# HEXOKINASE 2 IS IMPORTANT FOR TUMOR GROWTH AND METASTASIS IN PANCREATIC DUCTAL ADENOCARCINOMA

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology in the School of Medicine.

Chapel Hill
2016

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## ABSTRACT

Marybeth Anderson: Hexokinase 2 is important for tumor growth and metastasis in pancreatic ductal adenocarcinoma
(Under the direction of Jen Jen Yeh)

Pancreatic ductal adenocarcinoma (PDAC) is the 4$^{th}$ leading cause of cancer deaths in the United States. The majority of PDAC patients are diagnosed with metastatic disease, where treatment options are limited to cytotoxic chemotherapies that provide modest improvements in overall survival. Over 95% of PDAC tumors contain activating mutations in the oncogene *KRAS*, causing constitutive activation of key pathways promoting cancer cell proliferation, metabolism, and survival. Direct targeting of KRAS and its canonical effector pathways has proven ineffective for the treatment of advanced PDAC, suggesting that additional processes required for *KRAS*-driven tumorigenesis may be therapeutically beneficial. Metabolic reprogramming and increased glucose uptake were required for tumor growth in a genetically engineered mouse model of PDAC, suggesting that this pathway is important for PDAC tumorigenesis.

We found the glycolytic enzyme hexokinase 2 (HK2) to be significantly upregulated in primary PDAC tumors and PDAC metastases. Increased expression of HK2 was associated with poor overall survival after curative surgery, suggesting that HK2 promotes aggressive tumor biology. HK2 was shown to be both necessary and sufficient for regulating glycolysis, primary tumor growth and metastasis of PDAC cell lines. Pharmacologic inhibition of lactate production abrogated HK2-driven invasion in PDAC cell lines, while addition of extracellular lactate

promoted invasion, suggesting that HK2 promotes metastasis by regulating glycolysis. Given this, direct inhibition of HK2 or lactate production may be a promising approach for the treatment of advanced PDAC.

Because HK2 was a driver of PDAC tumor growth and metastasis, candidate microRNAs (miRNAs) were examined for their ability to function as tumor suppressors by inhibiting HK2 expression. miR-148a and miR-216b were negatively correlated with HK2 and down regulated in PDAC tumors. Both miR-148a and miR-216b interacted with the 3'UTR of HK2 and inhibited HK2 expression in PDAC cell lines, suggesting that these miRNAs directly regulate HK2 in PDAC. Restoration of miR-148a and miR-216b expression mimicked the effects of HK2 knockdown on anchorage independent growth and invasion of PDAC cell lines, suggesting a potential role for these miRNAs as inhibitors of PDAC tumor growth and metastasis via their regulation of HK2.

## ACKNOWLEDGEMENTS

I would first like to thank the many people who contributed to my professional development over the course of my graduate studies. My thesis advisor, Jen Jen Yeh, has provided me with much guidance, patience, and has been greatly supportive of my desire to pursue career in academic medicine. The members of her laboratory, both past and present, have also given me support, guidance, and enjoyment both inside and outside of the laboratory, without which I would not have been able to complete these studies. I would also like to thank the members of my thesis committee, Drs. William Kim, Kimryn Rathmell, Scott Hammond, and Praveen Sethupathy for their support and feedback throughout the development and completion of this work. The members of the Kim and Der laboratories, particularly Drs. Bhavani Krishnan, Tikvah Hayes, and Jeran Stratford, have provided invaluable resources, hands-on guidance, and much patience as I progressed through the years.

I would also like to thank the MD-PhD program directors and support staff, all of which have been incredibly supportive throughout my entire enrollment in UNC-Chapel Hill. They have helped me navigate my way through medical school, graduate school, and back. I would not have completed my graduate studies without the personal and professional support provided by Alison Reagan and Dr. Gene Orringer during my first years in the MD/PhD program. I cannot thank them enough and will always be indebted to them for their unwavering support.

I would next like to thank the many people who have contributed to my personal development throughout graduate school. The friendship and advice of Drs. Elizabeth Flate and Raoud Marayati have been instrumental to the completion of this work. Their guidance has given me the confidence to succeed in graduate school and each friend has kept me company during many weekends in the lab. Drs. Theresa Raphael-Grimm, Catherine Forneris, Samantha Meltzer-Brody and Laura Young have given me the tools necessary to for success in both medical and graduate school. Their unconditional support and guidance has been invaluable to my work both inside and outside of the classroom. I am forever grateful for their support.

I would like to thank my parents Dan, Maryann, Brad and Catherine. Without their support I would not have been able to put in the many long hours required to complete these studies. Last but not least, I would like to thank my husband, Steven, who is the most supportive man I have ever had the privilege of being with. He has given me all the help, inspiration, and unconditional love necessary to complete this work.

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| | |
|---|---|
| 3-BP | 3-Bromopyruvate |
| AIG | Anchorage independent growth |
| EGFR | Epidermal growth factor receptor |
| EMT | Epithelial-to-mesenchymal transition |
| ERK | extracellular signal-regulated kinase |
| FPKM | Fragments per kilo base per mega base |
| FTI | Farnesyltransferase inhibitor |
| GEMM | Genetically engineered mouse model |
| GLUT | Glucose transporter |
| GSEA | Gene set enrichment analysis |
| GTP | Guanosine triphosphate |
| HK | Hexokinase |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LDH | Lactate dehydrogenase |
| MAPK | mitogen activated protein kinase |
| MEK | MAPK/ERK kinase |
| miRNA | microRNA |
| mRNA | messenger RNA |
| mTOR | Mammalian target of rapamycin |
| NSCLC | Non-small cell lung cancer |
| PDAC | Pancreatic ductal adenocarcinoma |

| | |
|---|---|
| PI3K | Phophoinositide-3-kinase |
| PKB | Protein kinase B |
| shRNA | Small/short hairpin RNA |
| siRNA | Small interfering RNA |
| TPI | Triosephosphate isomerase |
| UTR | Untranslated region |
| VEGF | Vascular endothelial growth factor |
| QM | Quasi-mesenchymal |

# CHAPTER 1 : INTRODUCTION

## OVERVIEW OF PANCREATIC CANCER

### Clinical overview

Pancreatic cancer, 85% of which is pancreatic ductal adenocarcinoma (PDAC), is the 4$^{th}$ leading cause of cancer deaths in the United States. It is a devastating disease with an overall poor prognosis and a 5 year survival rate of around 7% (Howlader et al., 2013). Over half of all patients are initially diagnosed with metastatic disease, which greatly contributes to the high mortality observed in PDAC (Hidalgo, 2010). Significant risk factors for the development of this disease include a familial history of PDAC, a past medical history of chronic pancreatitis, long-standing diabetes mellitus, and personal history of cigarette use (Hidalgo, 2010, Maitra and Hruban, 2008, Ryan et al., 2014). Inherited mutations such as those in tumor suppressors genes like *p16* and *BRCA1*/2 account for a small percentage of PDAC patients but can significantly increase an individual's lifetime risk of developing PDAC if present, suggesting that these tumor suppressors may be biologically important for tumor development (Maitra and Hruban, 2008).

PDAC is considered a disease of the elderly, as it is rarely found in patients under the age of 45 and the median age at diagnosis is 71 years old (Howlader et al., 2013). Presenting symptoms depend upon the location and stage of the tumor at time of diagnosis (Hidalgo, 2010, Howlader et al., 2013, Ryan et al., 2014). Because 70% of tumors arise in the head of the pancreas and often cause obstruction of the common bile duct, a typical patient may present with

jaundice accompanied by nausea, vague abdominal pain, pancreatitis, and/or systemic symptoms such as weight loss (Bilimoria et al., 2007, Hidalgo, 2010, Ryan et al., 2014). Patients with tumors confined to the pancreas are eligible for curative surgery and have the highest rates of overall survival (Table 1-1, Hidalgo, 2010, Ryan et al., 2014). Unfortunately, only a small minority of PDAC patients are candidates for surgery and the majority present with unresectable or metastatic disease which have the worst overall survival rates (Table 1-1). Treatment of advanced PDAC is often palliative and limited to systemic chemotherapy with or without radiation therapy (Hidalgo, 2010). It is clear that treatment in advanced PDAC must to be improved, as the 5 year overall survival rate for patients with metastatic disease is <3% (Table 1-1, Howlader et al., 2013).

| | TMN Staging | Possible Treatment | Prevalence | Survival at 5 years |
|---|---|---|---|---|
| **Stage IA** | **T1** – Tumor localized within pancreas (<2cm diameter)<br>**N0** – No regional lymph node metastasis<br>**M0** - No distant metastases | Curative surgery with adjuvant chemotherapy | 9% | 27% |
| **Stage IB** | **T2** – Tumor localized within pancreas (>2cm diameter)<br>**N0**<br>**M0** | | | |
| **Stage IIA** | **T3** – Tumor extends past pancreas without involvement of celiac axis or superior mesenteric artery<br>**N0**<br>**M0** | | | |
| **Stage IIB** | **T1, 2, 3**<br>**N1** – Regional lymph node metastasis<br>**M0** | | 28% | 10.7% |
| **Stage III** | **T4** – Tumor involves celiac axis or the superior mesenteric artery<br>**N0**<br>**M0** | Chemotherapy +/- radiation therapy | | |
| **Stage IV** | **T1, 2, 3 or 4**<br>**N0, 1**<br>**M1** – Distant metastases | Palliative chemotherapy | 53% | 2% |

**Table 1-1: Staging, treatment and survival of patients with PDAC.**
Staging according to the most recent edition of the American Joint Committee on Cancer tumor-node-metastasis classification and possible treatments adapted from Ryan, et al. 2014. T = primary tumor, N = regional lymph nodes, and M = metastasis. Prevalence and overall survival at 5 years adapted from SEER 18 data for all races and sexes (Howlader, et al. 2013).

Gemcitabine, a nucleoside analog, has been the standard of care for the treatment of metastatic PDAC since a 1997 clinical trial showed it improved overall survival of metastatic PDAC patients relative to the previous standard of care, 5-fluorouracil (5-FU, Burris et al., 1997). Since 1997 multiple clinical trials have tested gemcitabine in combination with other cytotoxic or targeted chemotherapies in an attempt to improve outcomes for advanced PDAC patients. The vast majority of these trials produced modest, if any, improvements in overall survival until 2011 (Table 1-2). In 2011 a large clinical trial showed a four drug regimen consisting of folinic acid (leucovorin), 5-FU, irinotecan, and oxaliplatin (FOLFIRINOX) to significantly improve survival of metastatic PDAC patients (11.1 months) when compared to treatment with gemcitabine alone (6.8 months, Conroy et al., 2011). Unfortunately, due to increased toxicities with the FOLFIRINOX regimen it can only be given to patients with a high performance status (Conroy et al., 2011). A 2013 trial showed that treatment with gemcitabine plus the cytostatic chemotherapy nab-paclitaxel improved overall survival of metastatic PDAC patients (8.5 months) when compared to gemcitabine alone (6.7 months, Von Hoff et al., 2013). This regimen was well tolerated and represents an alternative to FOLFIRINOX for the treatment of advanced PDAC (Von Hoff et al., 2013).

While advancements in the treatment of metastatic PDAC have occurred in recent years, the improvements have been slow and incremental. With this in mind, much research has focused on understanding PDAC tumor biology. A better understanding of pathways required for tumor growth and metastasis will provide insight into pathways that can be targeted therapeutically for the treatment of advanced disease.

| | Total number of patients | Treatment | Median Survival (months) | P-Value |
|---|---|---|---|---|
| Burris, et al. 1997 | 126 | 5-FU<br>Gemcitabine | 4.4<br>5.7 | 0.002 |
| Herrmann, et al. 2007 | 319 | Gemcitabine<br>Gemcitabine + capecitabine | 7.2<br>8.4 | 0.234 |
| Moore, et al. 2007 | 569 | Gemcitabine<br>Gemcitabine + erlotinib | 5.9<br>6.2 | 0.038 |
| Kindler, et al. 2010 | 535 | Gemcitabine<br>Gemcitabine + bevacizumab | 5.9<br>5.8 | 0.950 |
| Conroy, et al. 2011 | 342 | Gemcitabine<br>FOLFIRINOX | 6.8<br>11.1 | <0.001 |
| Von Hoff, et al. 2013 | 861 | Gemcitabine<br>Gemcitabine + nab-paclitaxel | 6.7<br>8.5 | <0.001 |

**Table 1-2: Clinical trials examining the efficacy of multi-drug regimens to gemcitabine alone for the treatment of advanced stage PDAC.**
Cytotoxic chemotherapies examined for the treatment of PDAC includes 5-FU, gemcitabine, capecitabine, and the FOLFIRINOX regimen (Conroy et al., 2011, Herrmann et al., 2007). Nab-paclitaxel consists of the cytostatic paclitaxel conjugated to albumin for efficient tumor delivery (Von Hoff et al., 2013). Erlotinib is a targeted therapy against the endothelial growth factor receptor (Moore et al., 2007) and bevacizumab is a targeted therapy targeted against the vascular endothelial growth factor-A (Kindler et al., 2010).

## PDAC: an oncogenic *KRAS* driven cancer

Over 90% of PDAC tumors contain activating mutations in the proto-oncogene *kirsten rat sarcoma viral oncogene homolog* (*KRAS*), which encodes for a small GTPase that regulates key signaling cascades responsible for cell growth, metabolism, and survival (Eser et al., 2014, Yeh and Der, 2007). More than 95% of *KRAS* mutations occur in codon 12 and ultimately impair the ability of KRAS to hydrolyze GTP, thus causing constitutive activation of key pathways

necessary for oncogenesis (Eser et al., 2014). Work in genetically engineered mouse models (GEMMs) showed that expression an oncogenic *Kras* allele (*Kras*$^{G12D}$ or *Kras*$^{G12V}$) is sufficient to promote invasive PDAC over time (Guerra and Barbacid, 2013, Hingorani et al., 2003). Oncogenic Kras activity is also required for maintaining tumor growth *in vivo*, as its knockdown caused tumor regression in a PDAC GEMM (Ying et al., 2012). This is consistent with studies in human PDAC cell lines where genetic and pharmacologic inhibition of oncogenic KRAS activity was sufficient to inhibit cell growth (Collisson et al., 2011, Singh et al., 2009, Zimmermann et al., 2013).

The requirement of oncogenic KRAS for tumor initiation and maintenance in preclinical models has led many to believe it represents an ideal target for therapy in PDAC. Two different approaches have been used to inhibit oncogenic KRAS signaling (Figure 1-1). The first approach involved direct inhibition of oncogenic KRAS with small molecule inhibitors that compete for nucleotide binding or modifying the interaction of oncogenic KRAS with effector or regulatory proteins (Cox et al., 2014, Yeh and Der, 2007). While these efforts resulted in the discovery of molecules that successfully bind oncogenic KRAS *in vitro*, they are unlikely to be useful in the clinic due to low-affinity binding of their target (Cox et al., 2014). A second approach focused on preventing the proper localization and function of oncogenic KRAS by inhibiting the post translational modifications necessary for the targeting of KRAS to the cell membrane (Cox et al., 2014, Yeh and Der, 2007). One such class of drugs, farnesyltransferase inhibitors (FTIs), was tested in a phase III clinical trial for the treatment of advanced PDAC. Combination treatment with an FTI and gemcitabine ultimately showed no improvement in overall survival relative to gemcitabine alone, suggesting limited therapeutic benefit for this class of drugs in advanced PDAC (Van Cutsem et al., 2004).

Since efforts to directly target oncogenic KRAS have been unsuccessful, efforts have shifted to pharmacologic inhibition of canonical effector pathways upregulated by oncogenic KRAS signaling (Figure 1-1), including the phophoinositide-3-kinase (PI3K)/protein kinase B (PKB)/mammalian target of rapamycin (mTOR) pathway and the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (Cox et al., 2014, Eser et al., 2014). Targeting of these key effector pathways has been successful in preclinical mouse models of PDAC (Alagesan et al., 2014, Eser et al., 2013), prompting the clinical evaluation of drugs targeted against PI3K/PKB/mTOR and MAPK/ERK signaling (Figure 1-1, Cox et al., 2014, Eser et al., 2014). Unfortunately, phase II studies with inhibitors of mTOR, temsirolimus and everolimus, showed no anti-tumor activity in patients with metastatic PDAC (Javle et al., 2009), suggesting that these therapies are not clinically useful and other mechanisms to inhibit PI3K signaling should be clinically examined in PDAC. A phase II clinical trial with the oral MAPK/ERK kinase (MEK) inhibitor CI-1040 showed that the therapy did not have significant anti-tumor activity in PDAC, as none of the 15 patients examined exhibited a complete or partial response with MEK inhibitor treatment (Rinehart et al., 2004), suggesting that inhibition of the MAPK/ERK signaling pathway with CI-1040 is not clinically important and other inhibitors should be examined for their ability to improve outcomes in advanced PDAC.

While efforts to target canonical KRAS effector signaling have been unsuccessful thus far, studies examining the efficacy of additional pharmacologic inhibitors targeted against these pathways are still ongoing.

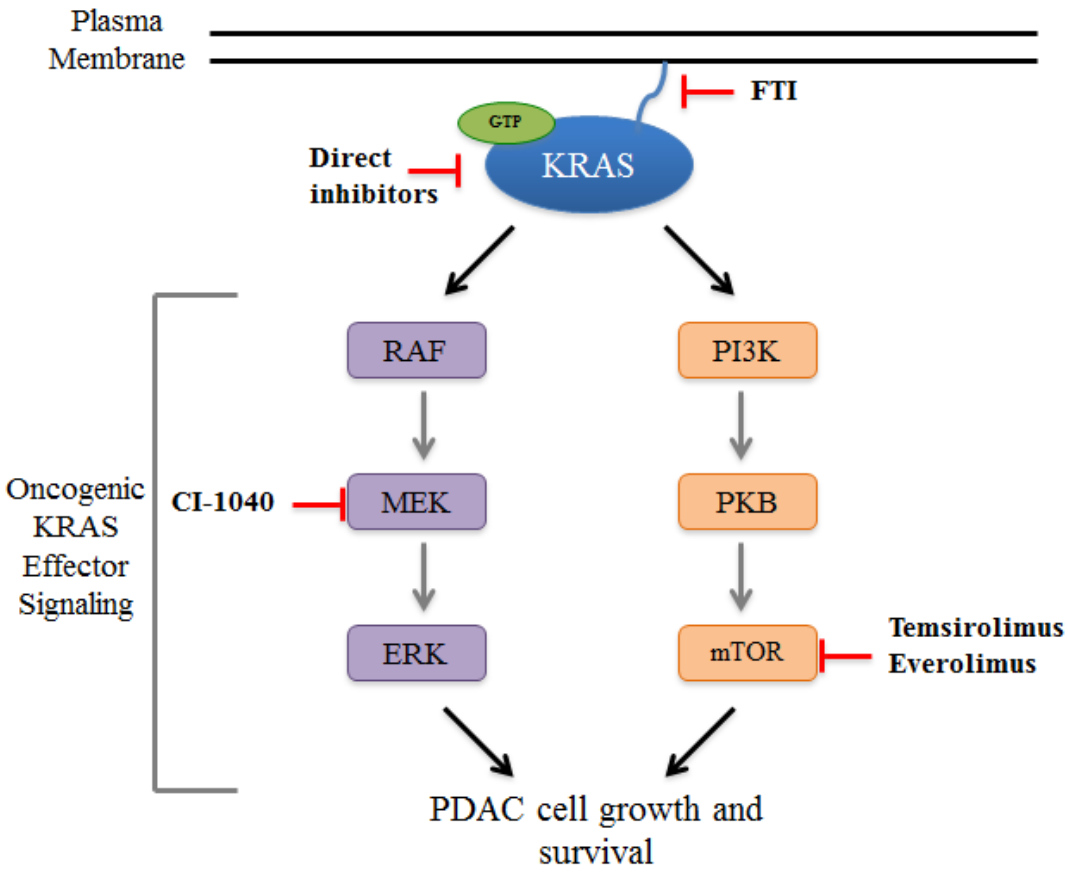



**Figure 1-1: Inhibition of oncogenic KRAS signaling for the treatment of advanced PDAC.**
Activating mutations in the KRAS result in a constitutively active KRAS protein bound to GTP. Oncogenic KRAS promotes PDAC cell growth and survival through activation of well characterized effector pathways including the ERK MAPK (purple) and PI3K/PKB/mTOR (orange) pathways. Efforts to inhibit KRAS signaling in cancer have focused on inhibiting KRAS itself, its membrane localization, and its downstream effector pathways, all of which have produced suboptimal results, suggesting that future efforts should focus on targeting non-canonical KRAS effector signaling.

## KRAS and molecular subtypes of PDAC

One hypothesis for the lack of clinical response to inhibition of canonical KRAS effector signaling is that PDAC is more genetically diverse disease than originally thought. Exome

sequencing of 109 PDAC tumors showed that in addition to oncogenic *KRAS* mutations, many tumors contain mutations in pathways important for tumorigenesis, including transforming growth factor-β signaling, transcriptional regulation, DNA repair, and stem cell signaling, which are not directly involved in KRAS effector signaling (Witkiewicz et al., 2015). Some of these mutations have targeted therapies currently in clinical trials and, therefore, represent novel targets for the treatment of advanced PDAC (Witkiewicz et al., 2015). The promise of targeting these additional mutations in PDAC is exciting and may help the field move away from the use of systemic cytotoxic chemotherapies toward a more targeted approach.

Further demonstrating the clinical importance of molecular diversity within PDAC are studies which examined gene expression of primary PDAC tumors to identify two molecularly distinct subtypes of PDAC, classical and quasi-mesenchymal (QM, (Collisson et al., 2011). The classical subtype exhibited gene expression profiles consistent with epithelial cells and predicted better overall survival in PDAC patients undergoing curative surgery, while the QM subtype exhibited high expression of mesenchymal genes and had worse overall survival (Collisson et al., 2011). The poor overall observed in the QM subtype suggests that this subtype represented tumors with an aggressive biology. Consistent with this, QM PDAC cell lines were resistant to genetic inhibition of oncogenic KRAS while classical subtypes were sensitive to KRAS inhibition (Collisson et al., 2011).

A panel of PDAC and non-small cell lung cancer (NSCLC) cell lines resistant to inhibition of oncogenic KRAS were observed to have gene expression profiles similar to the QM PDAC subtype and consistent with an epithelial to mesenchymal transition (EMT, Singh et al., 2009). EMT is a process associated with aggressive tumor biology and drives metastasis in cancer (Singh et al., 2009). PDAC and NSCLC cell lines resistant to oncogenic KRAS inhibition

were found to be sensitive to pharmacologic inhibition of SYK, a protein kinase upregulated in these cell lines and known to be important for EMT (Hong et al., 2014, Singh et al., 2009), suggesting that inhibition of pathways outside of canonical KRAS effector signaling may be important for treatment of the clinically aggressive QM PDAC subtype. This study further emphasizes the importance of understanding the molecular and genetic diversity within PDAC because these may dictate response to therapy.

While it is known that KRAS signaling drives tumorigenesis, it is clear from studies investigating genetic and molecular diversity of PDAC that pathways outside of canonical KRAS signaling are important for disease progression. Preclinical efforts should, therefore, focus on investigating novel pathways important for oncogenic *KRAS* tumor growth and how these pathways influence growth and survival of the multiple PDAC subtypes.

## REGULATION OF GLUCOSE METABOLISM IN PDAC

### Metabolic reprogramming in PDAC

In an effort to identify additional pathways important for promoting tumor growth in PDAC, a doxycycline inducible *Kras* driven mouse model of PDAC was used to examine the biological effects of oncogenic *Kras* activity (Ying et al., 2012). The authors first showed a requirement of oncogenic Kras for tumor maintenance, as inhibition of Kras expression caused tumor regression (Ying et al., 2012). Gene expression analysis and liquid chromatography-tandem mass spectrometry of PDAC tumors and cell lines after 24 hours of Kras inactivation showed down regulation of genes involved with glucose metabolism and decreased accumulation of glycolytic intermediates (Ying et al., 2012). Oncogenic Kras regulated transcription in this model by activation of MAPK/ERK signaling pathway, which led to increased activity of the c-

MYC transcription factor (Figure 1-2 (Ying et al., 2012). In the presence of oncogenic Kras, glycolytic intermediates were shuttled into anabolic pathways, including hexosamine biosynthesis and the pentose phosphate pathway, that are required for tumor growth *in vitro* and *in vivo* (Ying et al., 2012), suggesting that targeting of these individual biosynthetic pathways may be clinically important.

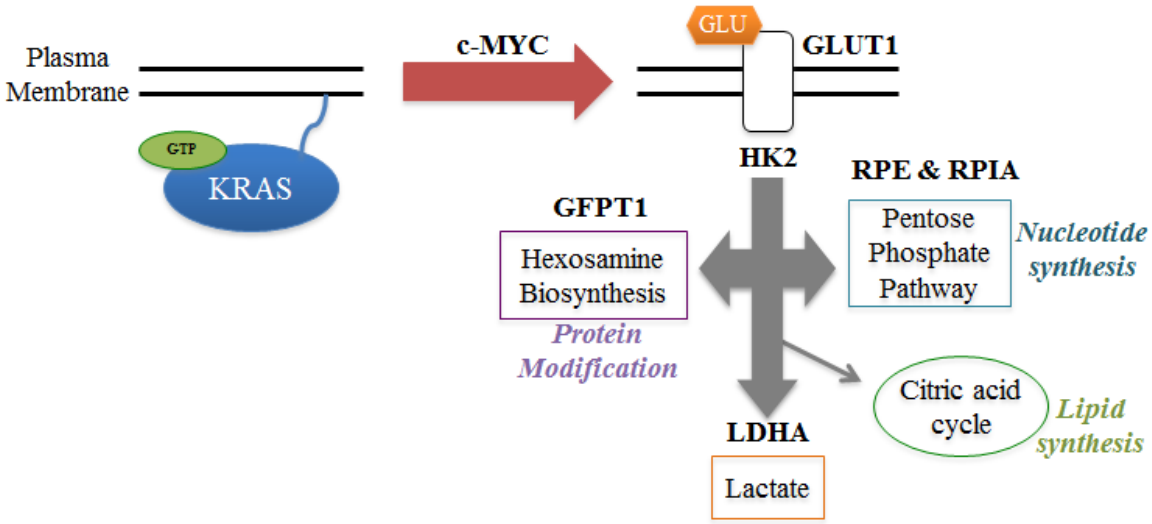



**Figure 1-2: Oncogenic KRAS activity promotes transcriptional upregulation of genes required for elevated rates of glucose metabolism and tumor growth in vivo.**
Activation of oncogenic KRAS causes transcriptional upregulation in a c-MYC dependent manner of key regulatory genes involved with anabolic glucose metabolism (shown in bold, right panel, (Ying et al., 2012). Genetic inhibition of GFPT1, regulating hexosamine biosynthesis, and RPE and RPIA, regulating the pentose phosphate pathway, is sufficient to inhibit growth, suggesting that targeting these pathways would be beneficial for the treatment of PDAC (Ying et al., 2012). GLUT1 and HK2 are required for glucose uptake (Glucose = GLU, orange) and are upstream of these important biosynthetic pathways required for oncogenesis.

To examine differences in glucose metabolism among the classical and QM PDAC subtypes, levels of metabolites in media collected during logarithmic growth across a panel of 38 PDAC cell lines were collected (Daemen et al., 2015). Media collected from classical cell lines was enriched with lipid metabolites, suggesting an increased rate of lipid synthesis, while media collected from QM cells was enriched with glycolytic intermediates and metabolites regulating redox potential (Daemen et al., 2015). Carbon isotope labeling showed that classical cell lines utilized glucose for lipid synthesis while QM utilized glucose for aerobic glycolysis (Daemen et al., 2015). Differences in metabolic demands between the PDAC subtypes suggested that they would respond differently to metabolic inhibitors. Indeed classical cell lines were sensitive to inhibitors of lipid synthesis while QM cell lines were sensitive to inhibitors of glycolysis, such as the pyruvate analog oxamate and a direct inhibitor of lactate dehydrogenase A (LDHA), further emphasizing the need to design therapies targeted against a genetically diverse PDAC with multiple subtypes (Figure 1-3, (Daemen et al., 2015).

These studies outline an important role for targeting glucose metabolism in PDAC, as multiple biosynthetic pathways, including glycolysis, lipid synthesis, and the pentose phosphate pathway, are required for growth in preclinical models of PDAC. The different subtypes of PDAC utilize glucose for different biosynthetic processes, emphasizing the importance of understanding the biological differences between the molecular subtypes. Direct inhibition of glucose uptake may prove to be an efficient way to treat both molecular PDAC subtypes, as it will impede the flow of glucose into pathway necessary for growth in one step (Figure 1-2).

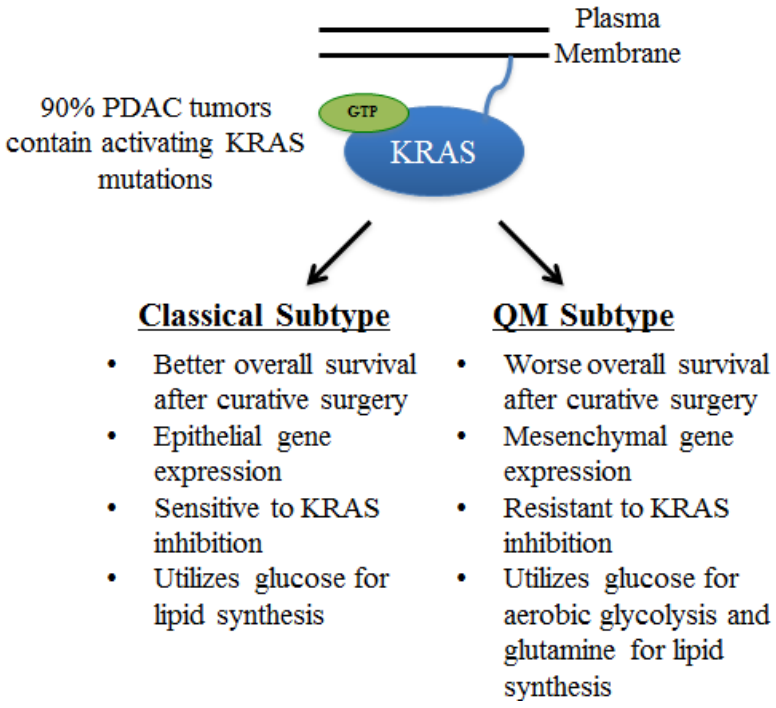



**Figure 1-3: Summary of the distinct molecular subtypes that exist within PDAC patient tumors and cell lines.**
PDAC subtypes were previously defined by Collisson et al (2011), while studies evaluating glucose metabolism in each subtype were performed by Daemen et al (2015).

**Tumor specific regulators of glucose uptake**

Oncogenic *Kras* transcriptionally upregulates multiple proteins required for glucose metabolism, including glucose transporter 1 (Glut1) and hexokinase 2 (Hk2) (Ying et al., 2012). Each of these proteins is required for glucose uptake and represents attractive targets for therapy because they can limit the flow of glucose into multiple pathways required for cancer cell growth and survival (Figure 1-2). GLUT1 belongs to a family of transmembrane proteins consisting of 13 different isoforms that are responsible for glucose entry and exit into the cell (Wood and Trayhurn, 2003). GLUT1 is expressed in many tissues throughout the body, but it is most highly expressed in erythrocytes and the endothelial cells making up the blood brain barrier (Wood and

Trayhurn, 2003). GLUT1 is often upregulated in cancer cells and its transcription is stimulated by low glucose, hypoxia, and oncogenes such as KRAS, ERBB2, and c-MYC (Macheda et al., 2005, Natsuizaka et al., 2007). The upregulation of GLUT1 in cancer suggests that it may be a good target for therapy, however its widespread expression in tissues throughout the body suggest that systemic GLUT1 inhibition may cause off-target effects in non-cancerous tissue.

HK2 belongs to a family of 4 proteins that are responsible for phosphorylation of glucose, a reaction necessary for keeping glucose in the cell and promoting its flow into downstream metabolic pathways (Wilson, 2003). HK2 is expressed at high levels during development but limited to skeletal muscle and fat in adult tissue (Vogt et al., 2000). HK2 is thought to be the "cancer-specific" hexokinase isoform as it is the main isoform upregulated in highly glycolytically active cancers (Katabi et al., 1999, Smith, 2000). This is observed in preclinical mouse models which show specific upregulation of Hk2, and not Hk1, in NSCLC and breast cancer GEMMs (Patra et al., 2013). Analysis of patient tissue also showed HK2, and not other isoforms, to be upregulated in tumors relative to normal tissue (Hamabe et al., 2014, Ogawa et al., 2015, Palmieri et al., 2009, Peng et al., 2008).

HK2 may be preferentially upregulated in cancer cells because it has a high affinity for glucose and, unlike HK1, it is not sensitive to inhibition with glucose-6-phosphate (Robey and Hay, 2006). In addition, HK2 is upregulated under environmental conditions commonly observed in solid tumors, including low glucose and hypoxia (Natsuizaka et al., 2007, Riddle et al., 2000, Zhao et al., 2011). The cancer specific expression of HK2 and its important role in regulation of glucose metabolism make it an attractive target for the treatment of glycolytically active cancers such as PDAC.

**HK2: an important regulator of cancer cell growth and glucose metabolism**

To determine if Hk2 is required for tumor growth *in vivo*, a recent study used GEMMs of NSCLC and breast cancer. Genetic deletion of Hk2 resulted in decreased tumor growth and increased overall survival of both GEMMs (Patra et al., 2013). HK2 is required for tumor growth of multiple cancer types, as its genetic inhibition resulted in decreased growth of cancer cell lines *in vitro* and *in vivo* (Gershon et al., 2013, Jiang et al., 2012, Wolf et al., 2011, Yoshino et al., 2013). The inhibitory effect of HK2 knockdown on tumor growth in these models is likely due to decreased glucose metabolism, as genetic inhibition of Hk2 in Kras and ErbB-2 mutant cells was sufficient to inhibit the flow of glucose into the pentose phosphate pathway and the citric acid cycle (Patra et al., 2013), supporting a role for the targeting of HK2 in PDAC as it has known that anabolic glucose metabolism is required for tumor growth *in vivo* (Ying et al., 2012). To examine the feasibility of targeting HK2 in the clinic, the effects of HK2 inhibition in normal tissue must be examined to ensure that systemic delivery of a potential HK2 inhibitor would not cause overwhelming toxicity. Genetic knockdown of Hk2 in the normal tissue of adult mice did not decrease overall survival, had no effect on body weight or growth, and had no effect on systemic glucose tolerance (Patra et al., 2013), suggesting that systemic targeting of HK2 would be well tolerated by patients. Even though HK2 has been shown to be required for tumor growth of multiple cancers, its role in promoting tumor growth in PDAC has yet to be established.

**MICRO RNA REGULATION OF HK2**

HK2 expression is upregulated in multiple cancer types and its function is important for tumor growth in preclinical models of cancer (Gershon et al., 2013, Patra et al., 2013, Wolf et al., 2011). While previous studies have shown HK2 to be upregulated in an oncogenic *Kras*

dependent manner, other mechanisms regulating HK2 expression may be important for driving oncogenesis. microRNAs (miRNAs) are short, noncoding RNA molecules which function as negative regulators of gene expression (Bartel and Chen, 2004). These endogenously expressed, single stranded RNA molecules bind to the 3' untranslated region (3'UTR) of target mRNA molecules in a sequence dependent manner (Huntzinger and Izaurralde, 2011). The interaction between a miRNA and its target mRNA result in recruitment of proteins required for mRNA degradation or inhibition of translation, an interaction that ultimately causes decreased target protein expression (Kim and Kim, 2012).

Since their discovery in the 1990s miRNAs have garnered much interest in the field of cancer research, because these molecules are commonly dysregulated in tumors and are regulators of a variety of biological processes, including glucose metabolism (Ruan et al., 2009, Singh et al., 2012). miRNAs are known to regulate HK2 expression in a variety of cancers (Fang et al., 2012, Jiang et al., 2012, Yoshino et al., 2013). In a GEMM of lung cancer, miR-143 was shown to be downregulated in Kras mutant tumors and was inversely correlated with HK2 expression (Fang et al., 2012). Restoration of miR-143 expression successfully inhibited aerobic glycolysis and tumor growth in colon, lung and renal cell carcinoma cell lines, suggesting that miRNA regulation of HK2 is important for suppressing tumor growth (Fang et al., 2012, Jiang et al., 2012, Yoshino et al., 2013). There are currently no direct inhibitors of HK2 available for use in the clinic and pharmacologic inhibition of HK2 will likely be difficult to achieve because of similarities in affinity for glucose and protein sequence between hexokinase isoforms (Robey and Hay, 2006, Wilson, 2003). miRNAs may, therefore, represent a unique opportunity to inhibit HK2 in the clinic. While miRNA regulation of HK2 in PDAC has not been studied, but existing

literature suggests that this interaction may be important for PDAC tumorigenesis and could be a useful mechanism for clinical inhibition of HK2.

## CONCLUSIONS AND DISSERTATION GOALS

### Does HK2 promote tumor growth in PDAC?

PDAC is a highly lethal disease with an overall poor prognosis (Ryan et al., 2014). Over half of all patients present to the clinic with existing metastatic disease, suggesting that PDAC tumors are biologically aggressive (Ryan et al., 2014). While 90% of patient tumors contain activating mutations in the oncogene *KRAS*, direct targeting of this gene and its downstream effector pathways was ineffective for the treatment of advanced PDAC, suggesting a need to target non-canonical KRAS signaling pathways in the clinic (Cox et al., 2014, Eser et al., 2014). Glucose metabolism was required for tumor maintenance in a preclinical model of PDAC and inhibition of key enzymes regulating biosynthetic pathways downstream of glucose uptake caused decreased tumor growth, suggesting that targeting glucose metabolism would be beneficial in PDAC (Ying et al., 2012). At least two biologically distinct subtypes of PDAC exist and these subtypes utilize glucose in different ways (Collisson et al., 2011, Daemen et al., 2015). Because of this, the most promising and efficient method for the treatment of molecularly diverse PDAC would include direct targeting of glucose uptake and, therefore, preventing its diversion into multiple downstream pathways required for growth.

HK2 has been shown to be important for glucose metabolism in preclinical models of *Kras* driven lung cancer (Patra et al., 2013) and we hypothesize that it is essential for tumor growth in PDAC. The studies outlined in chapter 2 examined the requirement of HK2 for PDAC disease progression. Analysis of gene expression in PDAC patient tumors suggested that HK2

may be required for both primary tumor growth and metastasis in PDAC. We used *in vitro* and *in vivo* techniques to show that HK2 is both necessary and sufficient to promote PDAC tumor and metastasis. The ability of HK2 to promote invasion of PDAC cell lines was dependent upon glycolysis, suggesting a direct link between elevated rates of glycolysis and increased metastatic potential in PDAC.

**Can miRNAs regulate tumor growth in PDAC by inhibiting HK2 expression?**

miRNAs are important regulators of gene expression and glucose metabolism in PDAC (Singh et al., 2012). These short, single stranded non-coding RNA molecules are dysregulated in PDAC tumors (Price and Chen, 2014) and may participate in tumorigenesis by regulating metabolic genes essential for tumor growth, such as HK2. The studies outlined in chapter 3 aimed to identify novel candidate miRNAs that may regulate HK2 in PDAC. Twenty four miRNAs were shown to be inversely correlated with HK2 expression, down regulated in PDAC tumors, and predicted to bind the 3'UTR of HK2. Two candidate miRNAs, miR-148a and miR-216b, directly interacted with the 3'UTR of HK2 and inhibited HK2 expression in PDAC cell lines. Overexpression of miR-148a and miR-216b mimicked the effect of HK2 knockdown on PDAC anchorage independent growth and invasion, suggesting that these miRNAs may function as tumor suppressors in PDAC.

# CHAPTER 2 : HEXOKINASE 2 PROMOTES TUMOR GROWTH AND METASATSIS BY REGULATING LACTATE PRODUCTION IN PANCREATIC CANCER[1]

## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy with a five-year overall survival of around 7% (Howlader et al., 2013). Over half of all patients initially present with metastatic disease, where treatment options are limited to cytotoxic chemotherapies that are not well tolerated and provide modest improvements in overall survival (Ryan et al., 2014). Over 90% of PDAC tumors contain activating mutations in the oncogene *KRAS,* suggesting that it may be an ideal target for therapy (Yeh and Der, 2007). While knockdown of KRAS inhibits PDAC cell growth *in vitro,* direct targeting of KRAS and its main effector pathways have proven unsuccessful in the clinic (Bryant et al., 2014, Yeh and Der, 2007). Much work has focused on identifying additional pathways promoting *KRAS* driven tumor growth in PDAC, with the hope of identifying new targets for therapy.

The importance of glucose metabolism in *KRAS* driven oncogenesis is well recognized (Blum and Kloog, 2014, Bryant et al., 2014, Guillaumond et al., 2014, Zhao et al., 2011). In a

[1] This chapter is adapted from a research article submitted for publication in the journal *Oncotarget*. The concept of the project was developed by myself and Jen Jen Yeh. I performed the *in vitro* experiments, while Raoud Marayati and the Animal Studies Core Facility at the University of North Carolina assisted with *in vivo* experiments. RNA sequencing data was processed by Richard Moffitt. Jen Jen Yeh and I prepared the manuscript for publication.

genetically engineered mouse model (GEMM) of PDAC, oncogenic Kras activity promoted transcriptional upregulation of key enzymes involved in glucose processing, including those regulating glycolysis, hexosamine biosynthesis and the pentose phosphate pathway (Ying et al., 2012). Activity of these enzymes was required for tumor growth, suggesting a role for targeting glucose uptake and anabolism in PDAC (Ying et al., 2012). Hexokinase 2 (HK2) is an enzyme responsible for phosphorylating glucose, a reaction necessary for glucose processing (Deeb et al., 1993, Wilson, 2003). Four hexokinase isoforms (HK1-HK4) are expressed at varying levels in tissues, but HK2 is the sole isoform overexpressed in cancer (Katabi et al., 1999, Mathupala et al., 2006, Mathupala et al., 2009, Patra et al., 2013, Smith, 2000). Genetic deletion of *Hk2* caused a decrease in tumor burden and increased overall survival of *Kras*-driven lung and *ErbB2*-driven breast cancer GEMMS (Patra et al., 2013). In addition, HK2 knockdown has been found to successfully inhibit tumor growth in glioblastoma, medulloblastoma and renal cell carcinoma (Gershon et al., Wolf et al., 2011, Yoshino et al., 2013).

While a direct role for HK2 in PDAC has yet to be reported, studies examining gene expression and PDAC patient outcomes have shown an association between increased expression of HK2 and more aggressive disease (Chaika et al., 2012, Ogawa et al., 2015). Anabolic glucose metabolism promoted disease progression in a PDAC GEMM, however analysis of human tissue revealed increased expression of genes involved in aerobic glycolysis, including HK2, in primary PDAC and PDAC metastases without changes in expression of anabolic genes, suggesting that glycolysis may be important in human disease (Chaika et al., 2012). PDAC cell lines with elevated rates of glycolysis showed increase expression of an epithelial-mesenchymal transition (EMT) gene signature and were classified as a quasi-mesenchymal, a PDAC subtype previously associated with shorter overall survival (Cheng et al., 2013, Collisson et al., 2011, Daemen et al.,

2015). Taken together these studies provide strong, indirect evidence suggesting a role for HK2 and glycolysis in promoting PDAC disease progression.

The current study shows that HK2 is required for primary tumor growth and metastasis in PDAC. By overexpressing HK2 in PDAC cell lines, we show that increased levels of HK2 are sufficient to promote cell proliferation, anchorage independent growth (AIG) and invasion, supporting a role for HK2 in driving disease progression. Pharmacologic inhibition of lactate production dampens the effects of HK2 on invasion while increased extracellular lactate is sufficient to promote invasion. Overall, this study provides a mechanistic link between HK2 and metastasis via regulation of lactate production and suggests that direct inhibition of HK2 may be a promising approach for treating PDAC.

## RESULTS

### Genes involved in glucose uptake and glycolysis are dysregulated in PDAC

We examined the expression of genes involved with glucose metabolism using a previously described dataset of tumors from primary and metastatic sites of 143 PDAC patients (GSE 71729) (Moffitt et al., 2015). A list of 153 unique genes of interest was compiled using existing KEGG and Reactome gene lists for glucose metabolism, glycolysis and gluconeogenesis, the pentose phosphate pathway and O-glycan biosynthesis (Subramanian et al., 2005). Also included were 14 genes belonging to the family of sugar transport facilitators (SLC2A/GLUT) that are responsible for glucose uptake (Joost et al., 2002, Wood and Trayhurn, 2003). To identify genes associated with tumorigenesis, we looked for those highly expressed in primary tumors compared to unmatched normal pancreas. To identify genes associated with metastasis, we looked for genes highly expressed in metastases compared to primary tumors.

Four genes – glucose transporter 1 (GLUT1), HK2, lactate dehydrogenase A (LDHA), and triosephosphate isomerase 1 (TPI1) – were upregulated in primary tumors compared to normal pancreas and in metastases compared to primary tumors ($P<0.001$, Figure 2-1). GLUT1 and HK2 both play a role in glucose uptake. LDHA is a key enzyme responsible for producing lactate from pyruvate, the final step in aerobic glycolysis (Adeva et al., 2013, Hsu and Sabatini, 2008). GLUT1, HK2, and LDHA were previously found to be regulated in an oncogenic Kras dependent manner, suggesting that they may be important for KRAS-driven tumor growth (Ying et al., 2012). TPI1 catalyzes an isomerization reaction in the glycolytic cascade but is not regulated in a Kras-dependent manner and was, therefore, not further studied (Ying et al., 2012).

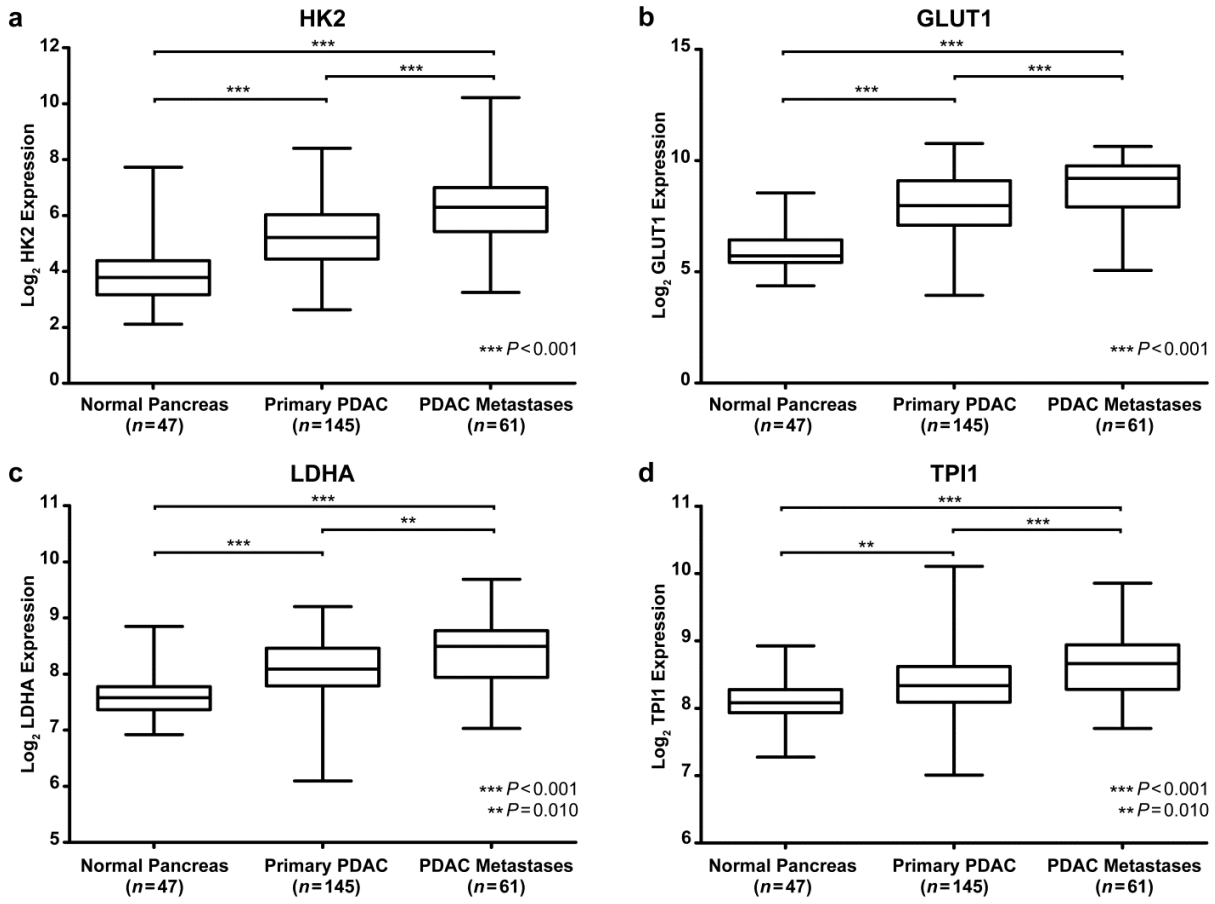
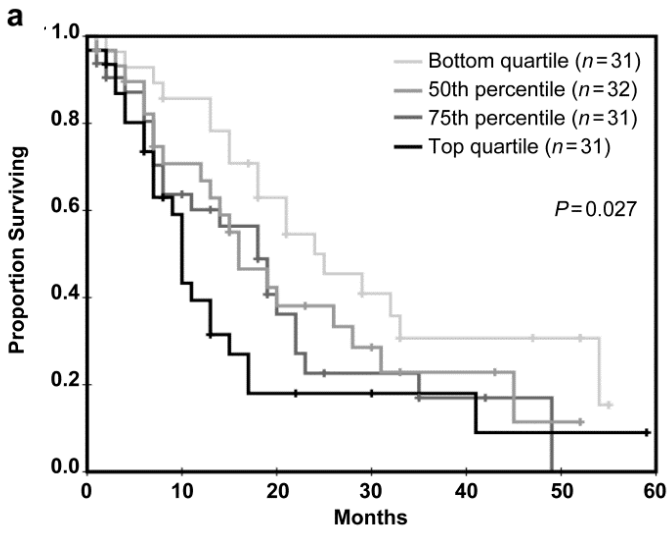



**Figure 2-1: HK2 (a), GLUT1 (b), LDHA (c) and TPI1 (d) are upregulated in PDAC primary tumors and metastases relative to normal pancreas.**
$Log_2$ of gene expression in normal pancreas, primary PDAC, and PDAC metastases. Box shows median expression with upper and lower quartiles and whiskers show maximum and minimum values. A one-way ANOVA with Bonferroni correction for multiple comparisons test determined statistical significance.

**Increased HK2 expression is associated with poor patient survival after surgery**

Increasing expression of key enzymes regulating glucose uptake and glycolysis in primary tumors and metastases suggest that these pathways are associated with aggressive tumor biology. To determine if GLUT1, LDHA, or HK2 was associated with clinical outcome, we evaluated the relationship between gene expression and patient survival. Patients with tumors containing high HK2 expression had a median survival of 13 months while patients with tumors containing low HK2 expression had a median survival of 21 months ($P$=0.027, Figure 2-2a). HK2 was also associated with shorter overall survival in patients with localized tumors who underwent curative surgery (hazard ratio (HR) 1.31 (1.07, 1.60), Figure 2-2b), suggesting that high HK2 expression may be associated with early disease relapse and metastasis. Neither GLUT1 nor LDHA expression was associated with patient survival (Figure 2-2b).




**b** **Cox Proportional Hazard Regression**

| | Hazard Ratio [95% CI] | *P* value |
|---|---|---|
| HK2 | 1.27 [1.04, 1.55] | 0.022 |
| GLUT1 | 1.11 [0.95, 1.29] | 0.196 |
| LDHA | 0.95 [0.65, 1.39] | 0.805 |

**Figure 2-2: HK2 expression is associated with poor overall survival.**
(**a**) Groups for Kaplan Meier survival analysis were based off HK2 expression in primary tumors. Lowest quartile showed median survival of 24 months (95% CI [14 ,34]) while highest quartile showed median survival of 10 months (95% CI [9, 11]).
(**b**) Correlation between HK2, GLUT1 and LDHA expression in primary tumors (n=125) and overall survival as determined by univariate Cox proportional regression. Hazard ratios and 95% CI shown.

## HK2 is necessary for AIG and invasion in PDAC cell lines

To directly assess the requirement of HK2 for promoting tumor growth and invasion, si- and shRNA were used to transiently and stably knockdown HK2 in two PDAC cell lines, CFPAC-1-LUC and PANC-1 (Figure 2-3). Both lines contain activating mutations in *KRAS* and exhibit the highest levels of HK2 expression relative to the normal immortalized epithelial cell

line HPNE across a panel of PDAC cell lines ($P$<0.001, Figure 2-4). Levels of HK1 were unaffected by transient and stable knockdown of HK2, suggesting that our constructs were HK2 specific and that there was no compensatory increase in HK1 expression (Figure 2-3).

To examine the effect of HK2 knockdown on PDAC cell growth, a soft agar assay was used to assess for AIG, a phenotype associated with both tumor growth and metastatic potential (Mori et al., 2009). Transient knockdown of HK2 in the CFPAC-1-LUC cell line caused a 72.7% decrease in colony growth compared to that observed for the nonspecific (NS) control ($P$<0.001, Figure 2-5b). Similar findings were observed with stable knockdown of HK2 ($P$<0.030, Figure 2-5b). Transient and stable knockdown in PANC-1 with siHK2 and shHK2#1 resulted in an 82.0% and 71.0% decrease in colony growth relative to NS, respectively ($P$<0.001, Figure 2-5b). Stable knockdown using shHK2#2 caused 30.1% growth inhibition relative to shNS ($P$=0.06, Figure 2-5b). The dampened effect on colony growth may be explained by inefficient knockdown of HK2 with the shHK2#2 construct (Figure 2-3). To assess the effect of HK2 knockdown on invasion, a Matrigel coated transwell invasion assay was used (Figure 2-6a). Transient and stable knockdown of HK2 caused an approximate 50% decrease in invasion in both cell lines ($P$<0.030, Figure 2-6b). These results show that HK2 is required for PDAC AIG and invasion.

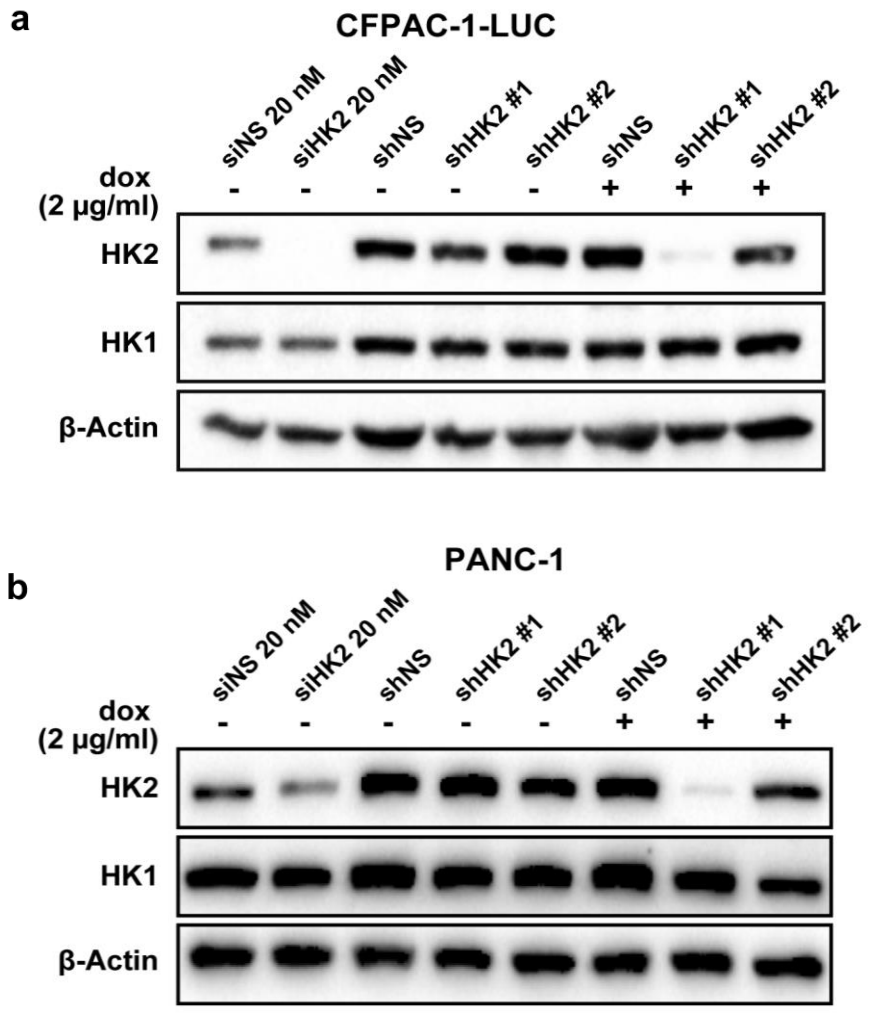



**Figure 2-3: Transient and stable knockdown of HK2 expression in PDAC cell lines.**
Transient and stable knockdown of HK2 was achieved using siRNA (siHK2, 20 nM) and a doxycycline inducible lentiviral shRNA construct (shHK2# and #2) in CFPAC-1-LUC (**a**) and PANC-1 (**b**) cell lines. Cells were isolated for western blot or phenotypic assay after 72 hours of doxycycline (2 µg/mL) exposure.

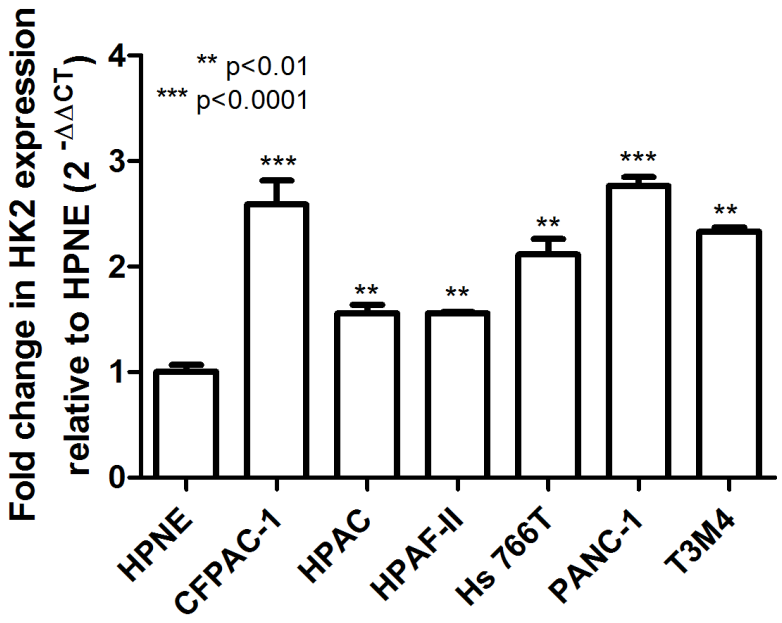



**Figure 2-4: HK2 is upregulated in PDAC cell lines relative to the normal immortalized pancreatic epithelial cell line HPNE.**
Fold change in HK2 expression across a panel of PDAC cell lines relative to the immortalized epithelial cell line HPNE. Fold change determined using the ΔΔCT method with mean and standard error of the mean (SEM) shown (n=3 technical replicates).

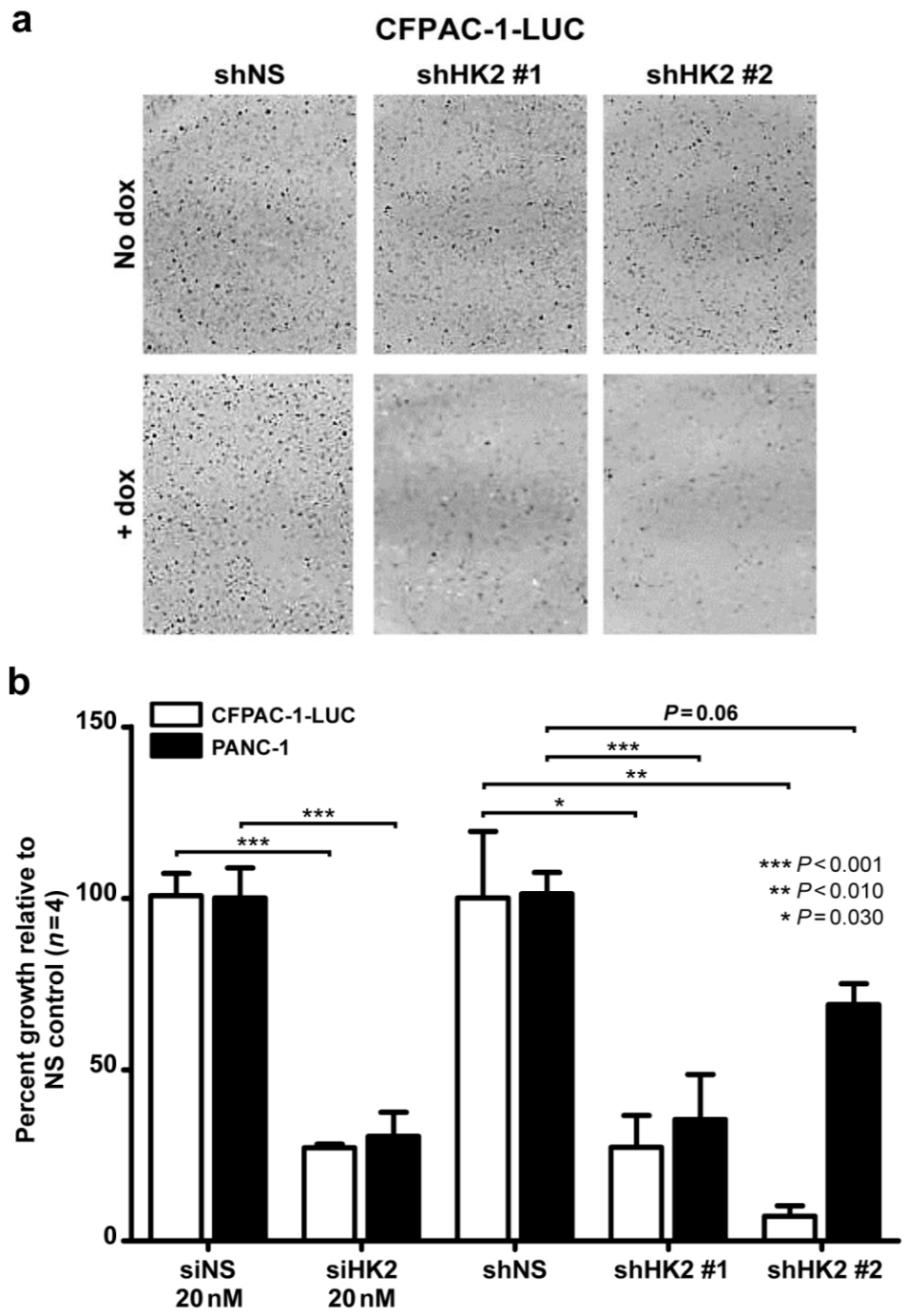



**Figure 2-5: HK2 is required for AIG of PDAC cell lines.**
(**a**) Representative images of colony formation in soft agar assays.
(**b**) Percent of growth with HK2 knockdown relative to control (siNS or shNS). Mean ± SEM of biological replicates shown (*n*=4).

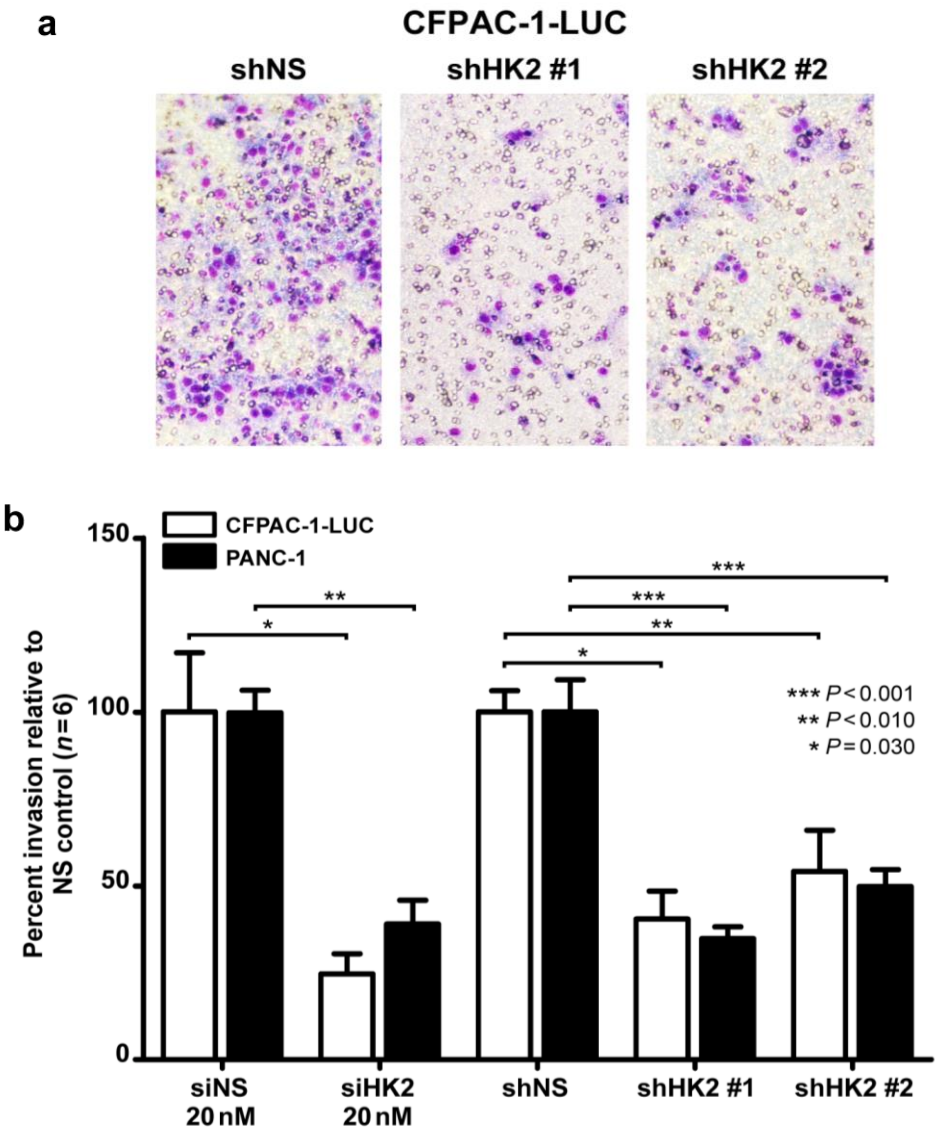



**Figure 2-6: HK2 is required for invasion of PDAC cell lines through a reconstituted matrix.**

(**a**) Representative images of colony formation in soft agar assays.

(**b**) Percent of invasion with HK2 knockdown relative to control (siNS or shNS). Average ± SEM for six biological replicates is shown.

**HK2 is sufficient to promote AIG and invasion in PDAC cell lines**

HK2 expression is limited in most normal tissues but preferentially upregulated in cancer cells (Katabi et al., 1999). Whether HK2 is sufficient to promote PDAC growth and metastasis remains unknown. To examine this, two PDAC cell lines stably overexpressing HK2 cDNA were generated (CFPAC-1-HK2 and PANC-1-HK2, Figure 2-7a). Hexokinase (HK) activity was measured to confirm that stable HK2 overexpression resulted in increased protein function. A 3.3-fold increase in HK activity was observed in CFPAC-1-HK2 and a 1.4-fold increase was observed for PANC-1-HK2 ($P<0.001$, Figure 2-7b). Additionally transient knockdown of HK2 with siRNA caused a significant decrease in HK when compared to the control ($P<0.010$, Figure 2-7b). No change in HK1 expression was found in the cell lines generated, suggesting that the increased HK activity can be solely attributed to changes in the level of HK2.

We next determined the effects of HK2 overexpression on AIG and invasion. A 5.1-fold increase in colony growth was observed with stable HK2 overexpression relative to the GFP control in CFPAC-1-HK2, while a 1.7-fold increase was observed in PANC-1-HK2 ($P=0.020$, Figure 2-8). We hypothesized that this increase in AIG results from an increased rate of cell proliferation, as HK2 overexpression caused an increase in anchorage dependent growth as well as AIG (Figure 2-8a). A 3.4-fold increase in invasion was observed for CFPAC-1-HK2 relative to the control while a 2.4-fold increase was observed for PANC-1-HK2 ($P<0.002$, Figure 2-9). Our data suggests that increased HK2 expression is sufficient to promote anchorage dependent and independent growth, as well as invasion.

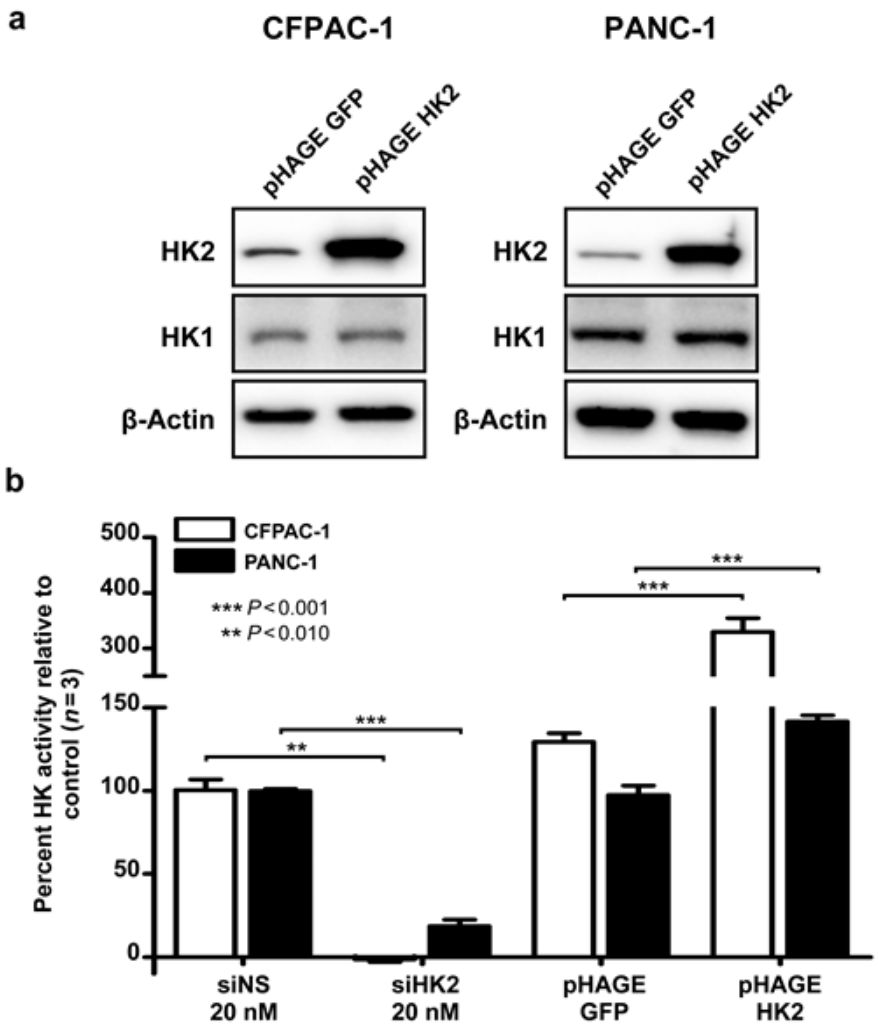



**Figure 2-7: Altered HK2 expression and activity in PDAC cell lines.**
(**a**) Stable overexpression of HK2 (pHAGE HK2) relative to control (pHAGE GFP) in CFPAC-1 and PANC-1 cell lines.
(**b**) Percent hexokinase activity of knockdown (siNS vs. siHK2) and overexpression (pHAGE GFP vs. pHAGE HK2) cell lines relative to control. Mean ± SEM of biological replicates (n=3) shown with student's t-tests for statistical significance.

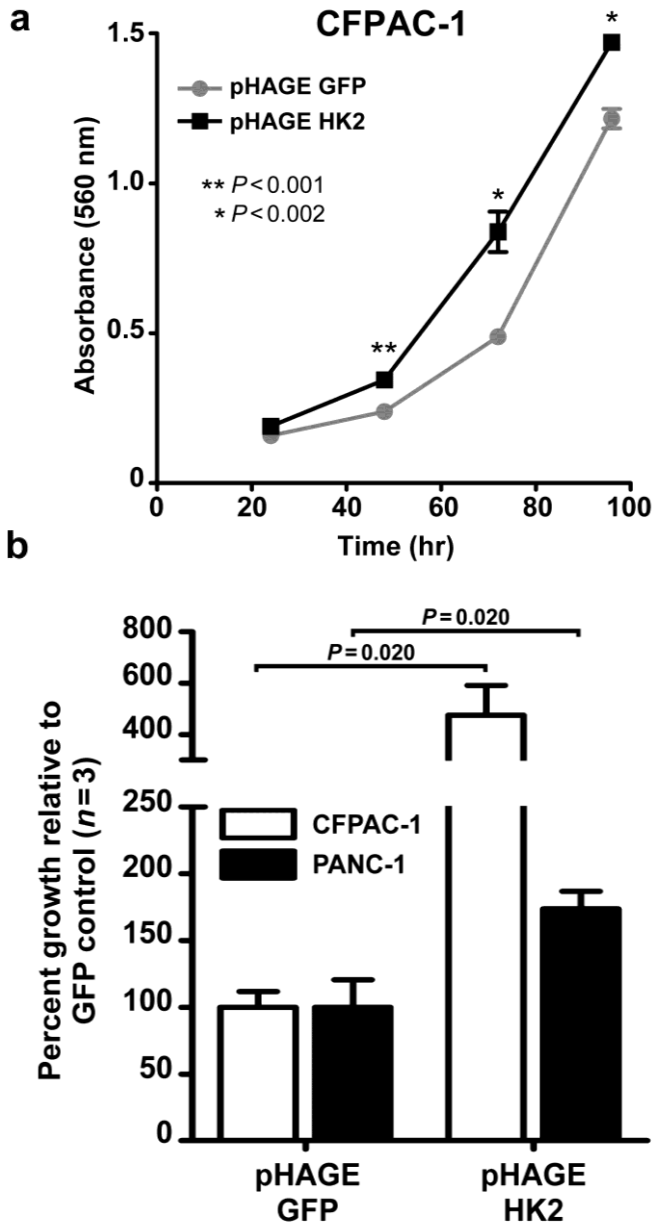



**Figure 2-8: HK2 is sufficient to promote AIG and cell proliferation in PDAC cell lines.**
(**a**) Cell proliferation in CFPAC-1-GFP and CFPAC-1-HK2 as determined using a MTT assay. Mean ± SEM of technical replicates (n=4) shown with student's t-tests for statistical significance at 48, 72, and 96 hours of growth.
(**b**) Percent colony growth with HK2 overexpression relative to control. Mean ± SEM of biological replicates (n=3) shown with student's t-tests for statistical significance.

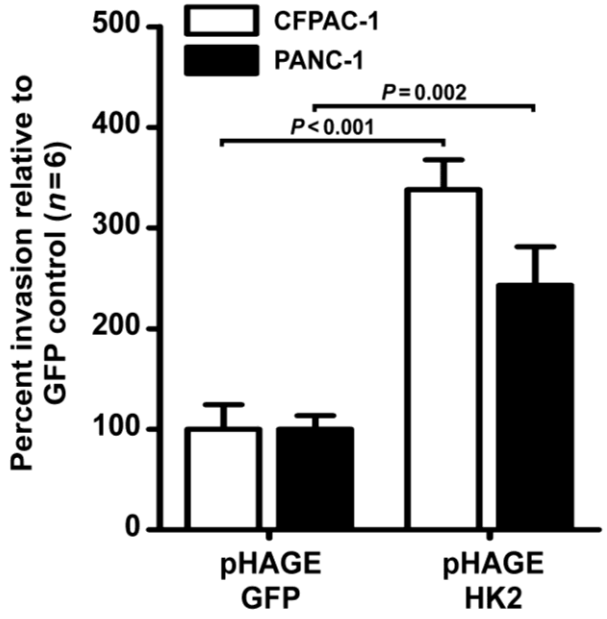



**Figure 2-9: HK2 overexpression is sufficient to promote invasion of PDAC cell lines.** Percent invasion with HK2 overexpression relative to GFP control. Mean ± SEM of biological replicates (n=6) shown with student's t-tests for statistical significance.

**HK2 promotes invasion by regulating lactate production**

We hypothesized that the HK2-driven changes in invasion observed result from changes in glycolysis, as elevated glycolysis has been previously linked to metastasis (Collisson et al., 2011, Daemen et al., 2015). To this end, we measured lactate production in cell lines with transient knockdown and stable overexpression of HK2. An approximate 20% decrease in lactate production was observed with HK2 knockdown relative to the NS control ($P$<0.003, Figure 2-10). Conversely, stable HK2 overexpression produced a 1.3-fold and 1.2-fold increase in lactate production for the CFPAC-1-HK2 and PANC-1-HK2 cell lines, respectively, suggesting that changes in HK2 are sufficient to alter glycolysis in PDAC cell lines ($P$<0.003, Figure 2-10). To determine if HK2 promotes invasion in a lactate dependent manner, the pharmacologic

inhibitor oxamate was used to inhibit glycolysis in cells with stable HK2 overexpression. Cells were pretreated with oxamate at the calculated IC50 prior to seeding into transwell invasion assay (Figure 2-11a). At the time of seeding, a 3.2 and 4.1-fold decrease in lactate production was observed with oxamate treatment relative in CFPAC-1-HK2 and PANC-1-HK2, respectively, confirming inhibition of glycolysis relative to control ($P$<0.001, Figure 2-11b).

An approximate 2.0-fold decrease in invasion was observed in oxamate treated cells with stable expression of HK2, suggesting that glycolysis is required for HK2 to promote invasion ($P$<0.010, Figure 2-11c). We next determined if the addition of extracellular lactate was sufficient to promote invasion. Cells were incubated with media supplemented with lactate (40 mM) for 24 hours, conditions that induce changes in histone acetylation and gene expression (Latham et al., 2012). The addition of lactate was sufficient to promote invasion in both cell lines, as a 3.5-fold increase in invasion for CFPAC-1-HK2 and 2.5-fold increase in invasion of PANC-1-HK2 were observed ($P$<0.001, Figure 2-12). These results suggest that HK2 regulates invasion in a lactate-dependent manner, supporting a direct link between elevated rates of glycolysis and increased metastatic potential.

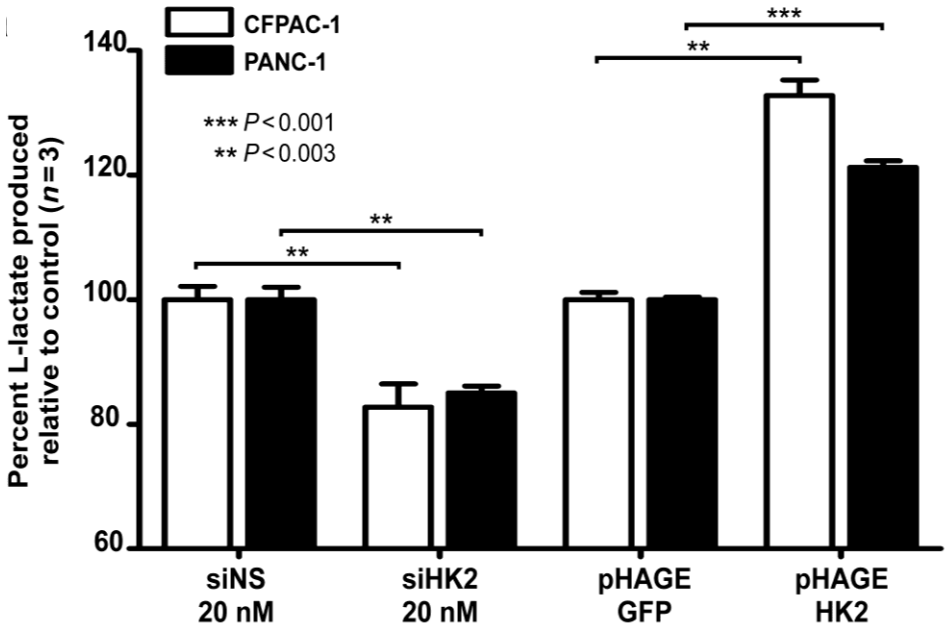



**Figure 2-10: HK2 regulates lactate production in PDAC cell lines.**
Relative lactate production in CFPAC-1 and PANC-1 with HK2 knockdown (siHK2 vs. siNS) and overexpression (pHAGE HK2 vs. pHAGE GFP). Mean ± SEM of biological replicates (n=3) shown with student's t-tests for statistical significance.

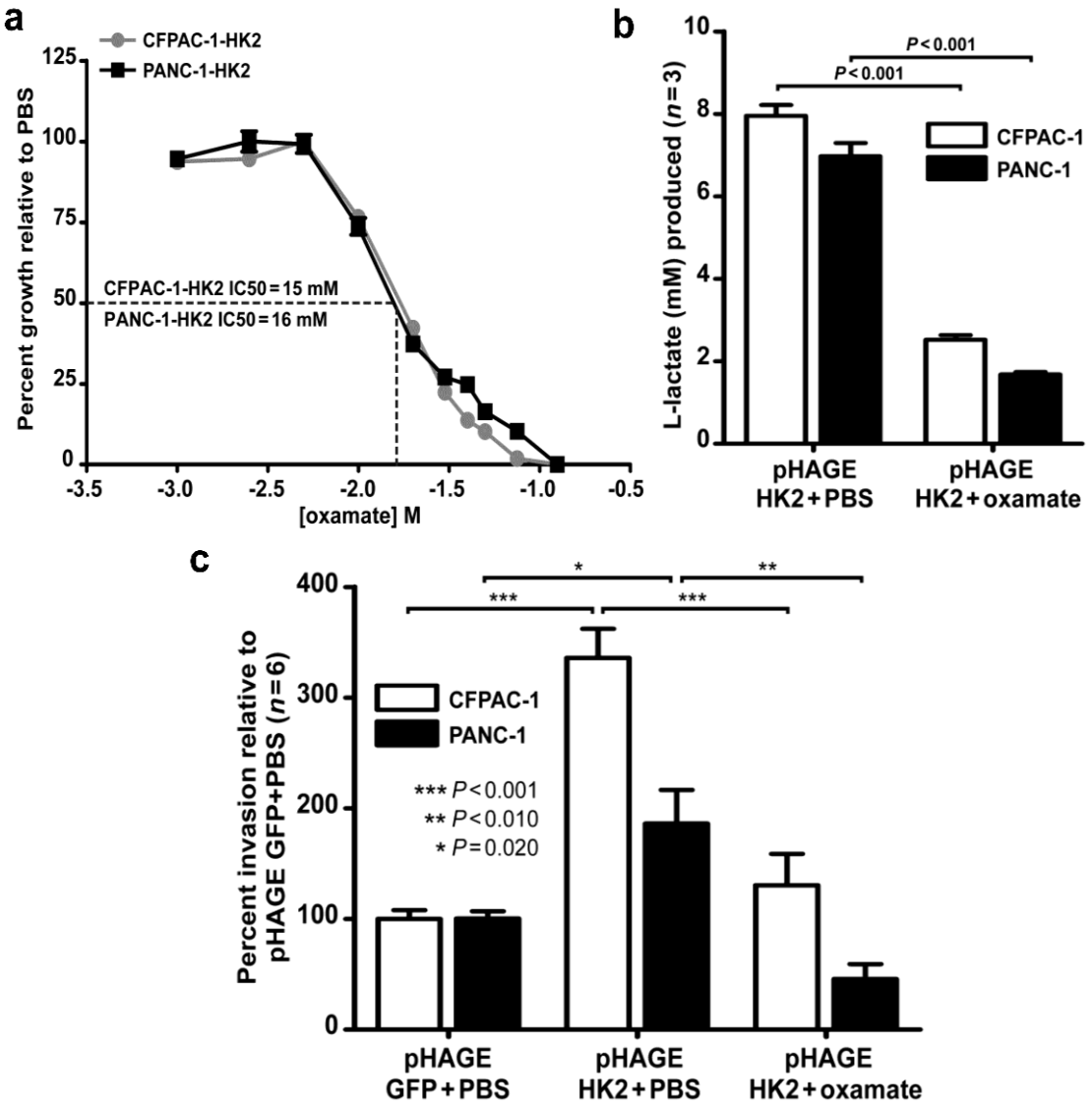



**Figure 2-11: The pyruvate analog oxamate inhibits lactate production and HK2 driven invasion of PDAC cell lines.**

(a) IC50 determination for CFPAC-1-HK2 and PANC-1-HK2 after 72 hours of oxamate treatment; Mean ± SEM of technical replicates (n=4) shown with student's t-tests for statistical significance. CFAPC-1 IC50 calculated to be 15 mM while PANC-1 determined to be 16mM using GraphPad Prism software (v.5, GraphPad Software, INC. La Jolla, CA, USA). (b) L-lactate produced (mM) in CFPAC-1-HK2 and PANC-1-HK2 cell lines treated with PBS or IC50 oxamate for 72 hours. Mean ± SEM of biological replicates (n=3) shown with student's t-tests for statistical significance. (c) Percent invasion in cells treated with PBS or IC50 oxamate. Mean ± SEM of biological replicates (n=6) shown with student's t-tests for statistical significance.

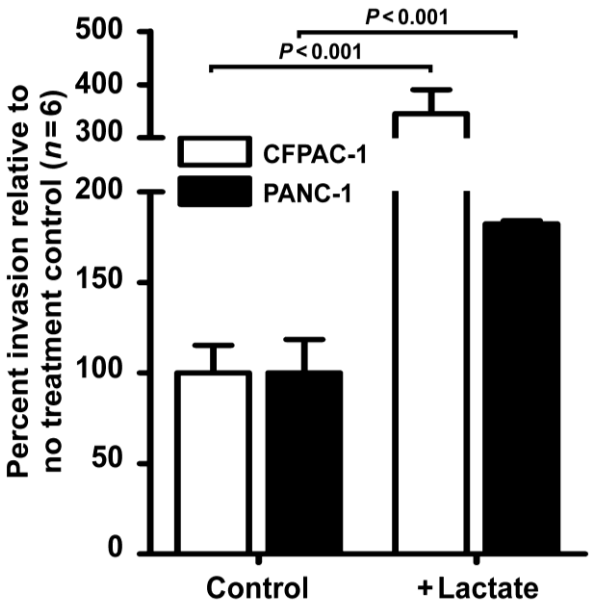



**Figure 2-12: Extracellular lactate enhances invasion of PDAC cell lines**
Percent invasion in PDAC cells incubated with extracellular lactate (40 mM). Mean ± SEM of biological replicates ($n$=6) shown with student's t-tests for statistical significance.

**HK2 is necessary for PDAC tumor growth and influences gene expression**

To examine the effects of HK2 knockdown on primary tumor growth, CFPAC-1-LUC cells containing a doxycycline-inducible shHK2#1 or shNS (control) were subcutaneously injected into immune-compromised mice. HK2 knockdown was confirmed in tumors expressing the shHK2#1 compared to those expressing shNS after 3 and 7 days of doxycycline administration (Figure 2-13a). To assess the effect of HK2 knockdown on long-term tumor growth, mice were given doxycycline or sucrose (control) once tumors reached an average volume of 152 $mm^3$ (standard deviation (SD) 46 $mm^3$). No change in growth was observed in the shNS tumors in mice treated with doxycycline, confirming that administration of doxycycline alone had no effect on tumor growth (Figure 2-14a). However, induction of shHK2#1 expression

resulted in tumor growth inhibition during the 30 day treatment period compared to the shNS controls ($P$<0.030, Figure 2-14b). At the end of treatment an average reduction in tumor volume of 57.5% was observed with HK2 knockdown ($P$=0.020, Figure 2-14b).

RNA sequencing was performed on tumors harvested at the end of the study to determine the effect of HK2 knockdown on gene expression *in vivo* (Figure 2-13b). Genes whose average reads per kilo base of transcript per million mapped reads (RPKM) was <10 were excluded from analysis, so that only genes with a high level of baseline expression were included ($n$=6 120). Gene set enrichment analysis (GSEA) was performed to identify differentially regulated gene sets between control (shNS, $n$=3) and HK2 knockdown (shHK2#1, $n$=4), with a focus on gene sets contained in the molecular signature database's (MSigDBv5) hallmark and oncogenic signatures gene lists (Subramanian et al., 2005).

We found 27 gene sets to be significantly enriched in shNS tumors relative to shHK2 tumors ($P$<0.02, false discovery rate (FDR)<0.100, Table 2-1) and 18 gene sets significantly enriched with HK2 knockdown relative to shNS (Supplementary table 4, $P$<0.02, FDR<0.100, Table 2-2) (Subramanian et al., 2005). Interestingly, one of the highest enriched pathways in the control tumors included genes upregulated with increased vascular endothelial growth factor-A (VEGF-A) activity (VEGF_A_UP.VI_UP, normalized enrichment score (NES) 2.2, $P$<0.001, FDR<0.001, Figure 2-15, Figure 2-16), a pathway important for promoting metastasis (Schoenfeld et al., 2004, Zetter, 1998). Furthermore, genes downregulated with VEGF-A signaling were enriched in the HK2 knockdown tumors (VEGF_A_UP.VI_DN, NES -2.4, $P$<0.001, FDR<0.001, Figure 2-15, Figure 2-16), suggesting that HK2 knockdown is associated with inhibition of VEGF-A signaling (Schoenfeld et al., 2004).

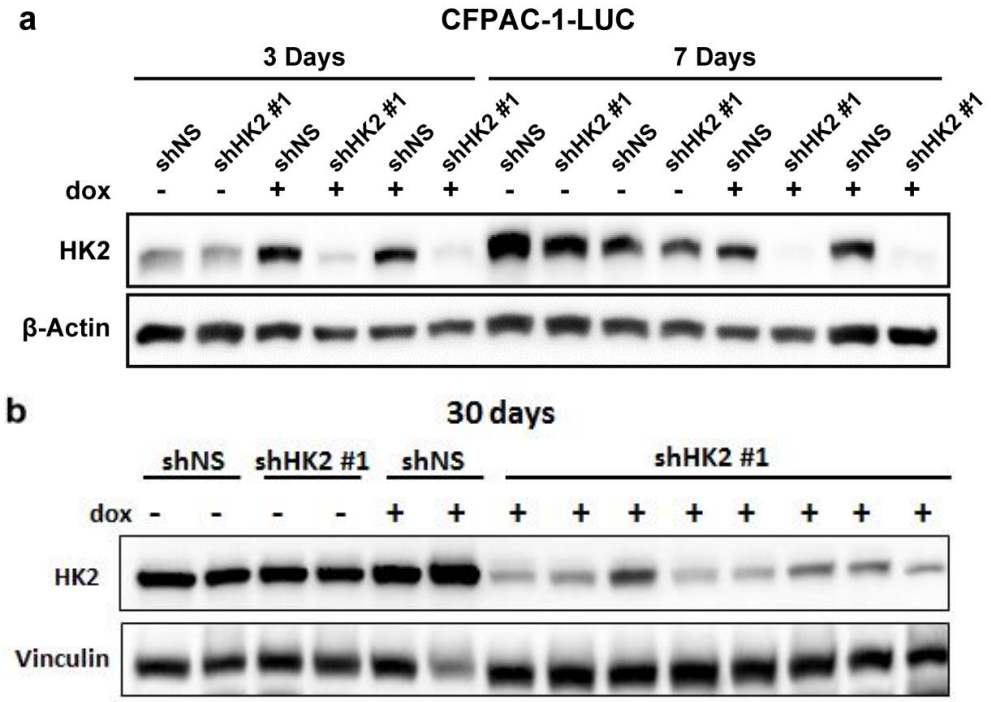



**Figure 2-13: Expression of HK2 in CFPAC-1-LUC xenografts containing stable expression of doxycycline inducible shRNA constructs.**
(**a**) Expression of HK2 in CFPAC-1-LUC tumors containing doxycycline inducible shNS or shHK2#1 treated with sucrose (control) or doxycycline for 3 or 7 days.
(**b**) Expression of HK2 in CFPAC-1-LUC tumors containing doxycycline inducible shNS or shHK2#1 after 30 days of treatment.

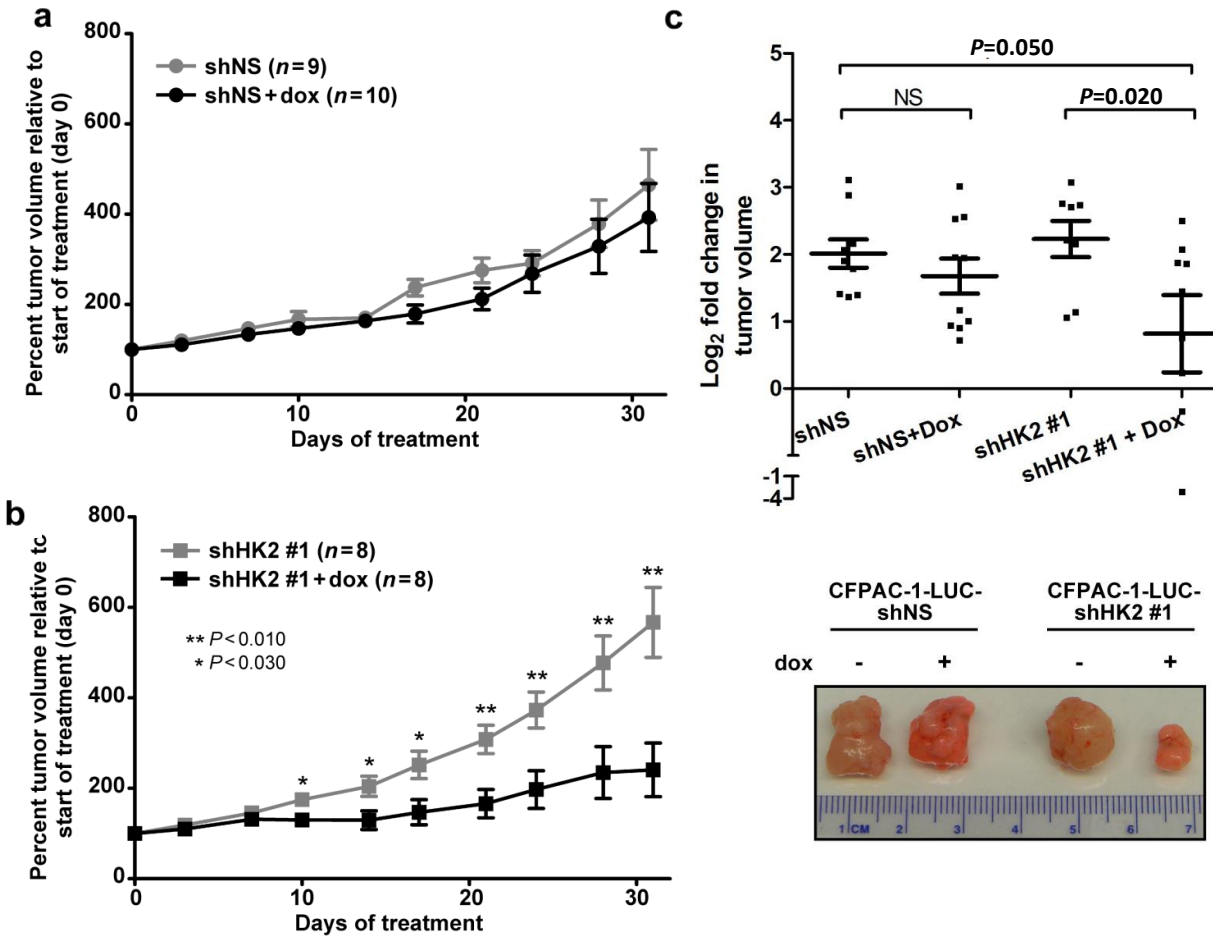



**Figure 2-14: HK2 is required for PDAC primary tumor growth *in vivo*.**
(**a**) Percent tumor volume relative to start of treatment for shNS tumors treated with sucrose (black, n=9) or doxycycline (grey, n=10) for 30 days. Average normalized tumor volume and SEM shown.
(**b**) Percent tumor volume relative to start of treatment for shHK2 tumors treated with sucrose (black, n=8) or doxycycline (grey, n=8) for 30 days. Average normalized tumor volume and SEM shown with student's t-tests for statistical significance at each time point.
(**c**) Statistical analysis of final tumor volumes for the four treatment groups. ANOVA analysis compared all four groups while t-tests compared treatment vs. control for shNS and shHK2#1. Each dot represents a single tumor and mean ± SEM shown. Representative images of tumors isolated after 30 days of treatment with sucrose or doxycycline.

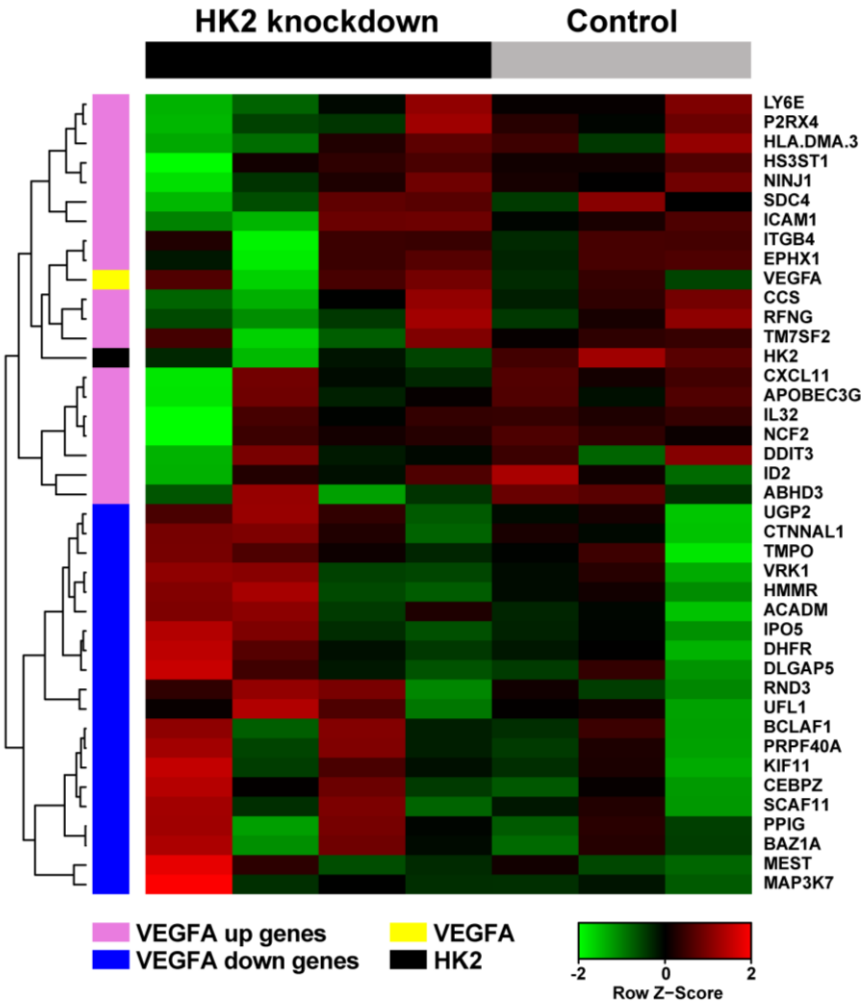



**Figure 2-15: Expression of genes regulated by VEGF-A expression in HK2 knockdown and control CFAPC-1-LUC xenografts.**

Heat map of the expression of the top 20 ranked genes from the gene lists by Schoenfeld, et al, 2004, including HK2 and VEGF-A, for shNS (control) and shHK2#1 (HK2 knockdown) tumors isolated at the end of treatment as determined by RNA-sequencing.

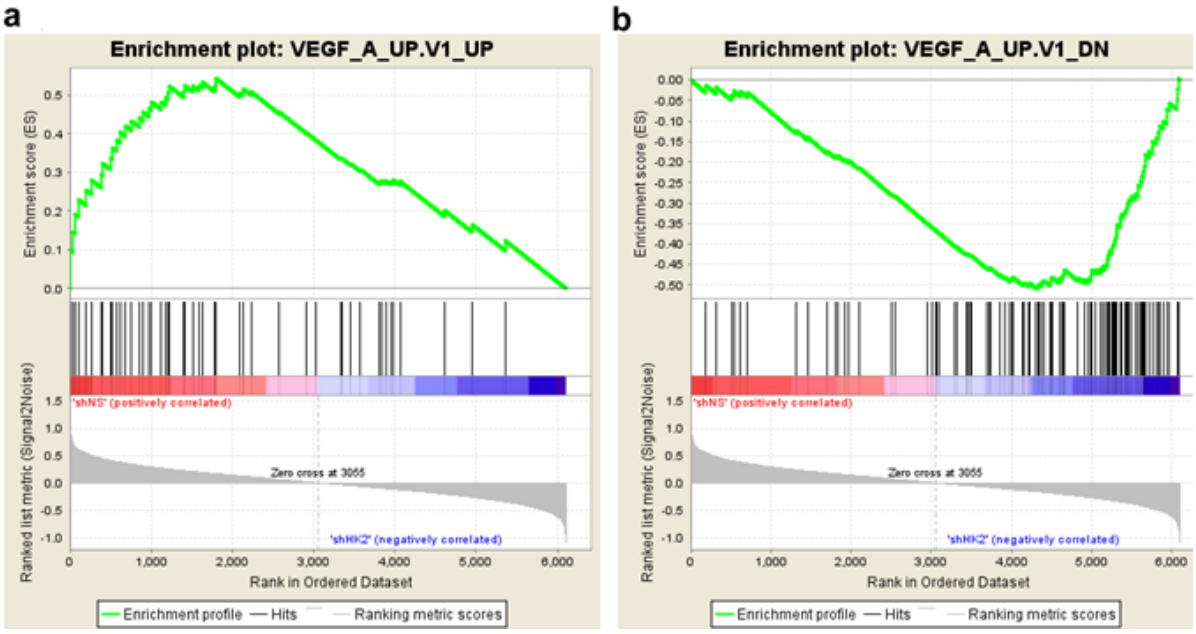



**Figure 2-16: GSEA enrichment plots for VEGF-A signaling**

(**a**) Enrichment plot for the genes identified by Schoenfield et al (2004) to be upregulated with ectopic expression of VEGF-A in human cell lines. This gene list is significantly enriched in control tumors relative to HK2 knockdown ($p<0.001$, FDR<0.001).

(**b**) Enrichment plot for the genes identified by Schoenfield et al (2004) to be downregulated with ectopic expression of VEGF-A in human cell lines. This gene list is significantly enriched in tumors containing HK2 knockdown relative to control ($p<0.001$, FDR<0.001).

| Gene List | # genes in list | NES | P-value | FDR |
|---|---|---|---|---|
| HALLMARK_INTERFERON_GAMMA_RESPONSE | 133 | 2.61 | P <0.001 | 0 |
| HALLMARK_INTERFERON_ALPHA_RESPONSE | 86 | 2.47 | P <0.001 | 0 |
| IL21_UP.V1_UP | 29 | 2.17 | P <0.001 | 3.11E-04 |
| VEGF_A_UP.V1_UP | 50 | 2.18 | P <0.001 | 4.15E-04 |
| KRAS.KIDNEY_UP.V1_DN | 27 | 2.11 | P <0.001 | 0.001 |
| ALK_DN.V1_DN | 31 | 1.97 | P <0.001 | 0.004 |
| HALLMARK_IL6_JAK_STAT3_SIGNALING | 37 | 1.98 | P <0.001 | 0.005 |
| SINGH_KRAS_DEPENDENCY_SIGNATURE_ | 17 | 1.94 | P <0.001 | 0.006 |
| HALLMARK_COAGULATION | 53 | 1.89 | 0.002 | 0.009 |
| SRC_UP.V1_UP | 35 | 1.82 | 0.002 | 0.017 |
| PKCA_DN.V1_UP | 27 | 1.79 | 0.000 | 0.020 |
| KRAS.LUNG_UP.V1_DN | 25 | 1.77 | 0.004 | 0.025 |
| HALLMARK_P53_PATHWAY | 117 | 1.73 | 0.000 | 0.032 |
| WNT_UP.V1_UP | 44 | 1.73 | 0.002 | 0.034 |
| HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY | 35 | 1.73 | 0.006 | 0.035 |
| KRAS.300_UP.V1_DN | 23 | 1.74 | 0.008 | 0.036 |
| BRCA1_DN.V1_DN | 19 | 1.70 | 0.004 | 0.037 |
| IL21_UP.V1_DN | 22 | 1.69 | 0.015 | 0.038 |
| HALLMARK_TNFA_SIGNALING_VIA_NFKB | 118 | 1.67 | 0.002 | 0.045 |
| KRAS.BREAST_UP.V1_UP | 26 | 1.60 | 0.018 | 0.075 |
| ATF2_UP.V1_UP | 43 | 1.58 | 0.019 | 0.082 |
| GCNP_SHH_UP_LATE.V1_DN | 69 | 1.54 | 0.006 | 0.092 |
| STK33_SKM_UP | 93 | 1.53 | 0.012 | 0.094 |
| ESC_J1_UP_EARLY.V1_DN | 73 | 1.54 | 0.014 | 0.095 |
| PRC1_BMI_UP.V1_UP | 27 | 1.54 | 0.033 | 0.097 |
| MEL18_DN.V1_DN | 53 | 1.55 | 0.014 | 0.097 |
| IL2_UP.V1_UP | 49 | 1.54 | 0.016 | 0.098 |

**Table 2-1: Gene sets enriched in shNS (n=3) relative to HK2 knockdown tumors (n=4).** This includes gene sets found to have a P<0.020 and an FDR<0.100. Gene sets analyzed were contained in the MSigDBv5 hallmark and oncogenic gene signature lists (Subramanian et al., 2005).

| Gene List | # genes in list | NES | P value | FDR |
|---|---|---|---|---|
| HALLMARK_MYC_TARGETS_V1 | 195 | -2.51 | P <0.001 | 0.000 |
| HALLMARK_E2F_TARGETS | 144 | -2.35 | P <0.001 | 0.000 |
| VEGF_A_UP.V1_DN | 108 | -2.34 | P <0.001 | 0.000 |
| HALLMARK_SPERMATOGENESIS | 27 | -2.30 | P <0.001 | 0.000 |
| PIGF_UP.V1_UP | 93 | -2.27 | P <0.001 | 0.000 |
| GCNP_SHH_UP_LATE.V1_UP | 98 | -2.14 | P <0.001 | 0.001 |
| HALLMARK_G2M_CHECKPOINT | 131 | -2.09 | P <0.001 | 0.001 |
| CSR_EARLY_UP.V1_UP | 90 | -1.90 | P <0.001 | 0.007 |
| ERB2_UP.V1_DN | 100 | -1.84 | P <0.001 | 0.011 |
| GCNP_SHH_UP_EARLY.V1_UP | 87 | -1.74 | P <0.001 | 0.025 |
| HALLMARK_MTORC1_SIGNALING | 168 | -1.69 | P <0.001 | 0.032 |
| TBK1.DF_DN | 146 | -1.62 | P <0.001 | 0.053 |
| CORDENONSI_YAP_CONSERVED_SIGNATURE | 35 | -1.75 | 0.002 | 0.025 |
| HALLMARK_MITOTIC_SPINDLE | 100 | -1.70 | 0.002 | 0.031 |
| HALLMARK_MYC_TARGETS_V2 | 42 | -1.66 | 0.004 | 0.038 |
| E2F1_UP.V1_UP | 90 | -1.55 | 0.006 | 0.076 |
| CSR_LATE_UP.V1_UP | 109 | -1.59 | 0.010 | 0.058 |
| PRC2_EZH2_UP.V1_UP | 51 | -1.61 | 0.014 | 0.053 |

**Table 2-2: Gene sets enriched in with HK2 knockdown (*n*=4) relative to shNS (*n*=3).** This includes gene sets found to have a P<0.020 and an FDR<0.100. Gene sets analyzed were contained in the MSigDBv5 hallmark and oncogenic gene signature lists (Subramanian et al., 2005).

**HK2 is required for PDAC metastasis in vivo**

To examine the requirement of HK2 for the promotion of metastasis, a tail vein assay was used (Elkin and Vlodavsky, 2001). CFPAC-1-LUC cells containing inducible shRNA constructs were treated with doxycycline prior to tail vein injection to induce shRNA expression (Figure 2-3a). Bioluminescence was used to monitor development of lung metastases and quantify tumor growth (Figure 2-17a). The average bioluminescence measured in the lungs of shNS injected mice was approximately 100-fold higher than that observed for the shHK2#1 injected mice ($P$=0.001, Figure 2-17b). The presence of metastatic disease was observed in 7 out of 7 mice injected with the shNS control compared to 3 out of 9 mice injected with shHK2#1 cells ($P$=0.011, Figure 2-17c). The presence of metastatic tumors was confirmed using hematoxylin and eosin (H&E) staining of sectioned lung tissue obtained from mice injected with shNS (Figure 2-18, top row). No histological evidence of tumor formation was observed with injection of HK2 knockdown cells (Figure 2-18, bottom row). These results show a requirement for HK2 in the extravasation and survival of cancer cells at distant organ sites, confirming an important role for HK2 in promoting metastasis.

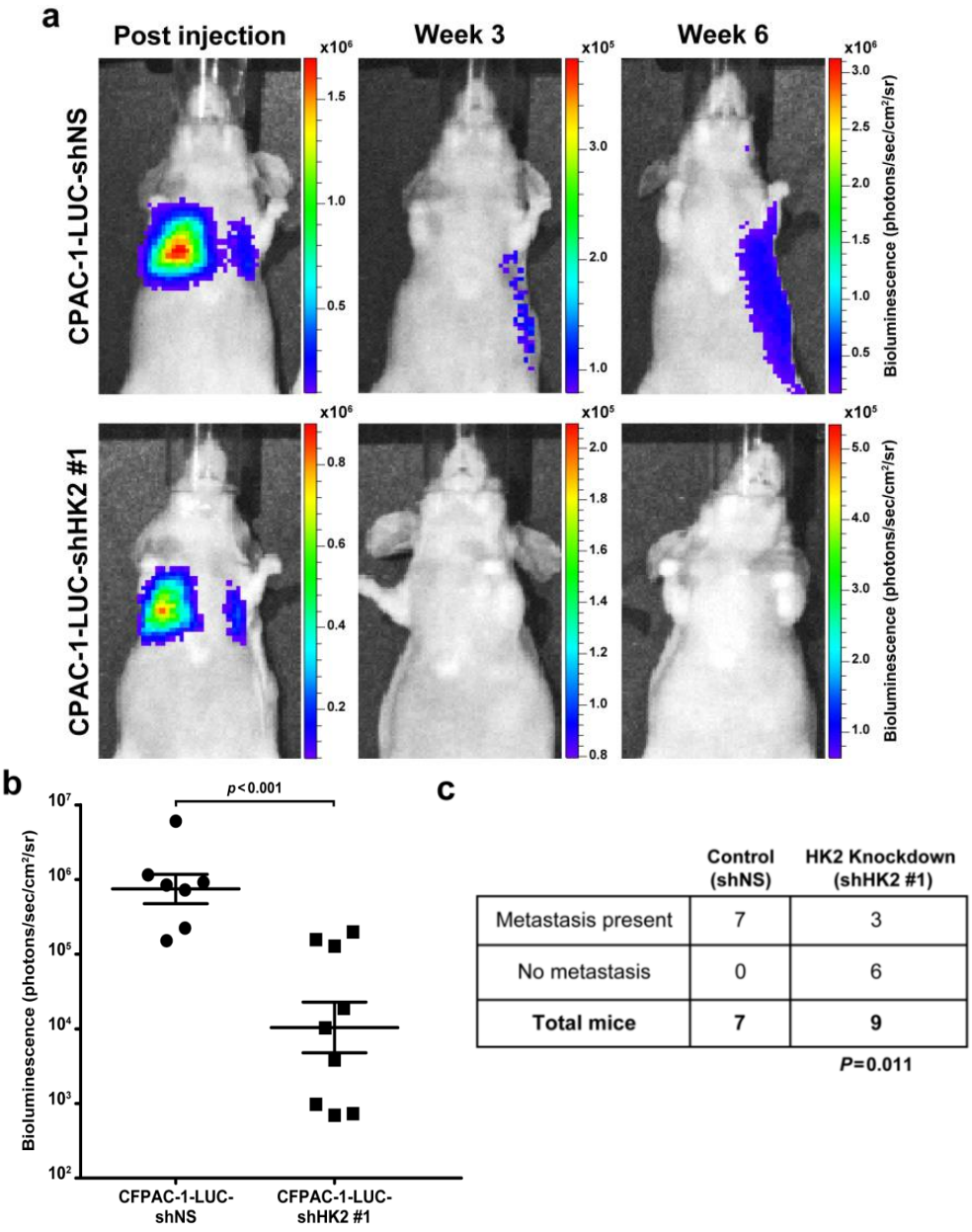



**Figure 2-17: HK2 is required for metastasis in PDAC.**

(**a**) Bioluminescence of mice injected with CFPAC-1-LUC shNS cells (top row) and cells with HK2 knockdown (CFPAC-1-shHK2 #1, bottom row) at the start of the study (column one) and at the end of the study (column 3).

(**b**) Bioluminescence measured for tumors observed in lungs obtained after autopsy. Each point represents luminescence of tumor foci (shNS, n=7 and shHK2, n=3) or an entire lung if no foci were observed (shHK2, n=6).

(**c**) Fisher's exact t-test showing a significant difference in formation of metastases with HK2 knockdown (P=0.011).

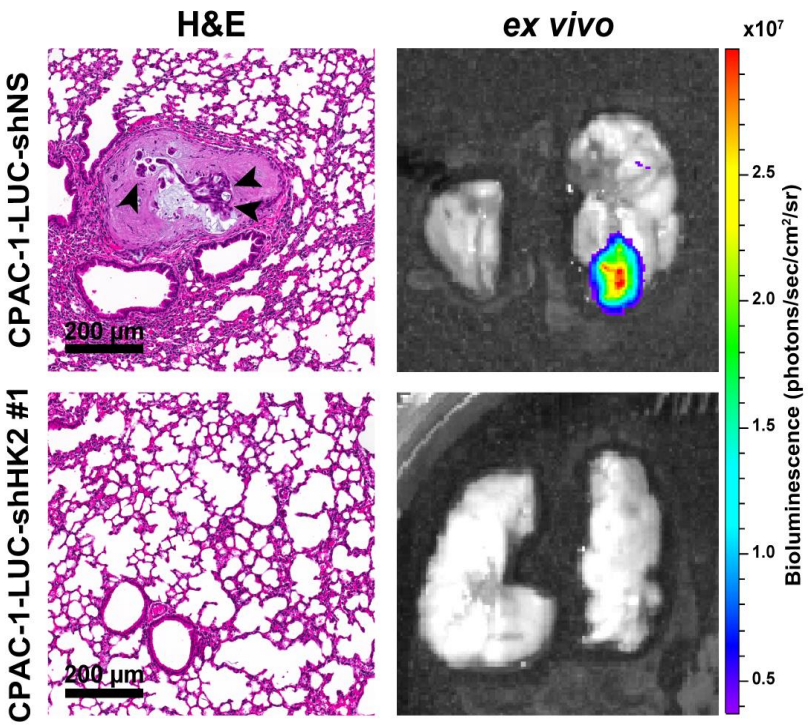



**Figure 2-18: Histological evidence of tumor formation in mice injected with control PDAC cell lines (shNS).**
Representative ex vivo images used for quantification of bioluminescence. H&E staining was used to confirm metastases formation. Arrowheads point to cancer cells in a metastatic lesion with surrounding normal lung tissue (top row). Lungs that did not exhibit bioluminescence showed no histological evidence of metastases (bottom row).

## DISCUSSION

PDAC is a highly lethal disease with an increased incidence of metastasis and an overall poor prognosis (Ryan et al., 2014). Similar to a GEMM which showed genes regulating glucose metabolism to be upregulated in PDAC (Ying et al., 2012), we saw increased expression of key glycolytic genes, including GLUT1, HK2 and LDHA, in primary PDAC tumors relative to normal pancreas, suggesting a role for these genes in human tumorigenesis. We show that knockdown of HK2 results in decreased PDAC growth *in vitro* and *in vivo*, in agreement with

what has been observed in other cancers (Gershon et al., 2013, Patra et al., 2013, Wolf et al., 2011, Yoshino et al., 2013). HK2 regulates glucose uptake, a process upstream of metabolic pathways including glycolysis, hexosamine biosynthesis, the pentose phosphate pathway and the citric acid cycle (Bryant et al., 2014, Ying et al., 2012). Direct targeting of HK2 can, therefore, impede the flow of glucose into multiple downstream pathways necessary for KRAS driven tumor growth (Daemen et al., 2015, Ying et al., 2012). Genetic deletion of HK2 in a preclinical model of Kras driven lung cancer was sufficient to alter glucose metabolism and improve overall survival (Patra et al., 2013), suggesting that direct targeting of HK2 would be beneficial in PDAC.

We also show that increased expression of HK2 in primary tumors is associated with shorter overall survival in PDAC patients undergoing curative surgery, in agreement with a smaller study that correlated increased HK2 protein expression with worse PDAC patient outcomes (Ogawa et al., 2015). This, along with the observation that HK2 is upregulated in PDAC metastases relative to primary tumors, supports a role for HK2 in the metastatic process. Associations between increased HK2 expression, metastatic disease, and poor clinical outcomes have been observed in other cancers however a direct link between HK2 and metastasis has not been shown (Hamabe et al., 2014, Ogawa et al., 2015, Palmieri et al., 2009).

Here we provide direct evidence that HK2 is necessary and sufficient to promote metastasis in PDAC, as its increased expression promotes invasion and its knockdown inhibits cancer cell extravasation and colonization at distant organ sites, important components of the metastatic cascade (Lee et al., 2015, Zetter, 1998). A study of PDAC cell lines correlated increased rates of glycolysis with aggressive tumor biology, suggesting that glycolysis may be important for metastasis (Daemen et al., 2015). We found that HK2 directly promotes metastasis

via regulation of glycolysis, as pharmacologic inhibition of lactate production prevents HK2-driven invasion and extracellular lactate is sufficient to enhance invasion. This result is in agreement with previous studies showing extracellular lactate enhances migration of breast cancer cell lines, encourages metastases seeding of breast cancer cell lines *in vivo* and promotes motility of glioblastoma cell lines (Baumann et al., 2009, Bonuccelli et al., 2010). As increased lactate production has also been linked to poor patient outcomes (Bonuccelli et al., 2010, Brizel et al., 2001, Daemen et al., 2015, Xian et al., 2015) we suggest that direct targeting of HK2 or inhibition of glycolysis may improve patient outcomes by limiting the formation of metastases.

Increased rates of glycolysis can promote invasion by altering the pH of the tumor microenvironment, enhancing cell signaling, influencing matrix metalloprotease activity and regulating gene expression (Baumann et al., 2009, Bonuccelli et al., 2010, Kato et al., 2005, Latham et al., 2012, Martinez-Outschoorn et al., 2011, Xu et al., 2002). We show that genes involved in VEGF-A signaling, a pathway important for angiogenesis and metastasis (Zetter, 1998), are significantly altered by HK2 knockdown. While a direct link between HK2 and VEGF-A was not assessed here, others have shown that lactate production influences VEGF-A signaling (Shi et al., 2001, Sonveaux et al., 2012, Xu et al., 2002). We show that extracellular lactate enhances PDAC cell invasion when present at concentrations known to influence gene expression (Latham et al., 2012). We hypothesize that HK2 regulates lactate production and this, in turn, promotes VEGF-A signaling and changes in gene expression necessary for metastasis. While our data supports this hypothesis, more studies are needed to confirm the connection between HK2 and VEGF-A.

In conclusion the present study supports a requirement for HK2 in PDAC tumorigenesis and metastasis that helps explain the associated findings of high HK2 expression in PDAC

patients with metastatic disease. We show that HK2 influences the invasive potential of PDAC cells by directly regulating glycolysis and that its knockdown induces changes in gene expression of pathways important for promoting metastasis, including VEGF-A signaling. Our data suggests that the targeting of HK2 may be a promising approach for treating metastatic disease.

## MATERIALS AND METHODS

*Cell culture and stable cell line generation*

Human cell lines CFPAC-1, HPAF-II, Hs 766T, T3M4, and PANC-1 were obtained from the American Type Culture Collection and authenticated via short–tandem repeat profiling (Genetica, Burlington, NC, USA). The HPNE cell line was described previously (Neel et al., 2014). CFPAC-1 was cultured in RPMI 1640, CFPAC-1-LUC was cultured in RPMI 1640 with geneticin (500 µg/mL, Invitrogen, Grand Island, NY, USA), and PANC-1 was cultured in DMEM. All were supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin (Invitrogen) and incubated at 37 °C in 5% CO2 atmosphere. For HK2 overexpression, the HK2 cDNA sequence from the pDONR-223-HK2 donor vector (Johannessen et al., 2010) (Addgene #23854) was cloned into a pHAGE puro destination vector (donated by the laboratory of William Kim, MD) using LR-clonase reaction as per the manufacturer's instructions (ThermoFisher Scientific #11791, Grand Island, NY, USA). For HK2 knockdown cell lines, shRNA sequences (below) were cloned into the pTRIPZ plasmid using EcoR1 and Xho1 restriction enzyme digestion of the pTRIPZ-shNS vector (donated by the laboratory of Channing Der, PhD). All constructs were verified by Sanger sequencing (Eton Biosciences, Research Triangle Park, NC, USA).

shHK2#1: CCGTAACATTCTCATCGATTT

shHK2#2: GCTACAAATCAAAGACAAGAA

A replication-incompetent lentivirus was generated in 293T cells using psPAX2 (Gag, Pol, Rev, Tat), pMD2.G (VSV-G), and target vector. For transduction, $1\times10^6$ cells were seeded in 100 mm plates with media containing lentivirus and polybrene (8 µg/mL, Invitrogen). After 24 hours, infected cells were selected with puromycin (2 µg/mL, Invitrogen). To induce shRNA

expression in pTRIPZ lines, cells were incubated for 72 hours with media containing 2 µg/mL doxycycline (Sigma-Aldrich, St. Lois, MO, USA).

*Transient knockdown with siRNA*

Reverse transfection in a six-well plate was performed with Lipofectamine RNAiMax (Invitrogen) as per the manufacturer's guidelines. 4-5x$10^5$ cells were seeded per well and a final concentration of 20 nM siNS (#4404021, ThermoFisher Scientific) or siHK2 (Catalog #S6560, Applied Biosystems, Grand Island, NY, USA) was used. Cells were incubated for 48 hours prior to use in assay or western blot.

*Western blot*

Samples were lysed in 200 µL RIPA buffer (pH 7.4) containing protease inhibitors (ThermoFisher Scientific). 20-25 µg protein suspended in SDS loading buffer was run on 10% SDS polyacrylamide gels and electro transferred to PVDF membranes. Membranes were blocked in 5% milk and incubated with 1:1 000 dilutions of primary antibodies in 5% BSA, including anti-HK1 (sc-#46695, Santa Cruz Biotechnology, Dallas, TX, USA), anti-HK2 (Catalog #2867, Cell Signaling Technology, Danvers, MA, USA), and the loading controls anti-β-Actin (sc-#47778, Santa Cruz Biotechnology) and anti-vinculin (Catalog #V9131, Sigma). Membranes were incubated with 1: 5000 dilutions of appropriate secondary antibodies in 5% milk (ThermoFisher Scientific). Incubations were for 1 hour at room temperature and Clarity Western ECL substrate with ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA) were used to detect immunoreactive bands.

*Anchorage independent growth*

1-2 x$10^4$ cells were seeded into a soft agar assay as was previously outlined in Martin et al (Martin et al., 2011). Briefly a six well plate was coated with 2 mL of a 0.6% bacto-agar and culture medium mixture. 500 µL of a cell and 0.4% bacto-agar mixture was added after solidification of the first layer. 300 µL media was added every four days for two-three weeks. If cells were treated with doxycycline prior to seeding, administration of media with doxycycline (2 µg/mL) continued. Colony growth was using Image J software (NIH, Bethesda, Maryland, USA). Percent growth was calculated by dividing the number of colonies observed by the average number of colonies in the corresponding control.

*Transwell invasion*

Uncoated inserts with 8-µm pores (Catalog #82050, Greiner Bio-One, Monroe, NC, USA) were coated with 100 µL of a diluted growth factor reduced Matrigel membrane matrix (300 µg/mL, ThermoFisher Scientific) and incubated at 37 $^oC$ for 2 hours. Coated inserts were then placed into a 24 well plate containing 750 µL normal culture media. 1-2 x$10^5$ cells were suspended in 250 µL media supplemented with 1% FBS and seeded into the upper chamber of insert. Cells invaded for 16 hours. Inserts were then cleaned, fixed, and stained with Diff Quik as per manufacturer's instructions (ThermoFisher Scientific). The number of cells invading was determined by counting five random fields per insert (counted by a blinded second party). Percent invasion was calculated by dividing the total number of cells invaded by the average number of cells invaded for the appropriate control.

*Hexokinase activity and lactate production*

To examine hexokinase activity a colorimetric assay was performed as per manufacturer's instructions (Hexokinase activity kit, Catalog #MAK091, Sigma). Sample preparation included lysis of $1x10^6$ cells in assay buffer, with a 1:10 dilution of lysate used in assay. A glycolysis cell-based assay was performed to measure l-lactate production as per manufacturer's instructions (Catalog #600450, Caymen Chemical, Ann Arbor, MI, USA). $1x10^4$ cells in 200 µL were seeded per well in a 96-well plate for 24 hours. 20 µL of medium was collected into a new 96 well plate for colorimetric detection. Absorbance at appropriate wavelengths was measured with a Synergy 2 microplate reader (BioTek, Winooski, VT, USA). Percent hexokinase activity and lactate production were determined by dividing the corrected absorbance reading for each replicate by the average corrected absorbance for the appropriate control.

*Cell proliferation*

To examine cell proliferation, $1x10^3$ cells in 200 µl were plated in quadruplicate into 96-well plates. After 24, 48, 72, or 96 hours of growth, 50 µl of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in PBS was added to each well. After 30 minutes the mixture was aspirated and 200 µl of dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly. $A_{560nm}$ was measured using a Synergy 2 microplate reader (BioTek). For IC50 determination $1x10^3$ cells in 200 µl were plated in quadruplicate into 96-well plates. After 24 hours, the medium was replaced with medium containing 75 mM PBS or oxamate (75 mM to 0.1 mM, Sigma). After 72 hour incubation, 50 µl MTT was added to each well and incubated for 30 minutes. The mixture was aspirated and 200 µl of DMSO was added to each

well, mixed thoroughly, and $A_{560\ nm}$ was measured using a Synergy 2 microplate reader. The IC50 was calculated using GraphPad Prism software (v5, GraphPad Software, INC. La Jolla, CA, USA).

*Gene Expression*

*qPCR*: RNA was isolated from CFPAC-1, HPNE, HPAF-II, Hs 766T, T3M4, and PANC-1 cell pellets using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). 2 µg of RNA was used for cDNA synthesis (Applied Biosystems) and 50 ng of RNA was used for real-time PCR (Applied Biosystems). Reactions were performed in triplicate on a 384 well plate using standard PCR settings on a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). HK2 expression was assessed with HK2 TaqMan qPCR array (Applied Biosystems, Hs00606086_m1) while β-actin was assessed with ACTB TaqMan qPCR array (Applied Biosystems, Hs01060665_g1). The ΔΔCT method was used for analysis.

*RNA sequencing*: 200-1000 ng of total RNA was used to prepare libraries with the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA). 75 base pair paired-end reads were sequenced on a NextSeq 500 Desktop Sequencer using a high output flow cell kit (Illumina). Reads were separated by species of origin using Xenome (Conway et al., 2012). Human specific reads were then aligned and quantified using Tophat2 (Kim et al., 2013), Cufflinks (Trapnell et al., 2012), hg19, mm10, and the UCSC transcript and gene definitions (genome.ucsc.edu). mRNA gene expression was analyzed with javaGSEAv2.2.1, and MSigDBv5.0 (Subramanian et al., 2005).

*Animal Studies:* All mouse studies were completed under protocols approved by the UNC Institutional Animal Care and Use Committee.

*Subcutaneous tumor injection*: $2x10^6$ CFPAC-1-LUC shRNA cells were subcutaneously injected into the flanks of 6-8 week old female nu/nu mice. When tumors grew to a mean of 152 $mm^3$ (SD 45.5 $mm^3$) mice were randomized and given either 2.5% sucrose or 2.5% sucrose + 1 mg/mL doxycycline in drinking water for the specified time period. Tumor volume was measured three times per week and calculated using the formula $(length \times width^2)/2$. Student's t-tests compared the growth of treatment versus control during the study and a one-way ANOVA with Bonferroni correction for multiple comparisons test determined statistical significance of final tumor volume.

*Tail vein injection*: CFPAC-1-LUC shNS and CFPAC-1-LUC shHK2#1 cells incubated with culture medium containing 2 µg/mL doxycycline for 72 hours prior to injection. Treatment continued via administration of a 2.5% sucrose + 1mg/mL doxycycline throughout the study. $2x10^6$ cells in 100 µL PBS were injected into the tail veins of 6-8 week old female nu/nu mice as previously described in Elkin, et al (Elkin and Vlodavsky, 2001). Mice were monitored for lung metastases weekly after the initial injection using an IVIS Lumina Kinetic optical imaging system with an EMCCD camera (PerkinElmer, Waltham, MA, USA). Lungs were collected upon autopsy, fixed in 10% formalin, paraffin embedded (FFPE), and sectioned into 10 µM slices at 100 µM intervals and stained with H&E. Fisher's exact t-test determined statistical significance.

# CHAPTER 3 : MICRO RNA REGULATION OF TUMOR GROWTH AND METASTASIS BY INHIBITION OF HEXOKINASE 2 IN PANCREATIC CANCER[2]

## INTRODUCTION

In chapter 2, we showed the metabolic enzyme hexokinase 2 (HK2) to be necessary and sufficient for promoting tumor growth and metastasis in pancreatic ductal adenocarcinoma (PDAC). This ability of HK2 to act as an oncogene in PDAC suggests that is regulation is critically important for PDAC disease progression. A variety of mechanisms are known to regulate HK2 expression, including hypoxia, low glucose, and oncogenic Kras activity (Natsuizaka et al., 2007, Ying et al., 2012). More recently microRNAs (miRNAs) have been shown to be important regulators of HK2 (Fang et al., 2012, Jiang et al., 2012, Yoshino et al., 2013), although their contribution to PDAC tumorigenesis by regulating HK2 has not yet been investigated.

miRNAs are short, non-coding RNA molecules that are endogenously expressed and function as negative regulators of gene expression (Bartel and Chen, 2004). miRNAs bind in a sequence dependent manner to the 3' untranslated region (3'UTR) of target mRNAs and this interaction results in the recruitment of proteins required for mRNA degradation and/or

[2] The concept of this project was developed by myself and Jen Jen Yeh. I completed the *in vitro* experiments found in this chapter, while Jannette Baren-Gale in the laboratory of Praveen Sethupathy completed the analysis of miRNA sequencing data.

inhibition of translation (Bartel and Chen, 2004, Huntzinger and Izaurralde, 2011). A single miRNA has the ability to target many different mRNAs and can regulate multiple biological processes important for cell growth and survival, including glucose metabolism (Singh et al., 2012). miRNAs are of growing interest to the field of cancer research because they are often dysregulated in tumor tissue and may be useful for the diagnosis or treatment of cancer (Ruan et al., 2009).

As discussed above, miRNA regulation of HK2 has been shown to be important for tumorigenesis. More specifically, miR-143, a miRNA down regulated in *Kras*-mutant lung tumors, inhibits tumor growth in lung cancer by negatively regulating HK2 (Fang et al., 2012). As similar regulatory relationship between miR-143 and HK2 has been observed in other cancer types, suggesting that miRNA regulation of HK2 is important for inhibiting tumorigenesis (Fang et al., 2012, Jiang et al., 2012, Yoshino et al., 2013). While miRNA regulation of HK2 in PDAC has not been studied, we hypothesize that it is critically important for inhibiting both primary tumor growth and metastasis in PDAC, two processes that require HK2. miRNAs regulating HK2 expression in PDAC may also be therapeutically important, as miRNA mimics may represent a novel approach to targeting HK2 in the clinic.

The current study sought to identify novel miRNAs regulating HK2 expression in PDAC. We observed miR-148a and miR-216b, miRNAs known to be downregulated in PDAC precursor lesions and primary tumors, to be negatively associated with HK2 expression. We showed these miRNAs to be important regulators of HK2 expression, anchorage independent growth (AIG), and invasion in PDAC cell lines. Our preliminary data supports a potential role for miR-148a and miR-216b as tumor suppressors in PDAC by regulating HK2 expression.

## RESULTS

### Identification of novel miRNAs regulating HK2 in PDAC

To identify miRNAs that regulate HK2 in PDAC, miRNA and mRNA expression in 58 primary PDAC tumors and 3 normal, tumor adjacent tissues were examined (the Cancer Genome Atlas, http://cancergenome.nih.gov). Because a direct interaction between a miRNA and its target mRNA results in decreased levels of target mRNA (Huntzinger and Izaurralde, 2011), we looked for miRNAs that are negatively correlated with HK2 expression in PDAC tumors. Seventy two miRNAs were negatively correlated with HK2 expression (Pearson correlation<-0.25, Figure 3-1), suggesting that they may inhibit HK2 in PDAC. To further narrow down the list of candidate miRNAs, we examined miRNA expression in PDAC tumor tissue relative to normal pancreas. Because HK2 is upregulated in PDAC (Figure 2-1), we looked for miRNAs that were downregulated in PDAC tissue relative to normal adjacent tissue. Of the 72 miRNAs negatively correlated with HK2, 49 miRNAs were downregulated in PDAC tumors relative to normal adjacent tissue (Figure 3-1).

Target prediction software was used to examine which of the 49 downregulated miRNAs were predicted to directly interact with the 3'UTR of HK2, an interaction essential for miRNA regulation of a target mRNA (Lewis et al., 2005). To limit the number of false positive miRNA-mRNA interactions identified, only miRNA-mRNA interactions predicted by at least 2 of 3 target prediction algorithms used in this analysis were considered to be a "true" prediction. TargetScan V6.2 (Agarwal et al., 2015), miRanda (Betel et al., 2008), and miRWalk (Dweep et al., 2011) were the three target predication algorithms used. Twenty four of the 49 miRNAs examined were predicted to interact with the 3'UTR of HK2 by at least 2 of the 3 algorithms (Figure 3-1). The resulting list of candidate miRNAs included those miRNAs which were

negatively correlated with HK2, downregulated in PDAC tumor tissue relative to normal pancreas, and predicted to interact to the 3'UTR of HK2 (Table 3-1).

Interestingly miR-143, a miRNA previously shown to be important in regulation of HK2 in other cancer types was not found to be negatively correlated with HK2 expression and was upregulated in patient tumors relative to normal pancreas (Bloomston et al., 2007), suggesting that this miRNA may not function as a tumor suppressor by directly regulating HK2 in PDAC. Included in the list are two miRNAs, miR-148a and miR-261b, previously shown to be diagnostic of PDAC and downregulated in PDAC tumors relative to normal pancreas in larger datasets (Bloomston et al., 2007, Schultz et al., 2012, Xue et al., 2013). Downregulation of miR-148a and miR-216b was an early event in PDAC tumorigenesis (Hanoun et al., 2010, Yu et al., 2012), further suggesting an important role for these miRNAs in driving PDAC disease progression. Because multiple studies showed an association between miR-148a and miR-216b and tumor development PDAC, we evaluated these two novel candidate miRNAs for their ability to inhibit tumor growth in PDAC by negatively regulating HK2 expression.

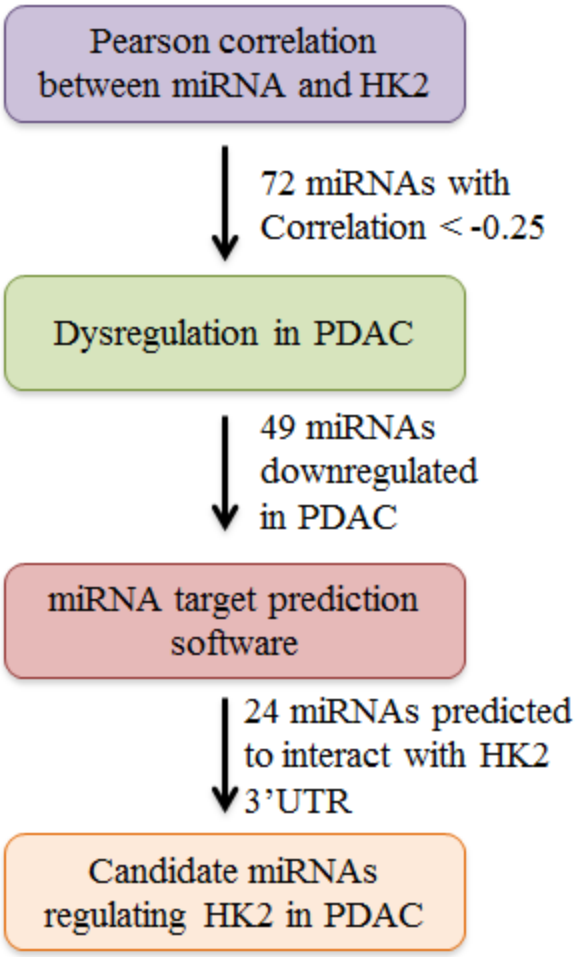



**Figure 3-1: Scheme for identification of novel miRNA regulators of HK2 in PDAC**
miRNA and mRNA expression from the Cancer Genome Atlas dataset for PDAC, which contained gene expression from 58 PDAC tumor samples and 3 normal adjacent tumor tissues, was used to identify 24 novel candidate miRNAs regulating HK2 expression in PDAC.

| | Stem-loop | Correlation with HK2 Expression | $Log_2$ fold change | P value for fold change | miRanda | miRWalk | TargetScan |
|---|---|---|---|---|---|---|---|
| **hsa-mir-140-3p** | hsa-mir-140 | -0.506 | -0.650 | 0.821 | Yes | Yes | Yes |
| **hsa-mir-381** | hsa-mir-381 | -0.492 | -0.336 | 0.803 | Yes | Yes | Yes |
| **hsa-mir-140-5p** | hsa-mir-140 | -0.438 | -1.721 | 0.035 | Yes | Yes | Yes |
| **hsa-mir-218-1-5p** | hsa-mir-218-1 | -0.430 | -0.839 | 0.300 | Yes | Yes | Yes |
| **hsa-mir-218-2-5p** | hsa-mir-218-2 | -0.429 | -0.839 | 0.295 | Yes | Yes | Yes |
| **hsa-mir-204-5p** | hsa-mir-204 | -0.413 | -1.712 | 0.615 | Yes | Yes | Yes |
| **hsa-mir-136-5p** | hsa-mir-136 | -0.410 | -1.633 | 0.091 | Yes | - | Yes |
| **hsa-mir-195-5p** | hsa-mir-195 | -0.370 | -1.250 | 0.345 | Yes | Yes | Yes |
| **hsa-mir-150-5p** | hsa-mir-150 | -0.354 | -2.368 | 0.445 | Yes | Yes | Yes |
| **hsa-mir-98** | hsa-mir-98 | -0.354 | -0.166 | 0.749 | Yes | Yes | Yes |
| **hsa-mir-30e-5p** | hsa-mir-30e | -0.346 | -1.515 | 0.056 | Yes | Yes | - |
| **hsa-mir-501-3p** | hsa-mir-501 | -0.337 | -1.187 | 0.126 | Yes | Yes | Yes |
| **hsa-mir-148a-3p** | hsa-mir-148a | -0.330 | -0.413 | 0.384 | Yes | Yes | Yes |
| **hsa-mir-199b-3p** | hsa-mir-199b | -0.327 | -0.182 | 0.892 | Yes | Yes | Yes |
| **hsa-mir-199a-1-3p** | hsa-mir-199a-1 | -0.327 | -0.182 | 0.893 | Yes | Yes | Yes |
| **hsa-mir-199a-2-3p** | hsa-mir-199a-2 | -0.326 | -0.187 | 0.906 | Yes | Yes | Yes |
| **hsa-mir-148b-3p** | hsa-mir-148b | -0.314 | -0.726 | 0.130 | Yes | Yes | Yes |
| **hsa-mir-134** | hsa-mir-134 | -0.288 | -0.584 | 0.242 | Yes | - | Yes |
| **hsa-mir-30e-3p** | hsa-mir-30e | -0.278 | -1.023 | 0.137 | Yes | Yes | - |
| **hsa-mir-216a** | hsa-mir-216a | -0.273 | -0.362 | 0.313 | Yes | Yes | Yes |
| **hsa-mir-185-5p** | hsa-mir-185 | -0.272 | -0.858 | 0.003 | Yes | Yes | Yes |
| **hsa-mir-216b** | hsa-mir-216b | -0.272 | -1.038 | 0.212 | Yes | Yes | Yes |
| **hsa-mir-217** | hsa-mir-217 | -0.271 | -0.476 | 0.936 | Yes | - | Yes |
| **hsa-mir-497-5p** | hsa-mir-497 | -0.271 | -0.872 | 0.357 | Yes | Yes | Yes |

**Table 3-1: 24 candidate miRNAs that are predicted to interact with the 3'UTR of HK2, negatively correlated with HK2 expression, and downregulated in PDAC tumor samples.**

**miR-148a and miR-216b directly interact with the 3'UTR of HK2**

To determine if miR-148a and miR-216b directly interacted with the 3'UTR of HK2, the entire 3'UTR sequence of HK2 was inserted into a luciferase reporter construct. The ability of a miRNA to suppress luciferase activity in cells containing this reporter construct suggests a direct interaction between a target miRNA and the 3'UTR of HK2. miR-143, a miRNA known to directly interact with the 3'UTR of HK2, was used as a positive control and caused a 42.4% decrease in luciferase activity (Figure 3-2). Similar results were shown with each candidate miRNA, as a 39.4% and 60.6% reduction in luciferase activity was observed with introduction of miR-148a and miR-216b, respectively (Figure 3-2). These results show a direct interaction between miR-148a and miR-216b with the 3'UTR of HK2.

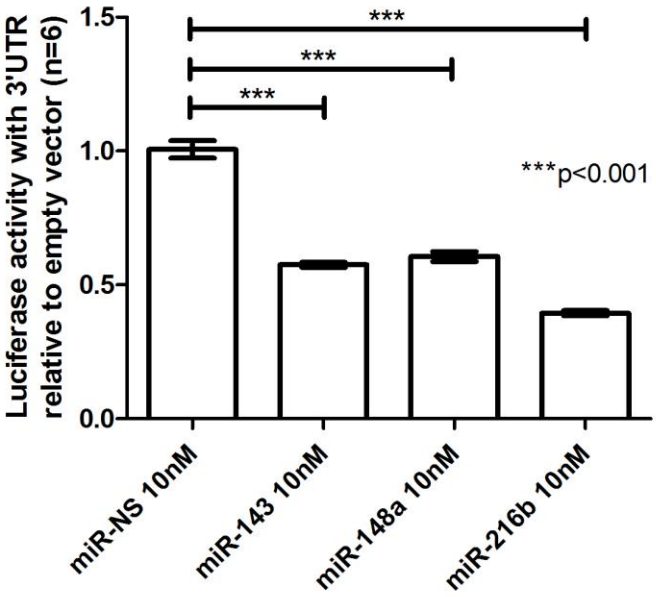



**Figure 3-2: Candidate miRNAs miR-148a and miR-216b directly bind the 3'UTR of HK2.** Luciferase activity was measured in 293T cells transfected with a luciferase reporter construct containing the 3'UTR of HK2 and 10 nM candidate miRNA mimics or a non-specific control (miR-NS). The luciferase activity was normalized to that measured for an empty vector control which was co-transfected with10 nM candidate miRNA mimic or miR-NS. Mean ± standard error of the mean (SEM) for biologic replicates shown (*n*=6).

**miR-148a and miR-216b inhibit HK2 expression in PDAC cell lines**

To determine if candidate miRNAs regulate HK2 expression in PDAC cell lines, miR-148a and miR-216b mimics were introduced into PDAC cell lines. siRNA targeted against HK2 (siHK2) and miR-143, a miRNA known to inhibit HK2 expression in other cancer cell lines, were also introduced into PDAC cell lines and served as a positive control to which the effects of candidate miRNA overexpression could be compared. Both HK2 mRNA and protein expression were examined upon introduction of miRNA mimics to determine if candidate miRNA-mRNA interactions resulted in either mRNA degradation or inhibition of translation of HK2.

Transient overexpression of miR-148a resulted in an approximate 30% decrease in HK2 mRNA expression in both PDAC cell lines examined ($P<0.003$, Figure 3-3a). This was similar to the effect of miR-143 overexpression, suggesting that miR-148a negatively regulates HK2 by promoting mRNA degradation in PDAC cell lines. Transient overexpression of miR-216b resulted in an approximate 20% and 60% decrease in mRNA expression in the CFPAC-1 and PANC-1 cell lines, respectively ($P<0.050$, Figure 3-3a), supporting the hypothesis that miR-216b negatively regulates HK2 by promoting mRNA degradation. Transient introduction of miR-148a and miR-216b inhibited HK2 protein expression in the PANC-1 cell line, as their overexpression resulted in an approximate 30% and 50% reduction in HK2 protein expression, respectively (Figure 3-3b). These results suggest that both miR-148a and miR-216b negatively regulate HK2 protein expression by promoting mRNA degradation in PDAC cell lines.

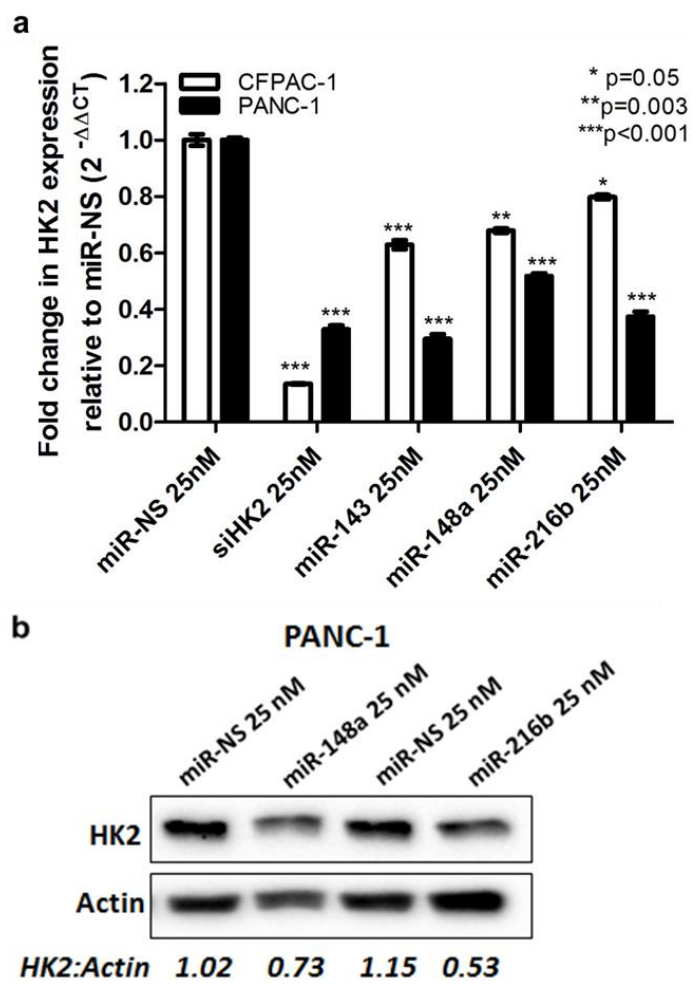



**Figure 3-3: miR-148a and miR-216b inhibit HK2 mRNA and protein expression in PDAC.**
(**a**) The fold change in expression of HK2 with introduction of siHK2 or candidate miRNA mimics relative to the non-specific control (miR-NS). Fold change determined using the ΔΔCT method with mean ± SEM shown (n=3 technical replicates).
(**b**) Change in HK2 expression after 48 hours of candidate miRNA overexpression in the PANC-1 cell line. HK2:Actin intensity was determined by densitometry and reported relative to the average ratio observed for miR-NS.

**miR-148a and miR-216b inhibit AIG and invasion of PDAC cell lines**

Since miR-148a and miR-216b inhibit HK2 expression in PDAC cell lines, we hypothesize that overexpression of these miRNAs in PDAC cell lines will phenocopy the effects of HK2 knockdown on AIG and invasion previously described. To directly assess the effects of miR-148a and miR-216b overexpression on AIG in PANC-1, a soft agar assay was used (Figure 3-4a). For comparison, siRNA targeted against HK2 (siHK2) was used to knockdown HK2 in PANC-1. Transient knockdown of HK2 with siRNA resulted in a 90.0% decrease in colony growth, consistent with previous results showing a requirement of HK2 for AIG of PDAC cell lines ($P<0.001$, Figure 3-4b). Transient overexpression of miR-148a and miR216b in PANC-1 resulted in a 77.9% and 38.8% decrease in colony growth on soft agar, respectively, suggesting that increased expression of both candidate miRNAs phenocopies the effect of HK2 knockdown on AIG in PDAC cell lines ($P<0.001$, Figure 3-4b).

To directly examine the effect of miR-148a and miR-216b on PDAC cell invasion, a transwell invasion assay was used (Figure 3-5a). For comparison, siHK2 was used to knockdown HK2 expression. Consistent with previous results showing a requirement of HK2 for invasion in PDAC cell lines, transient HK2 knockdown in PANC-1 caused a 58.7% decrease in invasion through a reconstituted matrix ($P<0.010$, Figure 3-5b). Transient overexpression of miR-148a resulted in a 68.0% decrease in invasion in PANC-1, while overexpression of miR-216b caused a 44.7% decrease in invasion ($P<0.040$, Figure 3-5b). These results show that candidate miRNA overexpression phenocopies HK2 knockdown in PDAC cell lines and that overexpression of either miR-148a or miR-216b is sufficient to inhibit invasion.

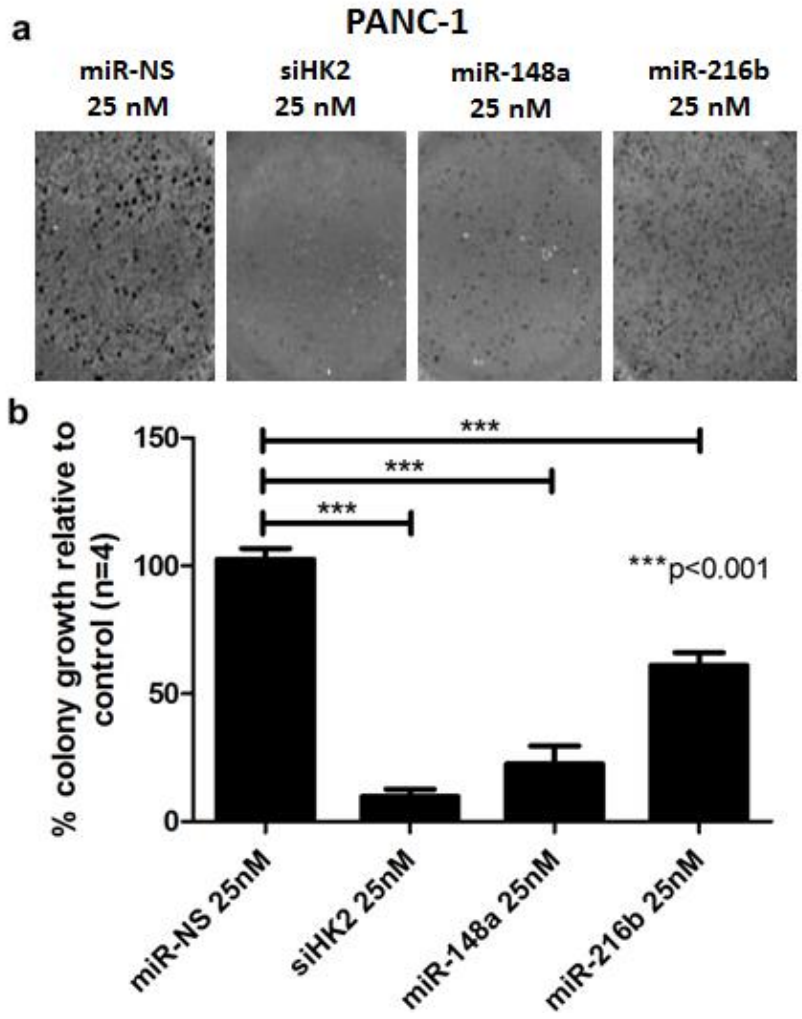



**Figure 3-4: miR-148a and miR-216b inhibit AIG of the PANC-1 cell line.**
(**a**) Representative images of colony formation in soft agar assays.
(**b**) Percent of growth with miRNA overexpression (25 nM) relative to control (miR-NS). Mean ± SEM of biological replicates shown (*n*=4).

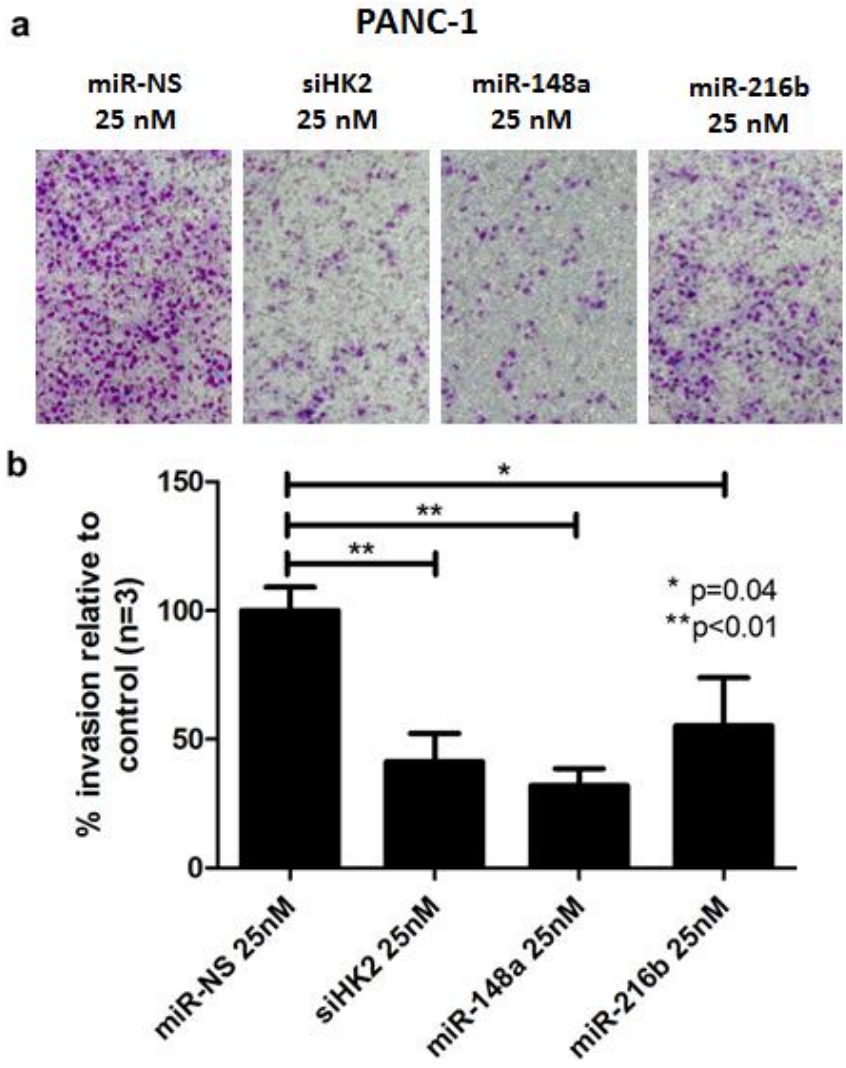



**Figure 3-5: miR-148a and miR-216b inhibit invasion of the PANC-1 cell line.**
(**a**) Representative images of invasion from transwell invasion assay
(**b**) Percent of growth with miRNA overexpression (25 nM) relative to control (miR-NS). Mean ± SEM of biological replicates shown (*n*=3).

## DISCUSSION

PDAC is a highly lethal disease with an increased incidence of metastases and overall poor prognosis (Ryan et al., 2014). We have previously shown that HK2 is an important driver of tumor growth and metastasis in PDAC and that inhibition of HK2 would be a good strategy for the treatment of advanced PDAC. While there are no pharmacologic inhibitors of HK2 currently available, others have found miRNAs to be useful inhibitors of HK2 expression in preclinical cancer models (Fang et al., 2012, Jiang et al., 2012, Yoshino et al., 2013). Analysis of miRNA and mRNA expression identified 24 novel miRNAs which are negatively correlated with HK2 expression in PDAC, downregulated in PDAC tumors relative to normal pancreas, and predicted to bind the 3'UTR of HK2. Importantly miR-143, a miRNA previously shown to be important for regulation of HK2 in other cancer types, was not included in this list of candidate miRNAs, suggesting that other miRNAs may play an important role in regulation of HK2 in PDAC.

miR-148a and miR-216b, two miRNAs known to be downregulated in PDAC precursor lesion and tumors, were included in the list of potential miRNAs regulating HK2 in PDAC (Bloomston et al., 2007, Hanoun et al., 2010, Yu et al., 2012). We show that miR-148a and miR-216b may function as tumor suppressors in PDAC by directly inhibiting HK2 expression, as transient overexpression of these miRNAs is sufficient to decrease HK2 mRNA, AIG, and invasion in PDAC cell lines. The importance of miR-148a and miR-216b for regulation of HK2 expression has not been previously shown, but it is known that these two miRNAs function as tumor suppressors in other cancer types. miR-148a suppresses invasion in hepatocellular carcinoma by inhibiting the epithelial to mesenchymal transition (Zhang et al., 2013) while miR-216b decreases invasion of *KRAS*-mutant cancer cell lines by negatively inhibiting oncogenic *KRAS* activity and its downstream signaling pathways (Deng et al., 2011).

It is clear that miR-148a and miR-216b have the potential to regulate many different pathways important for oncogenesis, but our data suggests that miR-148a and miR-216b regulation of HK2 may be especially important for inhibiting PDAC tumor growth and metastasis. Delivery of miRNA mimics and inhibitors is currently under investigation in preclinical models of cancer and include systemic delivery of modified nucleic acids or direct targeting of miRNAs to tumor tissue with nanoparticles (Price and Chen, 2014). Both of these strategies maybe feasible for the treatment of advanced PDAC, as systemic inhibition of HK2 in a GEMM causes no systemic defects in glucose tolerance and does not decrease growth or overall survival (Patra et al., 2013). Because miRNAs target many different mRNAs at once, it seems likely that systemic delivery of miR-148a and miR-216b mimics may cause off-target effects that may not be observed with direct pharmacologic inhibition of HK2. Thus, directed delivery of miRNAs to tumor tissue may be more clinically useful.

Our work outlines a potential role for miR-148a and miR-216b as tumor suppressors in PDAC by directly inhibiting the expression of HK2, a protein required for increased glycolysis, tumor growth and invasion of PDAC cell lines. More studies, however, are needed to verify the role of miR-148a and miR-216b as tumor suppressors in PDAC.

## MATERIALS AND METHODS

*Cell culture and plasmid generation*

The human cell lines HEK293T, CFPAC-1, and PANC-1 were obtained from the American Type Culture Collection and authenticated via short–tandem repeat profiling (Genetica, Burlington, NC, USA). CFPAC-1 was cultured in RPMI 1640 while HEK293T and PANC-1 were cultured in DMEM. All media was supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin (Invitrogen) and incubated at 37 °C in 5% CO2 atmosphere. For luciferase reporter constructs, the primers listed below were used to amplify the 3'UTR of HK2 from CFPAC-1 genomic DNA. Overnight Xho1 restriction enzyme digestion of the pPSI-CHECK2 vector was used to linearize plasmid DNA prior to ligation (donated by the laboratory of Al Baldwin, PhD). Ligation of the HK2 3'UTR PCR fragment and pPSI-CHECK2 vector was performed using the In-Fusion HD cloning system as per the manufacturer's instructions (Clontech, Mountain View, CA, USA). The final construct were verified by Sanger sequencing (Eton Biosciences, Research Triangle Park, NC, USA).

HK2 3'UTR US: TAGGCGATCGCTCGAAACCCCTGAAATCGGAAGG

HK2 3'UTR DS: AATTCCCGGGCTCGAAACATCTTCACTAGACTGAG

*Luciferase binding assay*

$1x10^4$ HEK293T cells were plated in each well of a 24 well plate. After 24 hours 100 ng vector DNA and 10nM miRNA mimics were co-transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). miRNA mimics were transfected with either an empty vector control or a vector containing the 3'UTR of HK2. 24 hours post transfection the cells were trypsinized and pelleted. Cells were suspended in 100 µL of PBS, lysed in 100 µL of the dual-

glo® luciferase reporter assay lysis buffer as per manufacturer's instructions, and the entire reaction was placed in a 96 well plate (Promega, Madison, WI, USA). Firefly luciferase activity was measured with a Synergy 2 microplate reader (BioTek, Winooski, VT, USA). 100 µL stop solution was added per well, incubated for 5 minutes, and renilla luciferase activity with the Synergy 2 microplate reader. The renilla luciferase activity, which varies with the interaction between a miRNA and the 3'UTR sequence, was normalized to firefly luciferase activity, which served as a transfection control for each biological replicate ($n$=6). The renilla:firefly luciferase activity for the construct containing the 3'UTR relative to the empty vector was determined for each miRNA and the ratio of 3'UTR:Emtpy vector for each candidate miRNA was normalized to that obtained for the miRNA negative control for each biological replicate (miR-NS).

*Expression of siRNA or miRNA mimics*

Reverse transfection in a six-well plate was performed with Lipofectamine RNAiMax (Invitrogen) as per the manufacturer's guidelines. 4-5x$10^5$ cells were seeded per well and a final concentration of 25 nM *mir*VANA mimic Negative Control #1 (miR-NS, Catalog #4464058, Ambion, Grand Island, NY, USA ), siHK2 (Catalog #S6560, Applied Biosystems, Grand Island, NY, USA), miR-148a *mir*VANA mimic (Catalog #MC10263, Applied Biosystems) or miR-216b *mir*VANA mimic (Catalog #MC12302, Applied Biosystems) was used. Cells were incubated for 48 hours prior to use in assay or western blot.

*Western blot*

Samples were lysed in 200 µL RIPA buffer (pH 7.4) containing protease inhibitors (ThermoFisher Scientific). 20-25 µg protein suspended in SDS loading buffer was run on 10%

SDS polyacrylamide gels and electro transferred to PVDF membranes. Membranes were blocked in 5% milk and incubated with 1:1 000 dilutions of primary antibodies in 5% BSA, including anti-HK2 (Catalog #2867, Cell Signaling Technology, Danvers, MA, USA) and the loading control anti-β-Actin (sc-#47778, Santa Cruz Biotechnology). Membranes were incubated with 1: 5000 dilutions of appropriate secondary antibodies in 5% milk (ThermoFisher Scientific). Incubations were for 1 hour at room temperature and Clarity Western ECL substrate with ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA) were used to detect immunoreactive bands.

*Quantitative PCR (qPCR)*

RNA was isolated from CFPAC-1 and PANC-1 cell lines 48 hours after introduction of siRNA or miRNA using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). 2 µg of RNA was used for cDNA synthesis (Applied Biosystems) and 50 ng of RNA was used for real-time PCR (Applied Biosystems). Reactions were performed in triplicate on a 384 well plate using standard PCR settings on a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). HK2 expression was assessed with HK2 TaqMan qPCR array (Applied Biosystems, Hs00606086_m1) while β-actin was assessed with ACTB TaqMan qPCR array (Applied Biosystems, Hs01060665_g1). The ΔΔCT method was used for analysis.

*Anchorage independent growth*

1-2 $x10^4$ cells were seeded into a soft agar assay after 48 hours of siRNA or miRNA overexpression as was previously outlined in Martin et al (Martin et al., 2011). Briefly a six well plate was coated with 2 mL of a 0.6% bacto-agar and culture medium mixture. 500 µL of a cell

and 0.4% bacto-agar mixture was added after solidification of the first layer. 300 µL media was added every four days for two-three weeks. Colony growth was quantified using Image J software (NIH, Bethesda, Maryland, USA). Percent growth was calculated by dividing the number of colonies for each biological replicate ($n$=4) observed by the average number of colonies in the control.

*Transwell invasion*

Uncoated inserts with 8-µm pores (Catalog #82050, Greiner Bio-One, Monroe, NC, USA) were coated with 100 µL of a diluted growth factor reduced Matrigel membrane matrix (300 µg/mL, ThermoFisher Scientific) and incubated at 37 $^{o}$C for 2 hours. Coated inserts were then placed into a 24 well plate containing 750 µL normal culture media. 48 hours after introduction of siRNA or miRNA, 1-2 x$10^5$ cells were suspended in 250 µL media supplemented with 1% FBS and seeded into the upper chamber of insert. Cells invaded for 16 hours. Inserts were then cleaned, fixed, and stained with Diff Quik as per manufacturer's instructions (ThermoFisher Scientific). The number of cells invading for each biological replicate ($n$=3) was determined by counting five random fields per insert. Percent invasion was calculated by dividing the total number of cells invaded by the average number of cells invaded for the appropriate control

# CHAPTER 4 : DISCUSSION AND FUTURE DIRECTIONS

## DISCUSSION

### Clinical relevance

Pancreatic cancer, over 85% of which is pancreatic ductal adenocarcinoma (PDAC), is a highly lethal malignancy with an increased incidence of metastasis and an overall poor prognosis (Howlader et al., 2013, Ryan et al., 2014). Metastatic PDAC patients are not considered candidates for curative surgery and their treatment is limited to cytotoxic chemotherapies, many of which produce modest, if any, response (Ryan et al., 2014). Many preclinical studies have focused on understanding pathways important for promoting tumor growth and metastasis in PDAC, with the hope that targeted therapies will improve clinical outcomes in metastatic PDAC. Over 95% of PDAC patient tumors contain activating mutations in the oncogene *KRAS* (Cox et al., 2014). Oncogenic *KRAS* mutations are sufficient to drive tumor growth in preclinical mouse models of PDAC, suggesting that activation of this signaling pathway is particularly important for driving disease progression (Guerra and Barbacid, 2013). Oncogenic Kras promotes increased glucose uptake, transcriptional upregulation of enzymes responsible for anabolic glucose metabolism and PDAC tumor maintenance (Ying et al., 2012). Genetic and pharmacologic inhibition of pathways involved with glucose metabolism decreases PDAC tumor growth (Daemen et al., 2015, Ying et al., 2012), suggesting that targeting glucose uptake may be therapeutically important in PDAC.

We show that genetic inhibition of HK2 results in decreased PDAC glycolysis, tumor growth and metastasis, suggesting that HK2 is an important driver of disease progression in PDAC. While a pharmacologic inhibitor of HK2 has not been tested in preclinical models, genetic inhibition of HK2 expression in an adult GEMM does not cause overt defects in glucose metabolism, weight loss, or decreased overall survival (Patra et al., 2013). This provides strong evidence that systemic targeting of HK2 may be well tolerated in the clinic and may be a good option in advanced PDAC.

**HK2 drives tumor growth in PDAC**

The importance of HK2 for in *Kras*-driven tumor growth was first shown in lung cancer GEMM, where genetic deletion of HK2 in *Kras* mutant lung cancer cells causes decreased tumor growth and an increase in overall survival (Patra et al., 2013). Prior to our study, the role of HK2 in driving tumor growth in PDAC was unknown. We have built upon existing literature by outlining an important role for HK2 in PDAC and showing that inhibition of HK2 is sufficient to decrease PDAC tumor growth. We also show that increased levels of HK2 are sufficient to promote anchorage independent growth (AIG) and invasion of PDAC cell lines, suggesting that HK2 functions as an oncogene in PDAC. This oncogenic activity has not been directly observed in the literature and suggests an important role for regulation of HK2 during PDAC oncogenesis.

PDAC is a molecularly diverse cancer with at least two subtypes that are associated with clinical outcomes (Collisson et al., 2011). It is clear that glucose metabolism differs between PDAC subtypes and each subtype has different sensitivities to metabolic inhibitors. The classical subtype is sensitive to inhibitors of lipid synthesis while the quasi-mesenchymal (QM) subtype is sensitive to inhibitors of glycolytic activity. We hypothesize that targeting of HK2 would be an

efficient way to treat the multiple subtypes of PDAC as direct inhibition of HK2 can reduce the flux of glucose into multiple pathways required for tumor growth in each of the two clinically important PDAC subtypes. Future studies should examine the effect of HK2 inhibition in both subtypes.

miRNAs are known to regulate HK2 expression in multiple cancer types and this regulation is important for inhibiting tumor growth in preclinical cancer models (Fang et al., 2012, Gregersen et al., 2012, Jiang et al., 2012). miR-143 functions as a tumor suppressor by regulating aerobic glycolysis via targeting HK2 (Fang et al., 2012, Jiang et al., 2012, Yoshino et al., 2013), however miR-143 is unlikely to function as a tumor suppressor in PDAC because it is upregulated in PDAC tumors relative to normal pancreas (Bloomston et al., 2007, Schultz et al., 2012). We identified two novel candidate miRNAs, miR-148a and miR-216b, that are negatively correlated with HK2 expression in PDAC, directly interact with the 3'UTR of HK2, inhibit HK2 mRNA expression, and AIG of PDAC cell lines. Decreased expression of each of these candidate miRNAs occurs in PDAC precursor lesions and tumor tissue (Xue et al., 2013, Yu et al., 2012), suggesting that their dysregulation contributes to oncogenesis. Our data shows that down regulation of miR-148a and miR-216b in PDAC precursor lesions may drive PDAC disease progression by regulating HK2 expression. Further studies are needed to confirm the tumor suppressive function of miR-148a and miR-216b, but the identification of these miRNAs as novel regulators of HK2 may provide new mechanisms for inhibition of HK2 in the clinic.

**HK2 promotes metastasis by regulating glycolysis**

Multiple studies have observed increased expression of HK2 in PDAC metastases (Chaika et al., 2012, Hamabe et al., 2014, Palmieri et al., 2009), suggesting an association

between increased HK2 activity and metastasis. We show that HK2 is required for PDAC cell invasion, extravasation, survival, and growth in distant organs. Overexpression of HK2 is sufficient to promote invasion of PDAC cell lines, suggesting that proper regulation of HK2 expression is important for inhibiting disease progression. Overexpression of miRNAs regulating HK2 in PDAC cell lines was sufficient to inhibit invasion, further supporting a role for miR-148a and miR-216b as tumor suppressors via their regulation of HK2.

We show that HK2 promotes invasion of PDAC cell lines by increasing glycolysis. Addition of the glycolytic inhibitor oxamate abrogated the effects of HK2 overexpression on invasion. Although an association between elevated rates of glycolysis and PDAC metastases has been shown (Chaika et al., 2012, Daemen et al., 2015), a direct relationship between these two has not been previously observed in PDAC. We, therefore, outline an important mechanistic link between elevated lactate production and cancer cell invasion. Given this, we hypothesize that inhibition of lactate production can prevent formation of metastases. Consistent with this, genetic and pharmacologic inhibition of lactate production via targeting of lactate-dehydrogenase A (LDHA) inhibits disease progression and metastasis in multiple preclinical models of cancer (Sheng et al., 2012, Xian et al., 2015). Future studies will be needed to determine if inhibition of lactate production is sufficient to prevent formation of PDAC metastases *in vivo*.

Elevated extracellular lactate can promote metastasis by multiple mechanisms, including influencing gene expression and altering the activity of enzymes important for matrix degradation (Baumann et al., 2009, Latham et al., 2012, Martinez-Outschoorn et al., 2011, Payen et al., 2015). While the effects of lactate production on gene expression or matrix degradation were not directly assessed here, we showed that knockdown of HK2 expression in PDAC cell line xenografts causes changes in gene expression. More specifically, we show vascular

endothelial growth factor A (VEGF-A) signaling to be altered with HK2 knockdown, suggesting that lactate-induced changes in gene expression may be at work in PDAC. Because activation of VEGF-A signaling occurs with an acidic extracellular pH (Xu et al., 2002), it is possible that HK2 knockdown in PDAC xenografts influences the pH of the tumor microenvironment and alters VEGF-A signaling by this mechanism. Future studies should directly assess exactly how the HK2-driven changes in lactate production influence metastatic potential in PDAC cell lines.

## FUTURE DIRECTIONS

### Targeting glucose metabolism in advanced PDAC

Our studies show that HK2 is both necessary and sufficient to promote primary tumor growth and metastasis in PDAC by regulating glycolysis. Direct inhibition of HK2 may be the most promising approach to treating PDAC, a disease that requires glucose for various metabolic processes including lipid synthesis, nucleotide synthesis, and protein glycosylation (Figure 1-2). While we hypothesize that HK2 would eliminate the need to target individual pathways required for PDAC tumorigenesis, we have yet to directly test this *in vitro*. Experiments examining the sensitivity of classical and QM PDAC cell lines to genetic inhibition of HK2 should be performed. To provide more insight into the metabolic consequences of HK2 inhibition in PDAC, future experiments could use carbon isotope labeling to examine how genetic inhibition of HK2 influences both glucose and glutamine metabolism in PDAC cell lines representing both subtypes. This experiment may be particularly useful in identifying additional metabolic pathways important for PDAC cell growth if it is found that the QM subtype is resistant to inhibition of HK2.

While there are currently no pharmacologic inhibitors of HK2 available, inhibitors of lactate production have been successfully utilized in preclinical cancer models to inhibit primary tumor growth (Granchi et al., 2014). Future studies could examine the ability of glycolytic inhibitors, including the well-tolerated LDHA inhibitor FX-11 (Le et al., 2010), to inhibit primary tumor growth and metastasis in a PDAC GEMM. Our results suggest that inhibiting glycolysis would limit both primary tumor growth and the development of metastases in this model. Inhibitors of glucose uptake, such as 3-bromopyruvate (3-BP) have been examined in preclinical models of cancer (Pedersen, 2012), but their efficacy for the treatment of advanced PDAC is unknown. Future studies could also examine the ability of 3-BP to inhibit primary tumor growth and metastasis in PDAC GEMMs.

**Investigating the link between lactate and PDAC metastasis**

While we showed that HK2 influences metastasis by regulating lactate production, we did not directly assess how lactate itself influences metastatic potential. Lactate can influence gene expression by regulating histone deacetylase activity in cancer cell lines (Latham et al., 2012). We hypothesized that the changes in gene expression that occurred with HK2 knockdown in PDAC cell line xenografts resulted from a change in lactate production and histone deacetylase activity. To directly examine this, histone acetylation in tumor cell lysates could be examined. If the mechanism described by Lathem et al., occurred in PDAC xenografts, control tumor lysates would show increased histone acetylation, as a result of increased lactate levels, when compared to tumors with HK2 knockdown. To compare lactate production in tumors with and without HK2 knockdown, a mass spectroscopy approach could be used to directly measure accumulation of this metabolite in the tumor.

To further assess if lactate accumulation influences gene expression in PDAC cell lines, RNA sequencing of cell lines after incubation with the glycolytic inhibitor, oxamate, could be performed. Changes in gene expression here should be similar to those observed in tumors with HK2 knockdown. Histone acetylation status in PDAC cell lines after incubation with extracellular lactate or treatment with oxamate would also provide support for the mechanism outlined by Latham, et al.

Extracellular lactate accumulation influences local pH and can, therefore, result in activation of signaling pathways required for metastasis, including VEGF-A. Since VEGF-A signaling was altered with HK2 knockdown, it is possible that HK2 influences VEGF-A expression via a pH-dependent mechanism. While we examined lactate production in PDAC cell lines with HK2 knockdown and overexpression, we did not directly examine if changes in HK2 expression influence extracellular pH. This important relationship could be examined using an assay which directly measures the extracellular acidification rate (ECAR) with HK2 knockdown and overexpression. Changes in ECAR with HK2 expression would provide evidence for a mechanism in which HK2 influences VEGF-A signaling via alteration of extracellular pH. To further test if this mechanism is important for PDAC, the relationship between cell culture pH and VEGF-A signaling could be examined in PDAC cell lines. An acidic pH is predicted to activate VEGF-A signaling, and we would predict similar changes in VEGF-A signaling with stable overexpression of HK2. Concordance between changes in VEGF-A signaling with an acidic pH and HK2 overexpression would suggest this mechanism is important for driving metastasis in PDAC.

**Exploring miRNA regulation of HK2 in PDAC**

The studies presented in chapter 3 provide preliminary evidence that miR-148a and miR-216b function as tumor suppressors in PDAC by negatively regulating HK2. Multiple studies must be performed to provide stronger evidence supporting a direct relationship between these candidate miRNAs and HK2. First, mutagenesis of the 3'UTR of HK2 at the predicted miRNA binding sites should be performed to confirm that decrease in luciferase activity observed with candidate miRNAs results from direct binding of a miRNA to a specific target sequence. While we examined the effects of miR-148a and miR-216b overexpression on HK2 expression in PDAC cell lines, hexokinase activity and lactate production in PDAC cell lines with miR-148a and miR-216b overexpression should also be examined. This would confirm that overexpression of miR-148a and miR-216b is sufficient to inhibit both the expression and activity of HK2 in PDAC cell lines.

Introduction of miR-148a and miR-216b inhibitors could be used to determine if inhibition of endogenous miRNA activity results in increased HK2 expression. A moderate to high level of baseline miRNA expression is needed for this experiment to be successful. Analysis of miRNA expression in PDAC tumors from chapter 3 suggests that while miR-148a is expressed in PDAC samples, miR-216b has a relatively low level of baseline expression in PDAC tumors. Given this, baseline miRNA expression should be taken into consideration when interpreting the effects of miR-216b inhibition on HK2 expression in PDAC cell lines. An alternative approach to inhibition of endogenous miRNA activity includes stable overexpression of the 3'UTR of HK2. Stable overexpression of the 3'UTR of HK2 would act as an endogenous miRNA "sponge" and effectively inhibit miRNAs from negatively regulating endogenous HK2 mRNA targets. This experiment is less desirable because it does not show which candidates are

directly responsible for regulating HK2 and only suggests that mature miRNAs are important for regulating HK2 expression in PDAC.

We showed that overexpression of miR-148a and miR-216b phenocopies HK2 knockdown, as it inhibited AIG and invasion of PDAC cell lines. While we suggest that miR-148a and miR-216b function as tumor suppressors by directly inhibiting HK2, our initial studies have not ruled out the possibility that these candidate miRNAs inhibit AIG and invasion of by targeting additional mRNAs required for these processes. To ensure that the effects of miR-148a and miR-216b overexpression on AIG and invasion are specific to their regulation of HK2, overexpression of the HK2 cDNA could be introduced into cells along with miRNA mimics. If HK2 cDNA abrogates the effects of miR-148a and miR-216b overexpression on AIG and invasion, it would be reasonable to conclude that these candidate miRNAs function as tumor suppressors by regulating HK2 expression in PDAC.

miR-148a and miR-216b are downregulated in PDAC tumors and precursor lesions, however the exact mechanism regarding their dysregulation in PDAC has not been investigated. One potential mechanism is that oncogenic KRAS activity, a process that occurs in early precursor lesions, suppresses the expression of miR-148a and miR-216b in PDAC (Guerra and Barbacid, 2013). This can be directly examined using genetic or pharmacologic inhibition of oncogenic KRAS in PDAC cell lines and measuring the resulting miRNA expression. If oncogenic KRAS activity downregulates miR-148a and miR-216b, then miRNA expression would increase upon KRAS inhibition. This experiment may provide new insights into how oncogenic KRAS activity promotes tumor formation in PDAC.

## CONCLUDING REMARKS

PDAC is a devastating disease with a high incidence of metastasis. We show that HK2 is both necessary and sufficient for primary tumor growth and metastasis, suggesting that HK2 functions as an oncogene and regulators of HK2 are critically important for inhibiting PDAC disease progression. miRNAs are negative regulators of gene expression and may, therefore, function as tumor suppressors in PDAC by inhibiting HK2. We identified miR-148a and miR-216b as regulators of HK2 and miRNA overexpression mimics the effect of HK2 knockdown on PDAC cell growth and invasion. Identification of miRNA regulators of HK2 provides new insights into how HK2 becomes dysregulated during PDAC tumor growth and metastasis. miRNAs also represent a novel mechanism to inhibit HK2 in PDAC that may prove useful in the clinic for the treatment of advanced disease.

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