.2 PKM2-siRNA Time Course in HEK293A

PKM2-specific siRNA knockdown was studied from whole cell homogenates of HEK293A cells transfected with 0.5 µg of siRNA for 0, 24, 48 and 72 hours. Cells were harvested after treatment for the specified time periods. Protein expression was detected by Western blotting with anti-PKM2 (1:2,500 dilution).

7.11.3 PKM2-siRNA Knockdown and Hypoxia Treatments in HEK293A

HEK293A protein expression was examined after cells were transfected with 0.5 µg PKM2-siRNA and treated with hypoxia for 0, 6, 16 and 24 hours by Western blotting with anti-PKM2 (1:2,500 dilution).

7.11.4 MTT Assay

Cell viability of HEK293A cells, that were under both knockdown of PKM2 and hypoxic exposure, was measured using the MTT assay which utilizes the dye 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. This dye is reduced in living cells to a purple, water insoluble formazan salt that can be measured using a spectrophotometer. The reduction occurs when mitochondrial dehydrogenases, only active in living cells, reduce the MTT to the formazan product which is solubilized by lysing the cells in 100% dimethyl sulfoxide (DMSO) (Mosmann, 1983). The colour was quantified by measuring the absorption using a spectrophotometric microplate reader (SpectroMax 340^{PC}, Molecular Devices, Sunnyvale, CA) at 570 nm with a background subtraction at 630 nm.

HEK293A cells were plated in 6-well plates at a density of 1.5×10^5 cells/mL and treated with 0.5 μ g PKM2-siRNA. After 24 hours, all cells in each well were transferred to individual 6 cm dishes and exposed to 0, 6, 16 and 24 hours of hypoxia. A stock solution of MTT was prepared at a concentration of 5 mg/mL in 1 X PBS and the volume of MTT added to each well was $1/10^{\text{th}}$ of the total volume of media in each well. After incubation at 21% O₂, 5% CO₂ and 37°C for 2 hours, the media with MTT was discarded and replaced with the same amount of the total volume of media in each well with DMSO and incubated at room temperature for another 10 minutes. One-hundred microlitres from each treatment was placed in a microplate and the absorbance was measured.

7.12 PKM2 Cloning

Recombinant PKM2 was cloned into a mammalian expression vector for *in vivo* transient expression of PKM2 in mammalian cell lines. The gene for human PKM2 (Open BioSystems Inc., Huntsville, AL) was initially cloned into a general shuttle vector (pCMV-SPORT6) and the gene was amplified from this vector using RT-PCR (Bio-Rad,

Hercules, CA) with Pfu (*Pseudomonas fluorescens*) proofreading DNA polymerase and gene-specific primers designed to contain *NdeI* and *EcoRI* restriction sites underlined respectively:

Forward Primer: 5'-CAGCAGCCCCATATGTCGAAGCCCCATAGTGAAGCC-3'

Reverse Primer: 5'-CTGAATTCTCACGGCACAGGAACAACACGC-3'

A N-terminal FLAG sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was introduced into the mammalian expression vector pCR3.1 (Invitrogen, Carlsbad, CA) for easy detection and purification with anti-FLAG antibodies and beads. The PCR products and vector were digested with *NdeI* and *EcoRI* and then ligated using T4 DNA ligase. After transformation into DH5 α competent bacterial cells and isolation of plasmids, constructs were screened for FLAG and proper insert orientation using *Hpy99I* and *PmlI* restriction enzymes respectively. Size verification was confirmed using High Ranger 1 kb DNA Ladder (Norgen Biotek Corporation, Thorold, ON) by agarose gel electrophoresis. Digests were analyzed using New England BioLab's NEBcutter v2.0 (<http://tools.neb.com/NEBcutter>), a software tool that takes submitted DNA sequences and produces detailed restriction maps. Plasmids from selected positive clones were purified using the GenElute kit (Sigma, St. Louis, MO). DNA was then sequenced by DNA LandMarks Inc. (Saint-Jean-sur-Richelieu, Quebec), confirming the identity of the cloned gene as PKM2 and the proper orientation in FLAG-pCR3.1.

7.13 Site-Directed Mutagenesis

Site-directed mutagenesis of the proline in the LXXLAP sequence would prevent oxygen-dependent hydroxylation of the protein and alter PKM2 function and/or stability under normoxic conditions. To compare the differences in protein turnover under normoxic and hypoxic conditions, wildtype and mutated PKM2 were transiently transfected into HEK293A cells. Site-directed mutagenesis of the proline 403 (CCC) to alanine (GCC) was performed using the rolling circle technique employed by Stratagene's QuikChange Site-Directed Mutagenesis Kit (La Jolla, CA) with specific mutagenesis primers:

Forward Primer: 5'-GCCGCCTGGCGGCCATTACCAGC-3'

Reverse Primer: 5'-GCTGGTAATGGCCGCCAGGCGGC-3'

After transformation in XL1-Blue Supercompetent cells, the resulting clones were purified with Promega's Wizard® Plus Minipreps DNA Purification System (Madison, WI), then sequenced (McGill University and Genome Quebec Innovation Centre, Montreal, QC) to confirm that the mutation was present.

7.14 Transient Transfections

To overexpress recombinant PKM2 in mammalian cell lines, transient transfections were performed as described by Invitrogen's Lipofectamine™ 2000 Reagent protocol (Carlsbad, CA) with a few modifications. Cells were washed with 1 X PBS, trypsinized, counted and plated at a density of 2.0×10^5 cells/mL in transfection

media (90% DMEM, 10% serum) 24 hours prior to transfection. Depending on the plate size used, the amount of DNA and Lipofectamine Reagent varied (according to the manufacturer's instructions for LipofectamineTM 2000 Reagent). On the day of transfection, the DNA was diluted with Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) and, in a separate tube, LipofectamineTM 2000 Reagent was diluted with the same amount of Opti-MEM medium. After 5 minutes of incubation at room temperature, the DNA and LipofectamineTM 2000 Reagent mixtures were combined and incubated for another 20 minutes at room temperature. The DNA/LipofectamineTM 2000 Reagent mixture was then directly added to the cells and incubated for another 6 hours at 37°C and 5% CO₂. After the 6 hours, all the media was discarded and replaced with regular growth media. At this time, cells were maintained under normoxic or hypoxic conditions.

7.15 DNA Optimization of Transient Transfections

To determine the optimal DNA concentration for overexpression of mature PKM2 (wildtype and site-directed mutants) and to prevent too much overexpression of the protein so that the cell system will not be overwhelmed and that differences could be observed between treatments, HEK293A cells were transiently transfected for 24 hours using LipofectamineTM 2000 Reagent according to the manufacturer's protocol. The concentrations of DNA transfected were: 0, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.5, 0.1, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 10.0 µg per 3.5 cm plate. SDS-PAGE and Western blotting with anti-FLAG (1:4,000 dilution, Sigma, St. Louis, MO).

7.16 Isoelectric Focusing (IEF)

To further study the assembly of PKM2 under hypoxic conditions, isoelectric focusing (IEF) and Western blotting were performed on whole cell homogenates of HEK293A cells after hypoxia exposure. The combination of IEF using Immobilized pH Gradient (IPG) strips (Bio-Rad, Hercules, CA) with SDS-PAGE allows for the separation of mixtures with different isoelectric points (pI). Proteins that are associated inside the glycolytic enzyme complex focus at a common isoelectric point that is different from the isoelectric points of the purified individual proteins (Mazurek *et al.*, 2005). This technique performed by Mazurek *et al.* (2005) was carried out with a linear gradient of glycerol (50 to 0% (v/v) and ampholines (pI 3.5 – 10.5). IEF will help in determining the assembly of PKM2 when exposed to hypoxic conditions. The IEF was performed under native conditions to prevent proteins from dissociating into their individual proteins. The IEF protocol used here is adapted from Bio-Rad's ReadyPrep™ 2-D Starter Kit Instruction Manual. Modifications were made to the protocol to keep the protein in its native form were: **1)** During preparation of the samples, cells were lysed with whole cell lysis buffer (described previously) instead of the urea lysis buffer; **2)** Focusing was performed at extremely low voltage (250 V for first step for two hours, 1,000 V for second step for four hours). The second dimension was run as a regular SDS-PAGE and Western blot procedure as described earlier using anti-PKM2 (1:2, 500 dilution, Cell Signalling Technology, Danvers, MA).

7.17 Statistical Data Analysis

Protein expression was quantified by densitometry (AlphaEaseFC v.3.1.2., Alpha Innotech Co., San Leandro, CA.). All results presented are expressed as means $\pm$ S.E.M. of at least three independent experiments except for optimization experiments and unless otherwise indicated. Data analysis was performed using the pairwise T-tests for two samples assuming unequal variance with a value of $p < 0.05$ (*), which was considered statistically significant in comparison to controls.

8. RESULTS

8.1 Database Search for LXXLAP Subsequence

The SWISS-PROT and TrEMBL databases (Boechmann *et al.*, 2003) were searched for proteins containing the conserved LXXLAP sequence of amino acids and the search resulted in over 5,000 different proteins containing the target sequence (from different organisms). The results were sorted using a PERL-based algorithm (Wall, 1987-2001) and the proteins were ranked according to **a)** their identity, **b)** the number of species in which the protein appeared and **c)** their possible involvement in oxygen-sensing pathways. An alignment of amino acid sequences of all human PK isoforms displayed that only PKM2 contained the specific oxygen-dependent HIF- α -like sequence (**Figure 16**). Also, this sequence is conserved in many mammalian species. Unlike HIF- α , the LXXLAP sequence only occurred once in PKM2.

8.2 Endogenous PK Activity in Different Cell Lines

To determine the best cell line to use as a model in studying PK, mammalian cells lines available in the Willmore Lab (rat skeletal-muscle myoblasts (L6E9), human embryonic kidney (HEK293A and HEK293T), human hepatoma (Hep3B), human cervical carcinoma (HeLa), human leukemia (K562), adherent clone of the rat pheochromocytoma (PC-12 AC), and monkey kidney fibroblast (COS7)) were scanned for total endogenous PK-specific activity. Cells were then harvested, lysed for whole cell homogenates and the total protein concentration determined. Lysates were then used to measure PK enzymatic activity. The activity analysis (**Figure 17**) shows that L6E9 cells

Figure 16: Multiple alignments of the different isoforms of human PK amino acid sequences as well as PKM2 amino acid sequences from multiple organisms showing conservation of the LXXLAP sequence.

		<u>LXXLAP</u>	
Human PK-R	448	RQLFEELRRRAAPLSRD	464
Human PK-L	427	RQLFEELRRRAAPLSRD	444
Human PK-M2	391	LQLFEELRRRLAPITSD	407
Human PK-M1	391	RKLFEELVRASSHSTD	407
Human PK-M2	391	LQLFEELRRRLAPITSD	407
Horse PK-M2	391	LQLFEELRRRLAPITSD	407
Mouse PK-M2	391	LQLFEELRRRLAPITSD	407
Rat PK-M2	347	LQLFEELRRRLAPITSD	363
Human HIF-1 alpha (a)	391	KKEPDALTLLAPAAGD	406
Human HIF-1 alpha (b)	553	QDTDLDLEMLAPYIPM	578

Figure 17: Enzyme activity of endogenous PK in different mammalian cell lines. Specific activity of PK in cells under normoxic conditions (21% O₂, 5% CO₂, and 37°C). Data represents one independent experiment.

had the highest PK specific activity followed by HEK293A cells (approximately 50% less).

8.3 Endogenous PKM2 Protein Levels in Different Cell Lines

To determine the cell line that expresses the most endogenous PKM2 protein levels, the mammalian cells lines utilized for PK specific activity (Section 8.2) were scanned for endogenous PKM2 protein levels. Cells were then harvested, lysed for whole cell homogenates and the total protein concentration determined. Lysates were then used for SDS-PAGE and Western blotting with PKM2-specific antibodies from two different sources (1:500 dilution (Staal *et al.*, 1988) and 1:2,500 dilution (Cell Signaling Technology, Danvers, MA)) that recognizes human, rat and mouse proteins. The immunoblot analysis (**Figure 18**) shows consistently that L6E9 cells have the highest levels of PKM2, expressing the PKM2 tetramer at a molecular weight of approximately 60 kDa, independent of which antibody was used.

8.4 Hypoxia and Hypoxic Mimetic Treatments on Endogenous PKM2 Protein Levels

PKM2 contains a similar LXXLAP sequence as found in HIF- α which is stabilized when exposed to hypoxic conditions (Safan and Kaelin, 2003). It was also shown previously that some metals can mimic hypoxia and stabilize HIF- α (Ho and Bunn, 1996). L6E9 cells were exposed to hypoxia and hypoxic mimetics such as cobalt chloride (CoCl₂) and proteasome inhibitor (PI) to determine whether these conditions stabilize PKM2 in a manner similar to HIF- α . The immunoblot analysis showed a band at

Figure 18: Western blot analysis of endogenous PKM2 protein levels in different mammalian cell lines. PKM2 protein expression from cells under normoxic conditions (21% O₂, 5% CO₂ and 37°C). Western blots were probed with anti-PKM2 antibodies: **A)** 1:500 dilution, Staal *et al.* (1988) and **C)** 1:2,500 dilution, Cell Signaling Technology. **B)** and **D)** Band densities were normalized against all proteins stained with Ponceau Red. **E)** Densitometry analysis of PKM2 protein expression. Data represent one independent experiment.

Figure 19: Western blot analysis of endogenous PKM2 protein levels in L6E9 cells. PKM2 protein expression of L6E9 cells after exposure to normoxia (21% O₂, 5% CO₂ and 37°C), hypoxia (1% O₂, 5% CO₂ and 37°C) and hypoxic mimetic (100 µM CoCl₂ and 10 µM PI at 21% O₂, 5% CO₂ and 37°C) treatments. **A)** and **B)** Representative Western blot of whole cell homogenates of PKM2 expressed in L6E9 cells using anti-PKM2 (1:500 dilution, Staal *et al.* (1988)) and normalized to β-actin (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) expression. **C)** Densitometry analysis of changes in PKM2 protein expression after treatments compared to normoxia. Data represent means ± S.E.M. from five independent experiments with * = p<0.05, compared to normoxia.

A

N(48h) PI(6h) CC(24h) H(6h) H(12h) H(24h) H(36h) H(48h)

B

C

approximately 60 kDa (**Figure 19A**). Visual inspection, along with densitometric analysis (**Figure 19C**), showed a significant increase of PKM2 expression of approximately 30 and 40% when exposed to 24 and 36 hours of hypoxia respectively, compared to normoxia. There was also an increase in PKM2 protein expression of approximately 20% after treatment with 100 μ M CoCl₂, while treatment with PI caused a 10% decrease in PKM2 protein expression.

8.5 Hypoxia and Hypoxic Mimetic Treatments on Endogenous PKM2 Gene Levels

A real-time RT-PCR approach was used to determine if changes in PKM2 protein levels exposed to hypoxia and hypoxic mimetics are due to changes in its transcript levels in L6E9 cells. Total RNA from treated L6E9 cells were used as a template to amplify a portion of the rat PKM2 gene by real-time RT-PCR and PKM2 gene-specific primers to determine a profile of PKM2 gene expression. Gene products were resolved via gel electrophoresis, revealing a single amplicon of correct molecular weight (*PKM2*, 213 bp; *β -actin*, 279 bp) (**Figure 20A and 20B**). The results of the relative gene expression after each treatment showed no significant changes (**Figure 20C**). All results were normalized to the internal reference gene of *β -actin* for real-time RT-PCR. These levels were analyzed by the Pfaffl method (Pfaffl, 2001) where the efficiency was set to 100% assuming *β -actin* levels were the same in all treatments.

Real-time RT-PCR analysis was also performed for PKM1 and PKLR gene expression using gene-specific primers. Analysis of gene products resolved via gel

Figure 20: Real-time RT-PCR analysis of endogenous *PKM2* gene expression in L6E9 cells. Total RNA from treated L6E9 cells treated with normoxia (21% O₂, 5% CO₂ and 37°C), hypoxia (1% O₂, 5% CO₂ and 37°C) and hypoxic mimetics (100 µM CoCl₂ and 10 µM PI at 21% O₂, 5% CO₂ and 37°C) were amplified for real-time RT-PCR analysis. **A)** and **B)** Representative *PKM2* and *β-actin* PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and viewed under UV light. Products of the correct size were amplified (*PKM2*, 213 bp; *β-actin*, 279 bp). **C)** Changes in *PKM2* gene expression were quantified by real-time RT-PCR where the level of *PKM2* was normalized by the amount of *β-actin* and represented as a relative gene expression ratio compared to normoxic cells. Data represent means ± S.E.M. from three independent experiments with * = p<0.05 compared to normoxia.

A

N(48h) PI(6h) CC(24h) H(6h) H(12h) H(24h) H(36h) H(48h)

B

C

electrophoresis showed no bands for these two isoforms in L6E9 cells (results not shown).

8.6 Hypoxia and Hypoxic Mimetic Treatments on Endogenous PKM2 Activity Levels

To investigate if the changes in endogenous PKM2 protein levels in L6E9 cells caused changes in PKM2 activity after exposure to hypoxia and hypoxia mimetics, the PKM2 enzymatic activity was assessed in whole cell homogenates of L6E9 cells after treatment. The activity analysis (**Figure 21**) showed a significant increase of PKM2 activity after short- and long-term hypoxia (~1.2- and 1.5-fold respectively) and CoCl₂ (~1.7-fold) exposure compared to normoxia. These findings are consistent with the results obtained for changes in PKM2 protein levels in L6E9 cells where long-term hypoxia (24 to 36 hours) and CoCl₂ treatments also caused a large significant increase.

8.7 FLAG-pCR3.1-PKM2 and Mutant Constructs

The correct ligation of the PKM2 gene into FLAG-pCR3.1 vector was verified by selective restriction digestion to ensure the proper cloned gene as PKM2 (*EcoRI* and *NdeI*), the in-frame fusion of FLAG (*Hpy99I*) and the orientation of the insert into the vector (*PmlI*) (**Figure 22**). Sequencing of the construct provided final confirmation of correct insertion (results not shown).

The clones resulting from the mutation of proline (CCC) to alanine (GCC) in the LXXLAP sequence in FLAG-pCR3.1-PKM2 was also confirmed by sequencing (results not shown).

Figure 21: Specific activity of endogenous PKM2 in L6E9 cells. Whole cell homogenates from L6E9 cells treated with normoxia (21% O₂, 5% CO₂ and 37°C), hypoxia (1% O₂, 5% CO₂ and 37°C) and hypoxic mimetics (100 µM CoCl₂ and 10 µM PI at 21% O₂, 5% CO₂ and 37°C) were assayed using the PKM2 enzyme assay. Data represent means ± S.E.M. from three independent experiments with * = p<0.05, compared to normoxia.

8.8 Optimization of DNA Concentration for Transient Transfection into HEK293A Cells

It is important to control the amount of DNA to be transiently transfected into cells. Overexpression of a particular protein can overwhelm the cell causing a decrease in cell viability (Miura and Yuan, 2000). Depending upon the function of the protein, different pathways may be overactivated leading to cell death. Various concentrations of DNA ranging from 0.0001 to 8 μg (in 3.5 cm plates at a density of 2.0×10^5 cells/mL) were transiently transfected into HEK293A cells for 24 hours and harvested. Whole cell homogenates were used for SDS-PAGE and Western blotting with anti-FLAG-HRP (1:4,000 dilution, Sigma, St. Louis, MO). HEK293A cells, containing 0 to 0.05 μg transfected DNA showed no expression whereas concentrations between 0.1 to 8 μg DNA produced significant expression (**Figure 23A and 23B**). Thus, 0.1 μg of plasmid was chosen as the optimum DNA concentration for transient transfections so that the cell system would not be overwhelmed with too much overexpressed protein and that differences in protein expression could be observed between treatments.

8.9 Hypoxia Treatments on Recombinant PKM2 Protein Levels

Wildtype and mutated PKM2 were transiently transfected into HEK293A cells to compare the differences in protein turnover after normoxic and hypoxic exposure. After treatments, cells were harvested and lysed using normal lysis buffer for the “soluble (S)” protein and urea extraction was performed on the pellet collected after normal lysis for the “insoluble

Figure 22: Verification of the ligation of N-terminal FLAG tagged PKM2 into the EcoRI/NdeI site of the pCR3.1 vector. Agarose gel (1%) stained with ethidium bromide and viewed under UV light. Selective restriction digests to ensure the proper cloned gene as: **Lane 1:** 1 Kb ladder (Norgen Biotek Corporation, Thorold, ON); **2:** digested PKM2 insert (*NdeI* and *EcoRI*, 1,596 bp); **3:** 1 Kb ladder (Norgen Biotek Corporation, Thorold, ON); **4:** undigested purified positive clone (6,653 bp); **5:** *EcoRI* (6,653 bp); **6:** in-frame fusion of FLAG (*Hpy99I*, 2,084 and 1,835 bp); **7:** *NdeI* (6,653 bp); **8:** orientation of the insert into pCR3.1 (*PmlI*, 4,835 and 1,818 bp).

Figure 23: Western blot of recombinant PKM2 transiently transfected into HEK293A cells. Recombinant PKM2 from whole cell homogenates was detected by anti-FLAG HRP (1:4,000 dilution, Sigma-Aldrich, St. Louis, MO). The concentrations were: **A) Lane 1:** 0 μ g; **2:** 0.5 μ g; **3:** 1.0 μ g; **4:** 2.0 μ g; **5:** 3.0 μ g; **6:** 4.0 μ g; **7:** 6.0 μ g; **8:** 8.0 μ g; **9:** 10.0 μ g and **B) Lane 1:** 0 μ g; **2:** 0.0001 μ g; **3:** 0.0005 μ g; **4:** 0.001 μ g; **5:** 0.005 μ g; **6:** 0.01 μ g; **7:** 0.05 μ g; **8:** 0.1 μ g; **9:** 0.5 μ g. Data represents one independent experiment.

A

B

(I)” protein. Protein expression was then analyzed using SDS-PAGE and Western blotting, probing with anti-FLAG-HRP. PKM2 expression was normalized to Ponceau Red staining on the same Western blot. Densitometric analysis of the recombinant PKM2 protein expression showed no significant difference for wildtype PKM2 after exposure to hypoxia, in both soluble and insoluble homogenates, compared to normoxia (**Figure 24**). Mutation of the proline in the LXXLAP sequence also did not produce significant differences in PKM2 protein expression.

To study the possibility of differences in the size or assembly of endogenous PKM2 and wildtype and mutant FLAG-tagged PKM2 after normoxia and hypoxia exposures in HEK239A cells, the native form of the protein was examined using non-denaturing polyacrylamide gels and Western blotting with anti-FLAG HRP and anti-PKM2 (1:500 dilution, Staal *et al.* (1988)). PKM2 protein expression with anti-FLAG of wildtype and mutant FLAG tagged PKM2 protein shows that all of the recombinant protein is at a large size and that it may be assembled as a tetramer with other proteins (> 250 kDa) (**Figure 25A**). This indicates the possibility that the recombinant protein may associate with the glycolytic complex since the tetramer is in its active form. No differences in PKM2 protein expression occurred between normoxic and hypoxic exposures. PKM2 protein expression with anti-PKM2 for all endogenous protein expressed by the non-transfected and transfected cells lines show more forms that are present (monomer, dimer and tetramer) (**Figure 25B**). Again, comparison of protein expression after normoxia and hypoxia exposure did not show any changes and the non-transfected cell line produced more endogenous protein than the overexpressed transfected cells.

Figure 24: Western blots of wildtype and mutant FLAG-tagged PKM2. HEK293A cells were transiently transfected with mammalian expression vectors containing wildtype or mutant PKM2 constructs and treated with normoxic (21% O₂, 5% CO₂ and 37°C) and hypoxic (1% O₂, 5% CO₂ and 37°C) conditions. **A) and C)** Representative Western blots of soluble and insoluble homogenates of HEK293A cells treated with normoxic and hypoxic conditions using anti-FLAG-HRP (1:4,000 dilution, Sigma, St. Louis, MO). **B and D)** Sample loading was normalized to Ponceau Red. **E)** Densitometry analysis of wildtype and mutant FLAG-tagged PKM2 protein expression under normoxic and hypoxic exposures. **Lane 1:** normoxia 48 hours “soluble” protein; **2:** hypoxia 24 hours “soluble” protein; **3:** hypoxia 48 hours “soluble” protein; **4:** normoxia 48 hours “insoluble” protein; **5:** hypoxia 24 hours “insoluble” protein; **6:** hypoxia 48 hours “insoluble” protein. Data represent means $\pm$ S.E.M. from three independent experiments with * = $p < 0.05$, compared to their respective controls (normoxia).

Figure 25: Western blot of native endogenous, wildtype and mutant FLAG-tagged PKM2. Untransfected HEK293A cells and cells transiently transfected with mammalian expression vectors containing wildtype or mutant PKM2 constructs were treated with normoxic (21% O₂, 5% CO₂ and 37°C) and hypoxic (1% O₂, 5% CO₂ and 37°C) conditions. Whole cell homogenates were run on non-denaturing polyacrylamide gels and Western blotting with: **A)** anti-FLAG-HRP (1:4,000 dilution, Sigma, St. Louis, MO) and **B)** anti-PKM2 (1:500 dilution, Staal *et al.* (1988)).

A

wildtype	-	-	-	+	+	+	-	-	-
mutant	-	-	-	-	-	-	+	+	+
normoxia (h)	48	-	-	48	-	-	48	-	-
hypoxia (h)	-	24	48	-	24	48	-	24	48

B

wildtype	-	-	-	+	+	+	-	-	-
mutant	-	-	-	-	-	-	+	+	+
normoxia (h)	48	-	-	48	-	-	48	-	-
hypoxia (h)	-	24	48	-	24	48	-	24	48

8.10 Isoelectric Focusing of Endogenous PKM2 After Hypoxic Exposure

To further study the assembly of PKM2 after normoxic and hypoxic exposure, isoelectric focusing (IEF) and Western blotting with anti-PKM2 (1:2,500 dilution, Cell Signaling Technology) on whole cell homogenates from HEK293A cells after treatments was investigated. The combination of IEF using Immobilized pH Gradient (IPG) strips (Bio-Rad, Hercules, CA) with SDS-PAGE allows for the separation of mixtures of proteins with different isoelectric points (pI). Proteins that are associated inside the glycolytic enzyme complex focus at a common isoelectric point that is different from the isoelectric points of the purified individual proteins (Mazurek *et al.*, 2005). The IEF was done under native conditions in order to prevent the large protein complexes from dissociating into individual proteins while the second dimension gel electrophoresis was performed under denaturing conditions as described earlier. Visual inspections of the Western blots indicate that the proteins are the correct size but the long protein streaks were not sufficiently focussed (**Figure 26**).

Figure 26: Western blot of endogenous PKM2 after isoelectric focusing. HEK293A cells were treated with normoxia (21% O₂, 5% CO₂ and 37°C) and hypoxic (1% O₂, 5% CO₂ and 37°C) conditions for 48 hours. Isoelectric focusing of whole cell homogenates of HEK293A cells after treatments and Western blotting with anti-PKM2 (1:2,500 dilution, Cell Signaling Technology). **A)** and **B)** Representative Western blots of HEK293A cells exposed to normoxic and hypoxic conditions for 48 hours respectively. Data represent one independent experiment.

8.11 RNA Interference (RNAi) of Endogenous PKM2

RNAi, using synthesized siRNA molecules designed to knockdown the human PKM2 gene, was carried out to determine the role of PKM2 on cell viability after exposure to hypoxia. Gene knockdown of proteins that play a large role in survival after exposure to hypoxia will show differences in cell viability.

Transfection optimization with 0, 0.25, 0.5 and 1 μ g of siRNA for 24 hours in L6E9 and HEK293A cells and Western blotting of whole cell homogenates with anti-PKM2 (1:2,500 dilution, Cell Signaling Technology, Danvers, MA) revealed that knockdown at all concentrations of siRNA produced a significant decrease in PKM2 protein expression in both cell lines compared to controls. A more complete knockdown of PKM2 occurred with 0.25 and 0.5 μ g siRNA in HEK293A cells (**Figure 27E**). Since 0.5 μ g of siRNA in HEK293A cells caused a significantly larger detectable knockdown of PKM2 at the level of the protein, this concentration and cell line was used for all subsequent knockdown experiments.

The amount of time required for knockdown of PKM2 was optimized by transfecting HEK293A cells with 0.5 μ g of siRNA and harvesting the cells at different times of incubation under normoxic conditions. While densitometry analysis (**Figure 28**) showed significant decrease of PKM2 protein expression at all time periods of incubation with siRNA compared to controls, 24 hours of incubation with siRNA produced the largest decrease and was therefore used for all subsequent knockdown experiments.

To determine if PKM2 is required for cell survival after exposure to hypoxia, cell viability of HEK293A cells under both knockdown with siRNA and exposure to hypoxia was examined using the MTT assay.

Figure 27: Western blot analysis of HEK293A and L6E9 cells transfected with PKM2-siRNA (0.25 to 1 μ g). Western blotting with whole cell homogenates from HEK293A and L6E9 cells with PKM2 knockdown under normoxic (21% O₂, 5% CO₂ and 37°C) conditions. **A)** and **C)** Representative Western blots of L6E9 and HEK293A cells after transfection with siRNA using anti-PKM2 (1:2,500 dilution, Cell Signaling Technology, Danvers, MA) respectively. **B)** and **D)** Sample loading was normalized to Ponceau Red on blots. **E)** Densitometry analysis of changes in PKM2 protein expression after transfection with different amounts of siRNA compared to controls (no siRNA). Data represent one independent experiment.

Figure 28: Western blot analysis of HEK293A cells transfected with PKM2-siRNA (0.5 μ g) for different time periods. Western blotting with whole cell homogenates from HEK293A cells under normoxic (21% O₂, 5% CO₂ and 37°C) conditions. **A)** and **B)** Representative Western blots of HEK293A cells with PKM2-knockdown and harvested at different time periods using anti-PKM2 (1:2,500 dilution, Cell Signaling Technology) and anti- β -actin (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) at 60 and 42 kDa respectively. **C)** Densitometry analysis of changes in PKM2 protein expression after transfection with 0.5 μ g siRNA for different time periods compared to controls (no siRNA). Data represent one independent experiment.

A

B

C

Cell viability decreased when exposed to hypoxia compared to normoxic cells without siRNA (**Figure 29A**). Cell viability also decreased only during long-term exposure to hypoxia in cells transfected with siRNA (**Figure 29B**). There were no significant differences in cell viability between hypoxic cells that did not contain siRNA to those that did (**Figure 29C**).

To study the effect on PKM2 protein expression after PKM2 knockdown and hypoxic exposure in HEK293A cells, whole cell lysates from the treated cells were analyzed by Western blotting with anti-PKM2 (1:2,500 dilution, Cell Signaling Technology). Densitometric analysis shows no significant differences in PKM2 protein expression between hypoxic cells containing siRNA and those that did not (**Figure 30**). As a result, hypoxia does not have an effect on PKM2 protein levels in HEK293A cells.

Figure 29: The effect of PKM2 knockdown and hypoxia on HEK293A cell viability. Viability of HEK293A cells transfected with 0.5 μ g siRNA and exposed to hypoxia for 0, 6, 16 and 24 hours of hypoxia (1% O₂, 5% CO₂ and 37°C) was measured by the MTT assay. Cell viability under normoxic conditions (21% O₂, 5% CO₂ and 37°C) without siRNA was used as a control and regarded as 100%. **I)** Absorbance vs. Treatment and **II)** Percent Cell Viability vs. Treatment. Data represent means $\pm$ S.E.M. from three independent experiments with letters **A**, **B** and **C** = $p < 0.05$ compared to their respective controls. **A** = no siRNA and hypoxia treatment, **B** = siRNA and hypoxia treatment and **C** = no siRNA vs. siRNA.

I

I

Figure 30: Changes in endogenous PKM2 protein levels in HEK293A cells transfected with PKM2-siRNA (0.5 μ g) and exposed to hypoxic (1% O₂, 5% CO₂ and 37°C) conditions. **A)** and **B)** Representative Western blots of whole cell homogenates of HEK293A cells after transfection with siRNA and hypoxic exposure using anti-PKM2 (1:2,500 dilution, Cell Signaling Technology) and anti- β -actin (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) respectively. **C)** Densitometry analysis of changes in PKM2 protein expression after transfection with 0.5 μ g siRNA and hypoxia exposure. Data represents means $\pm$ S.E.M. from three independent experiments with * = $p < 0.05$, compared to their respective controls (no siRNA).

A

B

C

9. DISCUSSION

There is little to no information regarding the involvement of hypoxia on the unique conserved LXXLAP amino acid sequence in PKM2. Reasons for why this isoform, and not any of the three other PKs, contains this sequence that is seen twice in HIF- α , could potentially be involved in the regulation of cell survival under hypoxic conditions, independent of HIF, is intriguing. PKM2 may be a direct target for HIF-induced gene expression based on the presence of a HRE located within the PK-M promoter (Park *et al.*, 2004). The results obtained from this present study provides evidence that the levels of the PKM2 mRNA are not significantly induced in response to hypoxia or hypoxic mimetics in L6E9 cells, but there may be oxygen-dependent post-translational modification of the LXXLAP sequence at the level of the protein under normal atmospheric oxygen conditions. In the absence of oxygen, hydroxylation of the conserved sequence in HIF- α cannot occur, thereby stabilizing the protein. Exposure of L6E9 cells to hypoxic and hypoxic mimetic conditions also resulted in stabilization of PKM2 thus also increasing its activity. However hypoxia does not stabilize PKM2 in HEK293A cells, thereby suggesting that hypoxia may not have an effect at the level of the protein and that it may not be posttranslationally modified in a similar manner to HIF- α . Furthermore, results indicate that PKM2 does not have a significant effect on reducing cell viability under PKM2-knockdown and exposure to hypoxic conditions, and thus may not play an essential role in protecting cells in response to hypoxic stressors in HEK293A cells.

9.1 LXXLAP-Containing Protein Search

Investigation of proteins containing the LXXLAP sequence of amino acids in the SWISS-PROT and TrEMBL databases produced over 5,000 different proteins that contained the conserved sequence. The purpose of this search was to discover other proteins that have the conserved LXXLAP sequence and that may be regulated in an oxygen-dependent manner similar to HIF- α . This is important because the sequence is found twice in HIF- α and is crucial in order for hydroxylation to occur, so that it can be ubiquitinated and targeted for degradation by the proteasome under normoxic conditions. It was found that PKM2, and not any of the other three isoforms of PK, contained the LXXLAP sequence. If this sequence is hydroxylated and the protein targeted for degradation after normoxic exposure while stabilized under hypoxic conditions, a new regulatory mechanism of PKM2 under different oxygen, independent of HIF, will have been discovered.

Examining the three dimensional structure of PKM2, it was found that the location of the LXXLAP sequence on the complete protein occurred internally at the interface of the two dimers, where the tetramer assembles (**Figure 31**). It was determined that hydroxylation may affect tetramer assembly, possibly occurring prior to dimer interaction. The possibility that oxygen affects the assembly of dimers into tetramer remains to be tested.

Figure 31: Three-dimensional structure of PKM2 showing the binding of FBP (orange) and the location of the LXXLAP sequence on each of the subunits (four of them). The LXXLAP sequences occurred at the interface between the two dimers in the fully assembled tetramer. PDB ID 1T5A is shown here drawn with PyMOL.

9.2 L6E9 and HEK293A Cells

L6E9 cells are undifferentiated rat skeletal muscle cells that propagate as mononucleated myoblasts. Results from our investigation show that these cells represent an ideal model system for studying PKM2. The response to hypoxia in L6E9 cells have not been previously investigated and, from the results obtained, L6E9 cells show highest endogenous PK activity and PKM2 protein expression under normoxic conditions (**Figure 17 and 18E respectively**). This is particularly true since real-time RT-PCR analysis showed no gene expression of the other isoforms of PK (PKM1 and PKLR) in L6E9 (results not shown). This makes L6E9 cells a good model system for further investigation of how endogenous PKM2 responds to various oxygen environments in a manner similar to HIF- α (i.e. at the level of the protein).

9.3 Endogenous PKM2 Expression and Activity

In the human PK-M gene, one short HRE consensus motif was found within the 1,300 bp region preceding the translational start codon (Shimizu *et al.*, 2004). The regulation of many proteins involved in hypoxia occurs at the level of the gene and the specific factor that controls most hypoxic gene expression is HIF (Semenza, 2003) which has been identified to have more than 60 direct target genes (Park *et al.*, 2004). Although the PK-M gene potentially contains one HIF binding site, we wanted to determine if PKM2 can be regulated by varying oxygen conditions, independent of HIF. Shimizu *et al.* (2004) demonstrated significant enhancement of PK mRNA levels after 6 hours of

hypoxia and levels reached a plateau at 48 hours. They also found that the PK protein levels increased after treatment with CoCl_2 and 24 hours hypoxia and increased further at 48 hours of hypoxia. These results demonstrated that PK may be upregulated in response to hypoxia via HIF activation in astrocytes. Our study utilized real-time RT-PCR to determine if changes occur at the level of the PKM2 gene in L6E9 cells under hypoxic and hypoxic mimetic conditions. From the results obtained, there were no significant changes in PKM2 gene expression under all hypoxic and hypoxic mimetic conditions compared to normoxia (**Figure 20C**). This therefore indicates that, from our observations, PKM2 is not regulated by HIF and that PKM2 is not affected at the level of the gene when exposed to hypoxic and hypoxic mimetic conditions in L6E9 cells.

PKM2 protein expression did not follow PKM2 gene expression after hypoxic and hypoxic mimetic exposure in L6E9 cells. The PKM2 protein contains a similar LXXLAP sequence as in HIF- α which is stabilized when exposed to hypoxia conditions (Safan and Kaelin, 2003). It was also shown previously that some metals can mimic hypoxia and stabilize HIF-1 α (Ho and Bunn, 1996). L6E9 cells were exposed to hypoxia and hypoxic mimetics such as cobalt chloride (CoCl_2) and proteasome inhibitor (PI) to determine whether these conditions stabilize PKM2 in a similar manner as HIF- α . PKM2 protein levels revealed a significant increase of approximately 30 and 40 % when exposed to long-term hypoxia (24 and 36 hours respectively) compared to normoxia (**Figure 19C**). PKM2 protein expression was also increased by about 20% after treatment with CoCl_2 while treatment with PI caused a 10% decrease in PKM2 protein expression. Therefore, changes in PKM2 protein levels are not due to changes in PKM2 transcript levels in L6E9 cells after hypoxia and hypoxic mimetic treatments. The increases in

PKM2 protein expression under hypoxia could indicate that the protein is posttranslationally modified in an oxygen-dependent manner and potentially destabilized and degradation by the proteasome under normoxic conditions. Hydroxylation of the LXXLAP sequence in HIF- α allows for association with vHL (the substrate recognition subunit of E3 ubiquitin ligase) and subsequent polyubiquitination, targeting it for degradation by the proteasome. To confirm the posttranslational modification, protein-protein interaction between PKM2 and vHL could be investigated by affinity purification of the tagged protein and Western blotting for vHL. The decrease in PKM2 expression after PI exposure, however, could indicate that the protein either is not destined to be degraded by the proteasome. Or, inhibition of the proteasome may cause accumulation of some of the protease which normally proteolytically cleaves PKM2, thereby increasing its degradation. Treatment of L6E9 cells with other protease inhibitors, which may stabilize PKM2, would confirm if the protein is destined to be degraded by the proteasome. Also, mutation of the proline in the PKM2 LXXLAP sequence may prevent oxygen-dependent post-translational modification and therefore stabilize the protein under normoxic conditions. This stabilization may be due to tetramer formation. PKM2 protein expression can then be analyzed, comparing with and without the mutation after normoxic exposure.

In conjunction with enhanced endogenous PKM2 protein expression under hypoxic and hypoxic mimetic conditions, endogenous PKM2 activity was also increased after short- and long-term exposure to hypoxia (~1.2- and 1.5-fold respectively) and CoCl₂ (~1.7-fold) exposure compared to normoxia (**Figure 21**). Enhancement of the activity of PKM2 can enable cells to survive under low oxygen conditions by converting

pyruvate to lactate and producing NAD^+ which is crucial in metabolism as they are electron carriers in energy transduction and can help in biosynthetic processes (Pollak *et al.*, 2007).

9.4 Recombinant PKM2 Expression

Expressing large amounts of the protein can aid in a closer study of a protein of interest. Fusion protein technology has become an important tool for producing proteins that can be used for easy detection, characterization and purification. This technique typically involves fusing a tag that can range from only a few amino acids to a complete protein attached to either the N- or C-terminus (or even both) of the target protein (Einhauer and Jungbauer, 2001). The tag can make identification and purification of the protein quick and easy, and there are a variety of expression vectors with different tag sequences that can be fused to almost any protein for different purposes (Einhauer and Jungbauer, 2001). A few requirements of the tag must be met in order to create a proper fusion protein: **1)** the tag must not interfere with the native folding of the protein to which it is attached and **2)** the tag should be water-soluble so that it is exposed on the surface of the protein in order for easy interaction with ligands such as antibodies (Einhauer and Jungbauer, 2001). We have chosen the small eight amino acid FLAG marker peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) as an N-terminal tag for PKM2. For strong antigen-antibody interactions, aromatic amino acids are known to be responsible for this and therefore Tyr is in position 2 of FLAG, flanked by charged amino acids (Einhauer and Jungbauer, 2001). The remainder of the peptide is highly hydrophilic and when the

protein is folded, this portion is highly exposed. There are many commercial anti-FLAG antibodies available. In our studies, fusion of FLAG with PKM2 (**Figure 22**) and mutant PKM2 (results not shown) was achieved and transfection in HEK293A cells with as little as 0.1 µg of DNA (per 3.5 cm dish) was detectable in 5 µg of total protein on a Western blot with the anti-FLAG antibody (**Figure 23B**).

HEK 293A cells are human embryonic kidney (HEK) cells which were produced by transformation of normal HEK cells with the sheared adenovirus 5 DNA (Graham *et al.*, 1977). This cell line was chosen for overexpression studies, not only because according to our results in Figure 17 and 18 HEK293A cells had the second highest PK activity and PKM2 protein expression respectively, but also because they are easily grown, easy to transfect and have been used widely in cell biology for many years. Transfection of DNA with Invitrogen's LipofectamineTM 2000 Reagent in HEK293A cells have been shown to produce 99% transfection efficiency (Invitrogen's LipofectamineTM 2000 Reagent Technical Bulletin, 2002). After optimization of the overexpression and confirmation that the fusion protein was present, hypoxia treatment of transiently transfected HEK293A cells produced no significant differences in wildtype FLAG-PKM2 expression compared to normoxia. Mutation of the proline in the LXXLAP sequence also did not produce any differences in PKM2 stability when compared to wildtype PKM2 protein expression (**Figure 24**).

There may be a possibility of differences in the size or assembly of endogenous PKM2 and wildtype and mutant FLAG-tagged PKM2 after normoxic and hypoxic exposure in HEK293A cells. Therefore the native forms of the protein were examined

using non-denaturing polyacrylamide gels and Western blotting with anti-FLAG-HRP and anti-PKM2 antibodies. Protein expression of wildtype and mutant PKM2, as determined with anti-FLAG, shows that all of the recombinant protein is present at a very high molecular weight (with no monomer seen) and that it may not only be assembled as a tetramer, but may also be associated with other proteins (> 250 kDa) (**Figure 25A**). This indicated the possibility that the recombinant protein may associate with the glycolytic complex in its active form. PKM2 protein expression (endogenous, overexpressed wildtype, overexpressed mutant), as assessed with anti-PKM2 antibody, showed more forms present (monomer, dimer and tetramer) on native gels, with the majority of the protein occurring at high molecular weight (> 250 kDa) (**Figure 25B**). This may be due to either a) the sensitivity or b) nonspecific binding of the anti-PKM2 antibody. Further investigation of the protein-protein interactions of both the endogenous and overexpressed PKM2 proteins, under normoxic and hypoxic conditions, is warranted.

To further study the assembly of PKM2 after normoxic and hypoxic exposure, isoelectric focusing (IEF) can be used as a technique that combines the use of Immobilized pH Gradient (IPG) strips with SDS-PAGE. Also, native isoelectric focusing utilizing sucrose gradient columns and ampholytes can be performed as well. Both techniques allow for the separation of mixtures of proteins with different isoelectric points (pI). Proteins that are associated inside the glycolytic enzyme complex focus at a common isoelectric point that is different from the isoelectric points of the purified individual proteins (Mazurek *et al.*, 2005). These studies could give rise to information regarding whether more or less active enzyme (more or less associated with the

glycolytic complex respectively) occurs after hypoxic exposure or if oxygen-dependent posttranslational modification is involved in the association or dissociation of PKM2 from the complex.

One could also perform single step affinity purification of recombinant PKM2 with anti-FLAG beads and mass spectroscopy (or Western blotting) to determine if PKM2 is associated with components of the glycolytic complex. These studies are currently being undertaken by the Willmore lab.

The results can also suggest that hypoxia may not have an effect on PKM2 and that the protein may not be post-translationally modified in a similar manner to HIF- α in HEK293A cells. There has been a study showing that overexpression of recombinant proteins in HEK293A cells are not a particularly a good model for normal cells and should be used in experiments in which the behaviour of the cell itself is not of interest (Shaw *et al.*, 2002). Shaw et al. (2002) demonstrated that HEK293 cells are not typical kidney cells, as they have attributes of developing neurons and neuronal progenitor cells.

9.5 Cell Viability

The use of RNA interference (RNAi) has been widely used in experimental biology to study the function of known and unknown genes. The decrease in the expression of a target gene can demonstrate the physiological role of the gene product. It has been shown that there are non-coding RNA molecules that can affect transcription, translation, replication and chromosome structure. The inhibition of gene activity can occur at the transcriptional level (transcriptional gene silencing (TGS)) or at the

posttranscriptional level (posttranscriptional gene silencing (PTGS)). In the early 1980s, it was demonstrated that small RNA molecules could bind to complementary sequences in mRNA and inhibit translation in *Escherichia coli* (Daneholt, 2006). Then in the 1990s, RNA silencing was revealed to occur naturally in plants. Subsequently in 1993, sense and antisense RNA was used to regulate translation in the worm *Caenorhabditis elegans* but the results were inconsistent. Finally in 1998, Andrew Fire and Craig Mello demonstrated that introduction of double stranded RNA (dsRNA) caused an efficient loss of the specific target mRNA that was homologous to the dsRNA in *Caenorhabditis elegans*. This led to the term "RNA Interference (RNAi)" and Fire and Mello received the 2006 Nobel Prize in Physiology or Medicine for this discovery (Daneholt, 2006).

The RNAi process is triggered by the introduction of a dsRNA, that contains a sequence complementary to the target gene to be knocked down, into the cell. A ribonuclease of the RNase III family, known as "dicer", shows specificity for dsRNA and catalyzes the cleavage of the dsRNA into small interfering RNAs (siRNAs) which are approximately 25 nucleotides long. A large multiprotein complex, known as the "RNA-induced silencing complex (RISC)", recognizes the siRNA and is activated by ATP to unwind and separate the strands (Wadhawa *et al.*, 2004). RISC then uses the antisense siRNA strand as a guide to find and bind to the target mRNA in a sequence specific manner (Wadhawa *et al.*, 2004). RISC contains a catalytic component which is a member of the argonaute protein family called "Slicer", an endonuclease that binds and cleaves the targeted mRNA (Daneholt, 2006). The cleaved mRNA is then recognized by the cell

Figure 32: The RNAi process. Introduction of dsRNA, that is sequence specific to the target gene, into the cell triggers the RNAi process. Dicer shows specificity for dsRNA and catalyzes the cleavage of the dsRNA into siRNA. RISC then recognizes the siRNA and unwinds it and separates its strands. RISC then uses the antisense siRNA strand as a guide to find and bind to the target mRNA in a sequence-specific manner. Slicer binds and cleaves the targeted mRNA, where the cleaved mRNA is then recognized by the cell as aberrant and is subsequently destroyed by RNases (Daneholt, 2006).

as aberrant and it is then destroyed (**Figure 32**). The destroyed mRNA prevents translation from occurring, silencing the expression of the specific gene from which mRNA was transcribed. Ultimately, it is the siRNA specific to the target gene that causes this effect. The RNAi method is robust and straightforward and, since its discovery, the technology has been used for identifying the function of unknown genes (Wadhawa *et al.*, 2004). It has also been demonstrated that RNAi is quick and sequence specific to the target (Hammond *et al.*, 2001). The disadvantages of this method of RNAi is that it only temporarily knocks down the target gene and (usually) does not provide complete "knock out". It does however provide comparative examination of the target gene and protein under different treatments. Thus, it provides a useful tool to determine the overall role of the target protein within the cell.

RNAi, using synthesized siRNA molecules designed to knock down the human PKM2 gene, was carried out to determine the role of PKM2 on cell viability after hypoxic exposure. Gene knock down of proteins that play a large role in the survival during hypoxia will result in differences in cell viability between cells with and without knock down, under hypoxic conditions.

There are a few methods used to measure cell viability and the most common technique is the trypan blue exclusion method which is a simple and quick way to evaluate cell membrane integrity (Altman *et al.*, 1993). Trypan blue is a dye that stains dead cells blue because the dye can be absorbed whereas live cells have their membranes intact. However studies have shown that this technique is limited as it can significantly overestimate cell viability and therefore has reduced accuracy since the number of blue-staining cells increases the longer the dye has been exposed to the cells (Altman *et al.*,

1993). Another technique is the MTT assay which utilizes the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. MTT reduced in living cells to a purple, water insoluble formazan salt that is soluble in DMSO. The reduction occurs when mitochondrial dehydrogenases, only active in living cells, reduce the MTT to the formazan product (Mosmann, 1983). The colour was then quantified by measuring the absorption using a spectrophotometer. This provides a measure which is directly proportional to the amount of living cells.

To determine if PKM2 is required for cell survival after exposure to hypoxia, cell viability of HEK29A cells under both knock down with siRNA and exposure to hypoxia was examined using the MTT assay. Cell viability decreased when cells were exposed to hypoxia, compared to normoxia, in cells without siRNA (**Figure 29A**). Cell viability also decreased only during long-term exposure to hypoxia in cells transfected with siRNA (**Figure 29B**). There were no significant decreases in cell viability when comparing cells that did not contain siRNA to those that did when treated with hypoxia (**Figure 29C**). These results indicate that knock down of PKM2 may not have a significant effect on reducing cell viability under hypoxic conditions and that PKM2 may not play an essential role in protecting cells in response to hypoxic stressors.

The present study was the first to examine the potential regulation of PKM2 at the level of the protein by posttranslational modification of the conserved LXXLAP sequence by hypoxia. Results shown in this thesis provide evidence that the PKM2 protein is stabilized and activity enhanced in response to hypoxia and hypoxic mimetics at the level of the protein but not at the level of the gene. However it is not possible to say with certainty that PKM2 is hydroxylated in a similar manner as HIF- α . Further

experiments to confirm this could include affinity purification of overexpressed recombinant wildtype and mutant PKM2 proteins, after exposure to normoxia and hypoxia, and determine if hydroxylation occurs on proline 403 under normoxic conditions (absent under hypoxic conditions).

Our interest in PKM2 stems from the fact that PKM2, is the only isoform of PK that contains this unique LXXLAP amino acid sequence found twice in HIF- α . Although this thesis presents only preliminary results of how PKM2 may be regulated, determination of the assembly of the protein before and after exposure to hypoxia can give rise as to more information on PKM2 and how this protein could be a potential therapeutic target for the treatment of tumors that experience varying oxygen conditions.

10. REFERENCES

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