INCREASED HEME FLUX AND MITOCHONDRIAL RESPIRATION ENHANCE TUMORIGENIC FUNCTIONS IN NON-SMALL CELL LUNG CANCER CELLS

by

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Dedicated to my Late Grandmother Sushila Narayan Sohoni

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The University of Texas at Dallas, 2019

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Lung cancer is the leading cause of cancer related deaths in United States, and about 85% of the cases are Non-small cell lung cancer (NSCLC). Many targeted therapies have been developed to treat lung cancer. Unfortunately, however statistical data over the past two decades suggest only a slight improvement in a patient's survival rate after diagnosis. Clonal evolution and tumor heterogeneity are the major obstacles in designing effective targeted treatments against cancer. To create more comprehensive treatments, emerging therapies target bioenergetic pathways of cancer cells. Like normal cells, cancer cells can generate energy only through glycolysis and oxidative phosphorylation. Notably, a number of studies have shown that many types of cancer cells rely heavily on mitochondrial respiration. Tumors of human non-small cell lung cancer (NSCLC) are heterogeneous but exhibit elevated glycolysis and glucose oxidation relative to benign lung. Heme is a central molecule for oxidative metabolism and ATP generation via mitochondrial oxidative phosphorylation (OXPHOS). Here, we have found that non-small cell lung cancer cells show elevated levels of heme synthesis and uptake, mitochondrial heme, oxygen-utilizing hemoproteins, oxygen consumption, ATP generation, and key mitochondrial

biogenesis regulators relative to non-tumorigenic cells. Likewise, proteins and enzymes relating to heme and mitochondrial functions are found to be upregulated in human NSCLC tissues relative to normal tissues.

Hence, we believe altering heme availability can be a useful strategy in treatment of highly heterogenous cancers. In order to seqester extra-cellular heme we engineered heme-sequestering peptides (HSPs). We observed that treatment with HSPs significantly reduce heme uptake, intracellular heme levels, and tumorigenic functions of NSCLC cells. This effect of HSPs is specific to heme as addition of heme largely reversed the effect of HSPs on tumorigenic functions. Furthermore, we also observed that HSP2 significantly suppresses the growth of human NSCLC xenograft tumors in mice. HSP2-treated tumors showed lowered oxygen consumption rates and ATP levels. To further verify the importance of heme in promoting tumorigenicity, we generated NSCLC cell lines with increased heme synthesis or uptake, which result from overexpression of the rate-limiting heme synthesis enzyme ALAS1 or uptake protein SLC48A1, respectively. These cells exhibited enhanced migration and invasion and accelerated tumor growth in mice. Notably, tumors formed by cells with increased heme synthesis or uptake also have elevated oxygen consumption rates and ATP levels. Our data show that elevated heme flux and function underlie enhanced OXPHOS and tumorigenicity of NSCLC cells. Therefore, targeting heme flux and function offers a novel strategy for developing lung cancer therapy.

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CHAPTER 1

INTRODUCTION

Cancer is a disease that exhibits accumulation of mutations in genes that are essential for cell survival and growth. Highly proliferating cancer cells have increased demands for both energy and macromolecule synthesis. Cancer cells are under tremendous metabolic stress. Thus, in order to survive, malignant cells reprogram their metabolic pathways in order to utilize available nutrients which can in turn promote growth and metastasis of tumor cells (Hanahan and Weinberg, 2011; Ward and Thompson, 2012). First studies in cancer metabolism date back to late 1920s when Otto Warburg proposed that tumor cells mainly rely on glucose and metabolize glucose by aerobic glycolysis (Warburg et al., 1927). Indeed, this phenomenon called aerobic glycolysis is exhibited by number of different tumors (Gillies and Gatenby, 2007; Pavlova and Thompson, 2016). This effect was subsequently used in tumor imaging and detection with the help of positron emission tomography scans, that use radiolabeled glucose analogs (Papathanassiou et al., 2009). These early findings laid the groundwork for existing cancer metabolism research. Cancer cells exhibit extremely complex intermingled network of pathways which enable cancer cells to metabolize various fuels and survive under metabolic stress. Numerous studies have demonstrated that malignant cells can toggle between glycolysis and TCA cycle, which allows cancer cells to metabolize glutamine and fatty acids as substrates to meet their high energy demands (Reitzer et al., 1978 and Chen and Russo, 2012) (Pavlova and Thompson, 2016). It has been confirmed that numerous cancer cells especially Myc driven cancer cells, use glutamine as a major carbon source (DeBerardinis and Cheng, 2010). Numerous studies point to the fact that lipid metabolism also plays a significant role in tumorigenesis of

different types of cancers. All these studies point to the fact that TCA cycle plays a very important role in cancer metabolism and tumorigenesis (Sajnani et al., 2017).

Cancer is one of the leading causes of death throughout the world, in which the main treatments involve surgery, chemotherapy, and/or radiotherapy (Shewach DS and Kuchta RD, 2009). Anticancer drugs can be classified according to their mechanism of action, such as DNA-interactive agents, antimetabolites, anti-tubulin agents, molecular targeting agents, hormones, monoclonal antibodies and other biological agents (Thurston, 2007). Chemotherapy involves the use of low molecular weight drugs to target rapidly proliferating tumor cells. Although, some chemotherapeutic agents are effective in controlling the proliferation of cancer cells, there are several side effects associated with them such as, bone marrow suppression, gastrointestinal tract lesions, hair loss, nausea, and the development of clinical resistance. These side effects occur because cytotoxic agents act on both tumor cells and healthy cells. Although several chemotherapeutic and targeted therapeutic agents are approved for treating different types of cancers, the five-year survival rate remains low. Thus, in order to improve the clinical outcomes for patients newly advanced sophisticated therapies are needed. Over the past two decades a substantial progress is made with advent of various targeted therapies and effective application of immunotherapy but, their effectiveness is limited because of presence of multiple driver genes and intra-tumoral genetic heterogeneity. Thus, alternative strategies are still needed for cancer treatment.

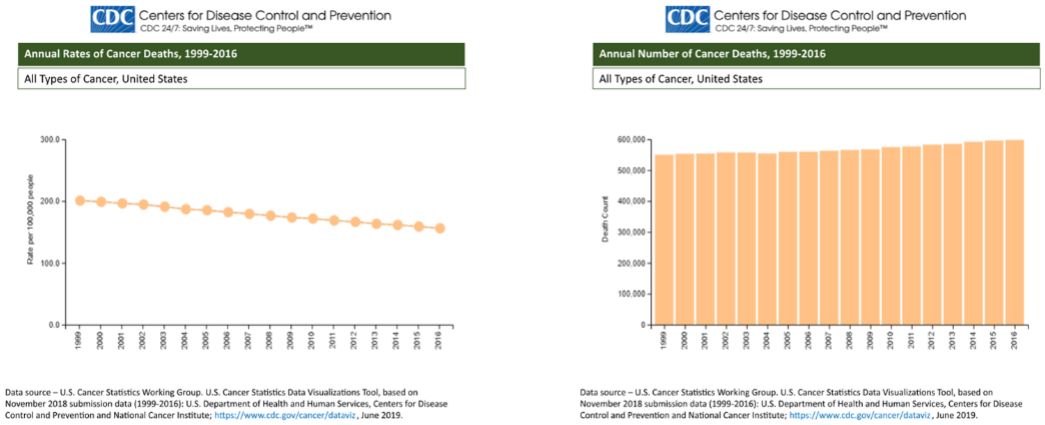

Figure 1.1: Annual Rates of Cancer Deaths, 1999-2016 and Annual Number of Cancer Deaths, 1999-2016

1.1 Targeting bioenergetics in tumor cells:

Tumor cells are versatile in their ability to adapt to their environment and support their proliferation and function. It has previously been shown that tumor cells can generate ATP by metabolizing an array of substrates importantly, glucose, glutamine and fatty acids. (Zaidi N et al., 2013; Menendez JA et al., 2007; Price DT et al., 2002; LiuY et al., 2010; Zha S et al., 2005; Comerford SA et al., 2014; and Mashimo et al., 2014) The two main pathways through which cells can generate ATP are glycolysis and oxidative phosphorylation and thus targeting these bioenergetic pathways offers a great potential for cancer therapy.

1.1.1 Targeting glycolysis:

Glucose is the most important energy-producing molecule for living organisms. Inside the cell, glucose can be metabolized by three mechanisms: glycolysis under anaerobic conditions, complete oxidation under aerobic conditions, and the pentose phosphate pathway. In glycolysis, glucose is broken down in to pyruvate generating 2 net molecules of ATP. In addition, glycolysis

also provides number of intermediates for other metabolic pathways. In 1920, Otto Warburg suggested that tumor cells and highly proliferative tissues metabolize glucose to lactate even in presence of ample amounts of oxygen, the phenomenon is called aerobic glycolysis (Warburg et al., 1927). It is believed that different tumor cells prefer aerobic glycolysis not only because of its high rate to generate ATP but also, it helps tumor cells in meeting their high demands for non-essential amino acids, fatty acids, and nucleotides (Pfeiffer et al., 2001).

Regulators of aerobic glycolysis

Many signaling pathways are involved in aerobic glycolysis process. Both the adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways are either directly or indirectly involved in the glycolysis of tumor cells (Chaube B et al.,2016). Famous oncoproteins c-Myc and hypoxia inducible factor 1 (HIF-1) are known to regulate key glycolytic enzymes. The oncogenic transcription factor c-Myc regulates glycolysis under normal oxygen conditions by directly activating lactate dehydrogenase (LDH) and other glycolytic genes. c-Myc can also activate genes associated with mitochondrial biogenesis. HIF-1 is a key transcription factor that regulates gene expression in the event of low oxygen or hypoxia (Shim H et al.,1997). It positively regulates GLUT1 and activates PDKs, which block the flow of pyruvate into the TCA cycle (Osthus RC et al., 2000). The tumor suppressor p53 also shows an inhibitory effect on glycolysis (Zawacka-Pankau J et al., 2011). p53 is known to directly stimulate oxidative phosphorylation and it was observed that the loss of p53 shifts metabolism from oxidative phosphorylation to glycolysis (Puzio-Kuter AM., 2011). Increasingly, research has focused on these regulators, which provide potential therapeutic targets for cancer treatment.

Drugs targeting glycolysis

1) 2-deoxyglucose (2-DG): 2DG is a synthetic glucose analog in which the C-2-hydroxyl group is replaced by hydrogen. 2DG has been extensively and thoroughly investigated in both scientific and clinical studies since the early 1950s. Upon internalization, 2-DG gets phosphorylated by hexokinase to produce 2-deoxyglucose-6-phosphate, which cannot be further metabolized by the cells. Accumulation of 2-deoxyglucose-6-phosphate leads to inhibition of hexokinase and eventually decrease in glucose uptake. Although early clinical trials of 2-DG showed promise in some patients, its use became limited because of toxicity associated with hypoglycemia symptoms (Landau et al., 1958) and lowered dose of 2-DG failed to limit disease progression (Raez et al., 2013; Stein et al., 2010). Although use of 2-DG in targeting glucose uptake or lactate production was somewhat successful in some clinical trials (Hay, 2016), its clinical success did not meet the expectation (Vander Heiden MG and DeBerardinis RJ, 2017). In addition to its main function of inhibition of glycolysis and glucose uptake it also exerts other extensive metabolic effects by interfering with various metabolic processes involving depletion of ATP, intensification of oxidative stress, interference with N-linked glycosylation, and induction of autophagy etc.

2) Inhibition of glucose transporters (GLUT): Glucose transport is the first step in the metabolism of glucose, which transports glucose across the plasma membrane. In humans, there are 14 glucose transporter proteins and they are dysregulated in most types of malignancies. It has been observed that cancer cells are more susceptible to glucose deprivation compared to normal cells. Various studies have demonstrated that inhibition of glucose transport results in apoptosis and can also decrease cancer cell proliferation (Zhang W et al., 2010).

GLUT1 is responsible for basal glucose transport in all cell types, and it has been shown that its level of expression correlates with the degree of invasion and metastatic potential of cancers (Zhang W et al., 2010). Treatment of various lung and breast cancer cell lines with anti-GLUT 1 antibodies was found to induce apoptosis. Anti-GLUT1 drugs combined with existing chemotherapeutic agents also found to show synergistic effects against different types of cancers (Zhang W et al., 2010). Given the widespread expression patterns of GLUT 1 and its role in transport of glucose across the blood–brain barrier, it is preferable to target transporters with far more limited expression profiles. (McBrayer SK et al.,2012). Studies on inhibition of GLUT started with GLUT1 antibodies, but recently several small molecule inhibitors of GLUT, including fasentin, phloretin, STF-31, and WZB117 were found to have anticancer effects. Fasentin is a small molecule that affects glucose transport thorough GLUT1 receptor (Wood TE et al., 2008). Studies by Wood et al. showed that fasentin and its analogues not only exhibit partial inhibition of the glucose transportation pathway but also break down the resistance of caspase activation, which is normally seen in malignant cells that are resistant to chemotherapy and other treatments (Wang S et al., 2003 and Mawji IA et al., 2007).

Polyphenol Phloretin (Ph) is isolated from apples and it targets GLUT2 in triple-negative breast cancer (TNBC). In addition to breast cancer Ph has been shown to be effective in treatments of bladder, liver, and colon cancer (Wu KH et al.,2018; Wu CH et al.,2009; Nelson JAS et al., 1993; and Yang KC et al., 2009). Compared to other GLUTs, GLUT1 plays a pivotal role in basal glucose uptake, but there is a lack of potent and selective inhibitors of GLUT1.

WZB117 is one of the few inhibitors that is selective for GLUT1 (IC50 = ~0.6 µM). In addition to inhibition of GLUT1, WZB117 also lowers the amount of intracellular ATP and causes stress

on the endoplastic reticulum (ER), which leads to cell cycle arrest (Liu Y et al., 2012). WZB117 significantly alters cancer cell proliferation in vitro and in vivo and it has shown synergistic effects with cisplatin and paclitaxel.

STF-31 is another compound that is a selective inhibitor of GLUT1 (IC50 = ~1.0 μM). Chan et al. used STF-31 in tumors that are deficient in von Hippel-Lindau (VHL) gene (Chan DA et al., 2011). STF-31 is a small molecule inhibitor of GLUT1 and its effect is mainly studied in renal cell carcinoma. STF-31 resulted in an inhibition of cancer cell growth and a decrease of tumor size in VHL-dependent models.

Ritonavir is a known antiretroviral medication. It functions as protease inhibitor, but recently it was discovered to have the potential utility as a noncompetitive inhibitor of GLUT4 for myeloma (Dalva-Aydemir S. et al., 2015). Ritonavir affected proliferation of multiple myeoloma cells and was used in combination with other drugs, such as metformin, to create synergistic effects. Targeting GLUT3 receptors has shown great promise in treatments of Temozolomide (TMZ) resistant glioblastoma tumors especially because, these tumors exhibit elevated levels of GLUT3 which contributes to their resistance to TMZ. (Le Calvé B et al., 2010). Although targeting glucose transport is very appealing strategy to combat different types of cancers, selective blockade of GLUTs in tumor cells can exhibit off target effects as GLUTs are ubiquitously expressed on all mammalian cells.

3) Inhibition of Hexokinase (HK): Hexokinase catalyzes first step in glycolysis and another very attractive potential target for cancer therapy. Hexokinase is tissue specific isoenzyme that phosphorylates glucose to glucose-6-phosphate (G-6-P). Importantly, G-6-P can feed to pentose phosphate pathway, which runs parallel to glycolysis and generates NADPH, and ribose 5-

phosphate which is a precursor for synthesis of nucleotides. There are four isoforms of this enzyme, but HKII is the major isoform that is heavily implicated in cancer because it plays major role in maintaining high glucose catabolism rates (Cárdenas ML et al., 1998). Many HK2 inhibitors such as 3-bromopyruvate, 2-deoxyglucose, GEN-27, benserazide, and lonidamine have shown promise in clinical trials (https://clinicaltrials.gov/ct2/show/NCT00633087). In lung cancer, interaction between HK and mitochondria is crucial not only to maintain high glycolytic rates but also for survival of tumors. A number of lung cancer cell lines show more than 40% activation of HK2.

3-bromopyruvate has been a subject of many studies for its anticancer activity against different types of cancers. 3-bromopyruvate was found to inhibit HK2, deplete ATP levels, and activate mitochondrial pathway of apoptosis. 3-bromopyruvate was also found to restore drug susceptibility in resistant tumors against variety of anticancer drugs. 3-Bromopyruvate has been hailed by many researchers as a potential breakthrough; however, it has yet to undergo formal clinical trials (Zhang Q et al., 2014; Jae HJ et al., 2009)

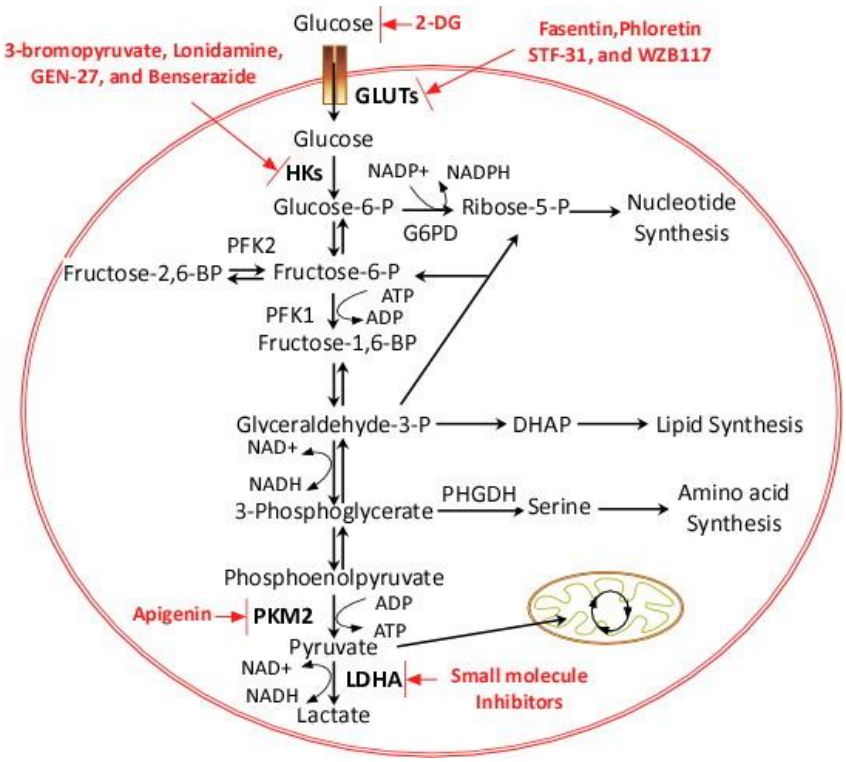

Figure 1.2: Drugs targeting glycolysis

Lonidamine is a derivative of indazole-3-carboxylic acid which inhibits HK2 activity and thus, downregulates aerobic glycolysis in cancer cells. Later studies in Ehrlich ascites tumor cells showed that lonidamine inhibits both respiration and glycolysis leading to a decrease in cellular ATP. Since its discovery over thirty years ago, lonidamine has gone through a multitude of clinical trials against many types of cancer including NSCLC (Di Cosimo S et al., 2003; Prabhakara S et al., 2008). Besides targeting HKII, subsequent studies have uncovered additional pharmacological targets for the drug, including the mitochondrial pyruvate carrier, the plasma membrane monocarboxylate transporters, the electron transport chain, and the mitochondrial permeability transition pore (Bhutia YD et al.,2016). These results have shown that the anti-cancer effects of lonidamine do not occur through a single target but rather at

multiple sites. Overall, the drug is capable of sensitizing tumors to chemotherapy, hyperthermia, and radiotherapy.

Genistein-27 (GEN-27) is a derivative of genistein, was synthesized and reported by Du et al. to exhibit potent anti-proliferation activity against colon cancer cells as well as prevention of colitis-associated tumorigenesis (Du Q et al., 2016). This anti-proliferative activity was observed across three human colorectal carcinoma cell lines with IC50's of less than 31μM. Studies in human breast cancer cells show that GEN-27 inhibits glycolysis in cancer cells by suppression of HKII ultimately targeting them for apoptosis. (Tao L et al., 2017). Several *in vitro* and *in vivo* studies show protective effect of Genistein on lung cancer carcinogenesis when compound is used alone or in combination with other compounds (Gadgeel SM et al., 2009; Wu TC et al., 2012 and Mahmood J et al., 2011)

Benserazide is a specific HKII inhibitor. Screened against a large number of targets, benserazide was identified to inhibit HKII in colorectal cancer cells. It induced apoptosis and suppress tumor growth in colorectal cancer xenograft models (Li W et al., 2017). Benserazide was also tested in breast cancer cell lines and was shown to decrease malignant properties associated with tumorigenesis, but via a different mechanism independent of HKII inhibition (Alli E et al., 2014). Recent studies have also discovered several natural products, including astragalin, resveratrol, and the flavonoid chrysin, possessing inhibitory efficacy against HKII and some specific cancer types. Astraglin was shown to reduce the activity of HKII by boosting microRNA-125b, and therefore inhibiting the proliferation of hepatocellular carcinoma (HCC) cells in vitro and in vivo (Li W et al., 2017). The increase in expression of microRNA-125b was shown to suppress the proliferation of the HCC cells via the microRNA-125b/HKII cascade. Inhibition of HKII by

chrysin and resveratrol were also shown to induce apoptosis in HCC cells, and therefore leading to a decrease in their proliferation (Li W et al., 2017 and Xu D et al., 2017). Overall, HK, especially HK II, is important for lung cancer cell survival, cell proliferation, and tumor initiation. Inhibition of HK might be a potential therapeutic target for NSCLC.

4) Inhibition of Pyruvate Kinase M2 (PKM2): Pyruvate kinase (PK) is the enzyme that catalyzes conversion of phosphoenolpyruvate to pyruvate in the last step of glycolysis. There are four isoforms of PK expressed in mammals, but the M2 isoform (PKM2) has been shown to be predominantly expressed in tumor cells and play a pivotal role in cancer metabolism and tumor growth (Muñoz-Pinedo C et al., 2012). PKM2 is involved in the process of tumorigenesis and tumor progression, including angiogenesis, cell cycle regulation, tumor microenvironment and metabolic abnormality (He X et al., 2017). PKM2 interacts with RAS-MAPK and mTOR pathways very efficiently (He CL et al., 2016). It is demonstrated that specific PKM2 inhibitors can inhibit the growth and survival of tumor cells (Vander Heiden MG et al., 2010). Shikonin, a small-molecule active chemical, serves as a PKM2 inhibitor and has been applied as an anti-cancer drug in human cancer models (Li W et al., 2014). Shikonin was found to suppress glycolytic rates in cancer cell lines that predominantly have higher expression of PKM2 (Chen J et al., 2011). Apart from the direct PKM2 inhibitors, there are some inhibitors that indirectly decrease the effect of PKM2 though intermediate molecule e.g. MEK/ERK inhibitors. Elumetinib is a MEK's inhibitor that reduces phosphorylation of ERK and decrease expression of PKM2. This results in decrease in tumor growth because of low levels of lactic acid. TRIM35 and FFJ-3 are few more examples of inhibitors of PKM2 altering glycolysis rates in cancer cells that ultimately triggers mitochondrial apoptosis signaling pathway (Li D et al., 2017). Few

studies have been published on small molecule inhibitors of PKM2. Resveratrol, a natural phytoalexin found in a variety of plants, was shown to reduce PKM2 expression in tumor cells, which resulted in an increase in the expression of ER stress and mitochondrial fission proteins but decrease in cell viability and the levels of fusion proteins (Wu H et al.,2016). Another natural compound, Apigenin, belonging to the flavone class, was recently found to restrain colon cancer cell proliferation via blocking of PKM2 (Shan S et al., 2017). These results further highlight the pivotal role of inhibition of PKM2 in cancer therapy. On the contrary, some reports also suggest that activity of PKM2 is important for tumor suppression, as pyruvate kinase can transfer the carbohydrate metabolic intermediates from biosynthesis to energy production. PKM2 activators aim to induce tetramerization of PKM2, which results in a decrease in glycolytic intermediates that are used as biosynthetic precursors. A class of quinolone sulfonamide activators has been reported to possess a distinctive mode of binding to PKM2 (Dong G et al., 2016). These activators bind to a site that is distinct from the FBP binding site, which results in the diversion of glycolytic intermediates away from the serine biosynthetic pathway, which produces serine that is required for continued cell proliferation.

5) Inhibition of Lactate Dehydrogenase (LDH): LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of nicotinamide adenine dinucleotide (NADH) and NAD+, when oxygen is absent or in short supply (Nelson DL et al., 2004). At high concentrations of lactate, the enzyme exhibits feedback inhibition and the rate of conversion of pyruvate to lactate is decreased. c-Myc and HIF1 directly regulate *LDHA* gene and LDH-5 (*LDHA*) under hypoxia respectively. LDHA genes are heavily overexpressed in lung cancer and hence have become important prognosis for lung cancer. Using a specific small molecule LDHA

inhibitor, Xie et al. reported that LDHA was essential for cancer-initiating cell survival and proliferation, which could be a potential therapeutic target for NSCLC (Xie H et al., 2014).

1.1.2 Targeting Mitochondria and OXPHOS

It is imperative to know that mitochondrial function and oxidative phosphorylation (OXPHOS) play critical roles in pathophysiology of cancer. Mitochondria are essential intracellular organelles that regulate energy metabolism, cell death, and signaling pathways that are important for cell proliferation and differentiation. Therefore, mitochondria are fundamentally implicated in cancer biology, including initiation, growth, metastasis, relapse, and acquired drug resistance. Based on these implications, mitochondria have been proposed as a major therapeutic target for cancer treatment. Mitochondria are mainly involved in production of ATP and intermediate metabolites necessary to fulfil bioenergetic and biosynthetic demands of cell. Mitochondria can use multiple carbon fuels to produce ATP and other intermediate metabolites, including pyruvate, which is generated from glycolysis; amino acids such as glutamine; and fatty acids. These carbon substrates feed into the TCA cycle in the matrix of mitochondria to generate the reducing equivalents NADH and FADH2. Further, NADH and FADH2 deliver their electrons to the electron transport chain (ETC) to generate high amounts of ATP by oxidative phosphorylation (OXPHOS). In addition, TCA cycle also generates different intermediates that are further used up in different biosynthetic pathways to generate different biomolecules such as glucose, amino acids, lipids, heme and nucleotides. Thus, mitochondria are central for both catabolic and anabolic processes in cell.

Mitochondrial Metabolism is required for tumor growth

Since last couple of decades there is a growing consensus in cancer biologists that tumor cells heavily rely on both glycolysis and mitochondrial metabolism that allows them access to different building blocks for synthesis of different macromolecules (nucleotides, lipids and amino acids) as well as ATP and NADPH, which are essential for cell proliferation (Ward PS et al., 2012). The role of activation of oncogenes such as MYC and KRAS in promoting glycolysis in tumors is well known (Lunt SY et al., 2011). Consequently, many tumors are addicted to glucose. Oncogenic activation also increases mitochondrial metabolism to generate ATP and TCA cycle intermediates that are used as precursors for macromolecule synthesis (Dang CV., 2012). For example, citrate from TCA cycle can contribute in lipids and nucleotides synthesis through its conversion to acetyl-coA and oxaloacetate respectively (Hatzivassiliou G et al.,2005). It is essential for cells to replenish TCA cycle intermediates that are exhausted for the biosynthesis of macromolecules. One mechanism by which cells restore their TCA cycle intermediates is by anaplerotic cycle involving stepwise oxidation of glutamine to α-ketoglutarate and then in turn to oxaloacetate. Subsequently, oxaloacetate can combine with acetyl-CoA to generate another molecule of citrate for macromolecule synthesis (DeBerardinis RJ et al., 2010). Thus, many cancer cells exhibit addiction to glutamine. The elevated oxidative phosphorylation and ETC generate copious amounts of ROS in cancer cells. The high levels of mitochondrial ROS activate signaling pathways proximal to the mitochondria to promote cancer cell proliferation and tumorigenesis (Schieber M et al., 2014). However, prolonged exposure to ROS can be damaging for cancer cells. Cancer cells tackle this problem by making abundance of NADPH which has high antioxidant activity (Lewis CA et al., 2014). Studies have

consistently demonstrated that increase in mitochondrial ROS aids therapies targeting TCA cycle intermediates or ETC proteins that contribute in tumorigenesis and metastasis (Porporato PE et al., 2014; Sullivan LB et al., 2013; Guzy RD et al., 2008; and Metallo CM et al., 2012). Taken together, these observations indicate that some tumors do not completely rely on glycolysis to meet the bioenergetics demands but, also use intact mitochondrial function to meet the other biosynthetic demands and ROS generation required for cell proliferation. The majority of ATP in tumor cells is produced by the mitochondria. Thus, targeting mitochondrial bioenergetics for cancer therapy can be an effective strategy mainly because of three reasons: (1) Centers of many tumors are poorly perfused and have limited access for nutrients especially glucose and oxygen. It is believed that ETC can function in very low oxygen concentrations, as low as 0.5%. Thus, limited amounts of nutrients can be metabolized by OXPHOS in these tumors. (2) There is a subset of tumors that show high dependency on oxidative phosphorylation mainly, B- cell lymphoma, melanoma and some NSCLC. and (3) Mitochondrial OXPHOS contributes in resistance to number of conventional chemotherapy treatments. It is also important to note that targeting mitochondrial bioenergetics will ultimately benefit therapies that target glycolysis.

Regulators of Mitochondrial metabolism in Cancer

Cancer cells are known to use diverse metabolites such as glucose, glutamine and fatty acids and TCA cycle plays a central role in their metabolism. Glucose is mainly metabolized by cancer cells through glycolysis after it is taken up by GLUT receptors. Although glycolysis is rapid, it cannot meet high energy demands of cancer cells hence, cancer cells metabolize glutamine through TCA cycle. Glutamine is the most abundant amino acid in human body. Glutamine also

serves as a substrate for biosynthesis of non-essential amino acids, purines, pyrimidines, and fatty acids which further enter TCA cycle to form α-ketoglutarate (α-KG) by anaplerosis. Glutaminolysis, breakdown of glutamine helps in replenishing the TCA cycle in cancer cells. Fatty acids undergo β-oxidation in TCA cycle to form acetyl-co-A that is used in TCA cycle and fatty acid synthesis. Tumor cells exhibit high levels of enzymes regulating the lipid biosynthesis to meet their high lipogenesis demands.

Metabolic reprograming in cancers is a result of mutations in oncogenes and tumor suppressor genes as they play major role in regulation of different metabolic pathways. The fate of cancer cells to follow a particular metabolic pathway depends on a specific mutation background as well as the tumor microenvironment factors. Several oncogenes and tumor suppressor genes such as MYC, HIF, P53, and RAS, are known regulators of bioenergetic pathways in tumors and play a critical role in determining how the TCA cycle is utilized in these cancer cells.

MYC is a proto-oncogene which controls a variety of cellular processes, such as cell proliferation, metabolism, cellular differentiation and genomic instability. MYC is severely implicated in various cancers and it is mainly responsible for driving transformation and progression of tumors. Aberrant MYC activity may result because of chromosomal translocations, gene amplifications or increased mRNA/protein stability (Gabay et al., 2014). Importantly, MYC is a central regulator of cellular metabolism, and can regulate an array of metabolic pathways ranging from aerobic glycolysis to OXPHOS. MYC has also been shown to regulate glutaminolysis, mitochondrial biogenesis, and in biosynthesis of amino acids and nucleotides (Adhikary S. and Eilers M., 2005; Gabay et al., 2014; Wahlström T and Arsenian

HM., 2015). MYC is a principal driver of glutamine anaplerosis and it is also involved in glutamine uptake. MYC can also upregulate fatty acid transporters (e.g., fatty acid-binding protein 4) and fatty acid oxidation genes such as hydroxyacyl-CoA dehydrogenase that generates acetyl-CoA which runs through TCA cycle (Wang R et al., 2011; Edmunds et al., 2015).

Hypoxia-inducible factors (HIFs) are transcription factors that play key role in regulation of bioenergetic pathways in response to low oxygen levels. HIFs are heterodimers consisting of oxygen sensing, α-subunit and a constitutively expressed β-subunit. Under normal oxygen conditions, the α-subunit is targeted for degradation upon hydroxylation by prolyl hydroxylases (PHD) and subsequent ubiquitination by von Hippel-Lindau (VHL) tumor suppressor. In most tumors, HIF1α expression is turned "ON" because of hypoxia resulting from poor vascularization or because of loss of VHL gene (Gordan JD and Simon MC, 2007). HIF1α activation shifts glucose catabolism towards aerobic glycolysis suppressing functioning of TCA cycle (Semenza GL, 2012). HIF1α is also responsible for upregulation of glucose transporters (SLC2A1 and SLC2A3) and glycolytic enzymes (Kim JW et al., 2006). It was observed that tumor cells with HIF1α activation often increase the usage of glutamine in order to compensate for lack of glucose feeding to the TCA cycle (Le A et al., 2012). Under hypoxia conditions, α-KG produced from glutamine undergoes reductive carboxylation to produces citrate that is used in lipid biosynthesis (Wise DR et al., 2008; Metallo CM et al., 2012; Gameiro PA et al., 2013).
p53 is a transcription factor and a known tumor suppressor that regulates many important cellular pathways, including cell survival, DNA repair, apoptosis, and senescence (Bensaad K et al., 2009). Wild-type p53 is crucial cell cycle regulator that is important to strike balance between bioenergetics and biosynthesis of macromolecules. P53 downregulates glycolysis and promotes

OXPHOS. p53 exerts its suppressive effects on glycolysis by decreasing expression of glucose transporters such as GLUT1 and GLUT4. p53 is also involved in indirect inhibition of two glycolytic enzymes, phosphofructokinase 1 and phosphoglycerate mutase (Kondoh H et al., 2005; Bensaad K et al., 2006, 2009; Zhang C et al., 2013). p53 first ensures availability of anapleurotic substrates, glucose, and glutamine, to the TCA cycle before activating OXPHOS. p53 is directly responsible for increasing expression of glutaminases, the enzyme that converts glutamine to glutamate that in turn enters TCA cycle (Zhang C et al., 2011). In most of the solid tumors, p53 is routinely found to be mutated. More than 50% of the malignancies show somatic mutations in p53 (Kruiswijk et al., 2015). A loss of p53 function has a significant impact on metabolic fate of these tumors. p53 mutations in these tumors trigger metabolic shift from oxidative phosphorylation to aerobic glycolysis.

The most frequently mutated RAS subfamily genes in cancer are K-RAS, N-RAS, and H-RAS, which play key roles in signal transduction following binding of extracellular ligand to receptor tyrosine kinases. (Pylayeva-Gupta et al., 2011; Stephen AG et al., 2014). RAS activates different scavenging pathways in certain types of cancers, but their main function is to promote nutrient uptake through both the extracellular and intracellular sources (Pylayeva-Gupta et al., 2011; Stephen et al., 2014). For example, KRAS driven pancreatic cells utilize extracellular glutamine to fuel the TCA cycle (Kamphorst JJ et al., 2015) and KRAS driven NSCLCs utilize glutamine released from cells which have undergone autophagy to promote TCA cycle (Guo JY et al., 2011; Strohecker AM and White E, 2014). Moreover, KRAS-driven cancer cells can generate acetyl-CoA from different branch chain amino acids (i.e., isoleucine, valine, and leucine) to fuel the TCA cycle (Mayers JR et al., 2014). A recent study demonstrated that copy

number gain of mutant KRAS responsible for tumor progression promotes glucose anaplerosis to generate intermediates of TCA cycle (Kerr EM et al., 2016).

Mutations in TCA cycle enzymes

Recent findings show that number of different cancers exhibit mutations or deregulations in different TCA cycle. These mutations and deregulations in enzymes ultimately result in characteristic metabolic and epigenetic changes that are correlated with disease transformation and progression.

1) Succinate dehydrogenase (SDH) is also known as complex II. It plays central role in both TCA cycle and ETC. SDH is a heterotetrametric enzyme complex consisting of 4 subunits (SDHA, SDHB, SDHC, and SDHD), which catalyzes the oxidation of succinate to fumarate in the TCA cycle, while simultaneously reducing ubiquinone to ubiquinol in the ETC (Chandel, 2015). SDH is classified as tumor suppressor gene. Mutations in SDH subunits and SDH assembly factor 2 (SDHAF2) have been identified mainly in hereditary paragangliomas (hPGLs) and pheochromocytomas (PCCs) (Baysal BE et al., 2002; Bayley JP et al., 2010; and Burnichon N et al., 2010). Neoplastic transformations result mainly because of loss of heterozygosity after a second mutation in the wild-type SDH allele (Gottlieb E and Tomlinson IP, 2005). Additionally, mutations in SDH have also been identified in gastrointestinal tumors, renal tumors, thyroid tumors, neuroblastoma, and testicular seminoma, implicating its importance in a wide range of cancer (Bardella C et al., 2011).

2) Fumarate hydratase (FH) is a homotetrameric cycle enzyme that catalyzes the stereospecific and reversible hydration of fumarate to L-malate. Beyond its mitochondrial role,

FH is also expressed in the cytoplasm where it participates in the urea cycle. FH is also important for synthesis of different amino acids and nucleotides (Adam J et al., 2014). Heterozygous mutations in FH have been found in multiple cutaneous and uterine leiomyomas (MCUL), as well as hereditary leiomyomatosis and renal cell cancer (HLRCC) (Launonen V et al., 2001; and Tomlinson IP et al., 2002). Additionally, mutations in FH have been identified in bladder, breast and testicular cancer (Carvajal-Carmona LG et al., 2006; Ylisaukko-oja SK et al., 2006).

3) Isocitrate dehydrogenase (IDH) The IDH family consists of three isoforms (IDH1, IDH2, and IDH3) that catalyze conversion of isocitrate to α-KG. IDH2 and IDH3 are expressed in the mitochondria, whereas IDH1 is expressed in the cytoplasm. IDH1 and IDH2 function as homodimers that require NADP+ as a co-factor for conversion of isocitrate to α-KG, whereas IDH3 is a heterodimer (IDH3A, IDH3B, and IDH3G) that can only oxidize isocitrate to α-KG and requires NAD+ as a co-factor (Chandel NS, 2015). IDH 1/2 mutations are mainly heterozygous missense mutations in active arginine residues involved in isocitrate binding. IDH1/2 mutations are mainly found in low-grade glioma and secondary glioblastoma (around 80%) but other malignancies such as acute myeloid leukemia (20%), angioimmunoblastic T-cell lymphomas (20%) have also been shown to have IDH1/2 mutations. Other malignancies such as thyroid, colorectal, and prostate cancer also have alterations in IDH1/2 (Yan H et al., 2009; and Yen KE et al., 2017). These neomorphic mutations lead to gain of function that enable conversion of α-KG to 2-hydroxyglutarate (2-HG) which is an oncometabolite. Beyond mutations detected for cycle enzymes, several studies have demonstrated that other cycle enzymes, Citrate synthase (CS), Aconitase (AH), and α-ketoglutarate dehydrogenase complex (KGDHC) are deregulated in different types of cancers. (Table 1.1)

Table 1.1: Other TCA cycle enzymes involved in different types of cancers

Other Enzymes in TCA Cycle	Function	Types of Cancer	References
Citrate Synthas (CS)	condensation of acetyl-CoA and oxaloacetate to form citrate	Pancreatic Ovarian Renal	Schlichtholz et al., 2005; Lin et al., 2012; Chen et al., 2014
Aconitase (AH)	conversion of citrate to isocitrate	Gastric Prostrate	Singh et al., 2006; Wang et al., 2013
α-ketoglutarate dehydrogenase complex (KGDHC)	conversion of α-ketoglutarate to succinyl-CoA and NADH	colorectal breast lung esophageal cervical, pancreatic	Hoque et al., 2008; Ostrow et al., 2009; Fedorova et al., 2015 Snezhkina et al., 2016

Drugs targeting mitochondrial bioenergetics

Metformin: Metformin is routinely used in treating diabetes as it is very effective in decreasing hepatic gluconeogenesis and diminish insulin levels in circulation. (Bailey CJ and Turner RC, 1996). Interestingly, few epidemiological studies have suggested that patients taking metformin to control their blood glucose levels are less likely to develop cancer (Evans JM et al., 2005). In addition, metformin also found to increase survival rate of patients who have developed cancer (Dowling RJ et al., 2012). Furthermore, multiple laboratory-based studies have also provided evidence that metformin may serve as an anticancer agent (Buzzai M et al.,2007; Memmott RM et al., 2010; and Tomimoto A et al., 2008). Strong preclinical and laboratory experiment data is available about effects of metformin on different types of tumors. Metformin is currently being used in several clinical trials in combination with current standard treatments. There are two main mechanisms of action for antitumor effects of metformin. First is through limiting circulatory insulin levels (Pollak M et al., 2014). Insulin is a known mitogen and it helps in

stimulation of protooncogenic PI3K pathway in most of the cancer cells. However, not all cancers are sensitive to insulin and thus reduction of insulin levels may not work in all types of cancers. Second mechanism by which metformin exerts antitumor effects is by blocking ETC complex I (El-Mir MY et al., 2000; and Wheaton WW et al., 2014) and directly affecting mitochondrial ATP production inducing cancer cell death by apoptosis (Bridges HR et al., 2014). Metformin also inhibits the biosynthetic capacity of the mitochondria to generate macromolecules (lipids, amino acids, and nucleotides) within cancer cells. The metformin uptake is facilitated by organic cation transporters (OCTs) that are present on few tissues such as liver and kidney. OCTs confer both safety profile and also limitations to metformin treatment for cancer therapy since only few types of tumors express OCTs (Emami Riedmaier A et al., 2013). Current clinical trials including metformin are focused on configuring active dose required to inhibit mitochondrial complex I.

Phenformin: Phenformin as another biguanide which is used as alternative to metformin for inhibition of ETC complex I. Phenformin is less polar and more lipid soluble hence exhibits higher specificity to mitochondrial membranes than metformin. Compared to metformin, phenformin gets readily transported into the cancer cells thus it shows greater antitumor activity. Recently, it has been demonstrated that phenformin also inhibits mitochondrial complex I to exert its antitumor effects in experimental models of cancer. Additionally, cells impaired in glucose utilization were most sensitive to phenformin (Birsoy K et al.,2014). An important drawback of phenformin clinically is that phenformin increases the incidence of lactic acidosis, and thus it has been withdrawn for use in humans in most parts of the world. Phenformin has showed great promise in experimental models of NSCLC driven by KRAS and LKB1 mutations

(Shackelford DB et al., 2013). Phenformin when used in combination with BRAF inhibitors showed great effect treatment of melanoma by inhibition of mitochondrial respiration.(Yuan P et al., 2013)

VLX600 is a lipophilic cation-based triazinoindolyl-hydrazone compound and mitochondrial oxidative phosphorylation (OXPHOS) inhibitor, with potential antitumor activity. In tumor cells, inhibition of OXPHOS by VLX600 induces a HIF1 a dependent shift to glycolysis (Zhang X et al., 2014). Thus, VLX600 showed great promise in treatment of tumors which have limiting glucose availability.

Cyclopamine, a known inhibitor of Hedgehog signaling pathway, has been shown to exhibit anti-carcinogenic properties. Cyclopamine tartrate (CycT), a water-soluble analog of cyclopamine, and is therefore, a better potential therapeutic agent. Cyclopamine and cyclopamine tartrate inhibit smoothened (SMO), which facilitates Hedgehog signaling. CycT imposes its anticancer effects through generation of ROS in mitochondria of cancer cells. It also induces mitochondrial fission and fragmentation in some NSCLC cell lines impeding mitochondrial respiration. It has shown to affect heme metabolism and mitochondrial function in vitro and in vivo in some NSCLC (Kalainayakan SP et al., 2018).

Heme, an iron metalloporphyrin IX performs diverse functions in mammalian cells, most importantly it acts as a cofactor or prosthetic group for number of complexes involved in ETC. Thus, heme plays a central role in energy generation by regulating mitochondrial function. Different NSCLC cells exhibit high heme flux and thus, targeting extracellular heme by engineered heme sequestering proteins (HSPs) has shown a great promise in controlling proliferation of different NSCLC lines in vitro and in vivo (Sohoni et al., 2019).

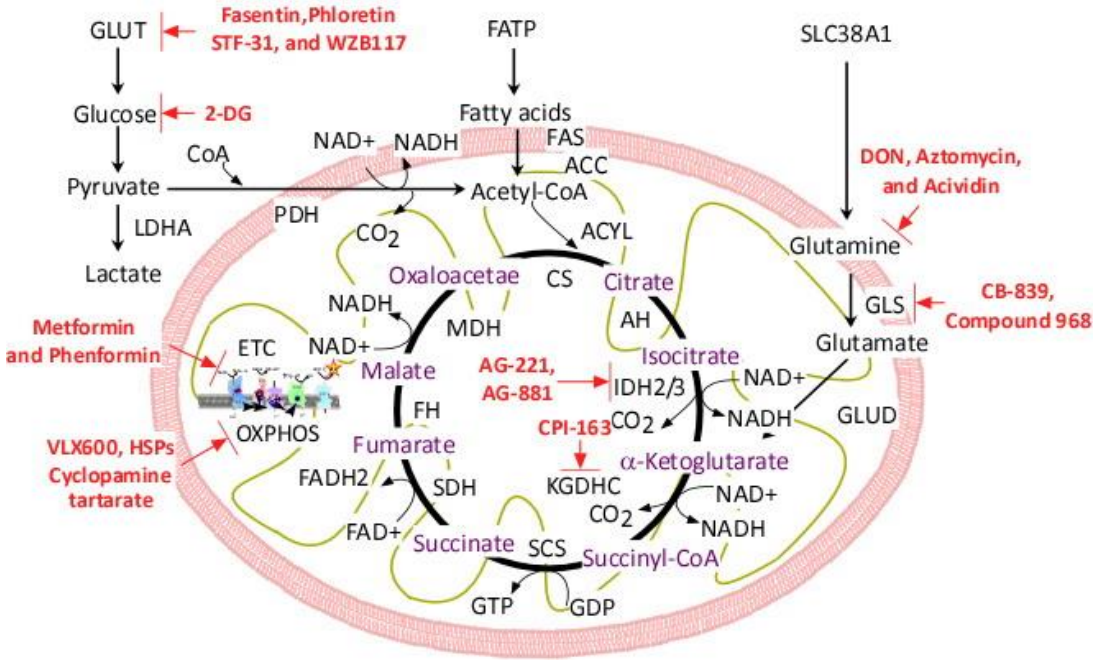

Figure 1.3: Drugs targeting Mitochondria and TCA Cycle

1) Drugs targeting mitochondrial metabolism and TCA cycle:

Glutamine analogs: Many tumors use glutamine as an alternative fuel source to feed TCA cycle. Small molecule inhibitors offer attractive strategy for therapeutically targeting these tumors. Analogs of glutamine, 6-diazo-5-oxo-L-norleucine (DON), azotomycin and acivicin isolated are originally from Streptomyces. DON binds different glutamine using enzymes irreversibly rendering them inactive. DON has been shown to inactivate glutaminase at low micromolar levels (Thomas AG et al., 2013) as well as multiple glutamine amidotransferases (Cervantes-Madrid D et al., 2015) involved in de novo purine and pyrimidine synthesis (Hartman SC et al., 1963; Livingston RB et al., 1970), coenzyme synthesis (La Ronde-Le Blanc N et al.,2009), amino acid synthesis (Jayaram HN et al.,1976), and hexosamine production (Walker B et al.,

2000). The kinetics of inhibition and inactivation have been described for some, though not all, of DON's target enzymes. At far higher concentrations DON also serves as a substrate and an inhibitor of several amino acid transporters and transglutaminases (Livingston RB et al., 1970), as well as a number of amino acid synthesis reactions more relevant in prokaryotic systems (Goto Y et al., 1976). Azotomycin and acivicin share similar mechanism of action as DON since they are also structural analogs of glutamine. (Ovejera et al., 1979; Ahluwalia et al., 1990; Griffiths et al., 1993). These compounds although effective have failed to enter clinics because of their tissue toxicities. Another approach to target glutamine limitation is by blocking glutamine transport by using Sulfasalazine (Oettgen HF et al., 1967; Lo M et al., 2008; Chan WK et al., 2014; Parmentier JH et al., 2015; Rodman SN et al., 2016; Roh JL et al., 2016; and Shitara K et al., 2017).

GLS inhibitors: Glutaminase (GLS), which is responsible for the conversion of glutamine to glutamate, plays a vital role in up-regulating cell metabolism for tumor cell growth and is considered to be a valuable therapeutic target for cancer treatment. Based on this important function of glutaminase in cancer, several GLS inhibitors have been developed. CB-839 directly targets glutaminase and affects the conversion of glutamine to glutamate which in turn alters several downstream pathways, including the TCA cycle, glutathione production, and amino acid synthesis (Gross S et al., 2010 and Jacque N et al., 2015). CB-839 has been tested for the treatment of various cancers, and it is currently being evaluated in phase 1 and 2 clinical trials for treatment of hematological and solid tumors. Other compounds compound 968 (Wang JB et al., 2010) and bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide81, diminish glutamine catabolism and delay tumor growth in experimental models of cancer. Use of some

non-specific inhibitors targeting conversion of glutamine to a-ketoglutarate by aminotransferases have also showed promise in some studies. (Thornburg, J.M. et al.,2008 and Qing G et al., 2012)

CPI-163: Pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDHC) both enzymes require lipoate as co-factor. CPI-613 is an analog of lipoate thus, it can simultaneously inhibit both PDH and KGDHC. CPI-613 stimulates phosphorylation and inhibition of PDH via activation of PDK (Zachar Z et al., 2011), CPI-613 acts on DLD (the E3 component of KGDHC) and also leads to suppression of the E2 subunit of KGDHC, DLST (Stuart SD et al., 2014) these effects induce a large amount of mitochondrial ROS. Currently, CPI-613 is being tested in phase I and II clinical trials, as a single agent or in combination with standard chemotherapy, to treat cancers (NCT02168140, NCT01902381, NCT02232152, and NCT01766219).

Small molecule inhibitors for IDH1/2: Mutations in TCA cycle gene IDH2 provide a unique opportunity for therapeutic intervention. Several small molecule inhibitors of mutant IDH2 are currently in clinical development out of which small molecule, Enasidenib (AG-221) that inhibits mutant IDH2 and compound AG-881 that targets both IDH1 and IDH2 have shown great outcomes in initial trials. These compounds directly bind to the active site of mIDH1/2 enzymes and locks the enzyme in inactive state thus, preventing conformational change required to convert α-KG into 2-HG. AG-221 can be orally administered for mutant IDH2-R140 and IDH2-R172 (Yen K et al., 2017), and is currently undergoing phase I/II clinical trials as a single agent for the treatment of AML and solid tumors (e.g., glioma and angioimmunoblastic T-cell lymphoma; NCT01915498 and NCT02273739, respectively. AG-881, which targets both IDH1 and IDH2 can be orally administered inhibitor. It has been shown to cross blood-brain barrier and hence, may serve as a better option for glioma patients (Medeiros BC et al., 2017). Currently,

AG-881 is in phase I clinical trial for AML patients with mutant IDH1/2, and a clinical trial for patients with glioma will begin soon (NCT02492737 and NCT02481154).

2) Drugs targeting mitochondrial redox: The high levels of mitochondrial ROS produced during ETC can play significant role in tumorigenesis and proliferation of cancer cells by regulating downstream pathways. Excess of ROS is toxic for the cancer cells hence to negate toxic effects of ROS, cancer cells increase their antioxidant capacity (DeNicola GM. et al., 2011). Mitochondria are proximal to the signaling pathways that are responsive to ROS. Thus, mitochondrially targeted antioxidants effectively reduce cell proliferation in vitro and tumorigenesis in vivo (Weinberg, F et al., 2010; Cheng G et al., 2012; and Nazarewicz, RR et al., 2013). Elesclomol is known to induce oxidative stress in cancer cells, creating high levels of reactive oxygen species (ROS), such as hydrogen peroxide, in both cancer cells and normal cells (Kirshner JR et al., 2008) The increase in oxidative stress beyond baseline levels elevates ROS beyond sustainable levels, exhausting tumor cell antioxidant capacity, which may result in the induction of the mitochondrial apoptosis pathway. Normal cells are spared because the increase in the level of oxidative stress induced by this agent is below the threshold at which apoptosis is induced. Elesclomol is currently in phase 1 clinical trial for the treatment of different solid tumors.

Phenethyl isothiocyanate (PEITC) is present as gluconasturtiin in many cruciferous vegetables with remarkable anti-cancer effects. PEITC is known to exert its antic cancer effects by blocking the initiation phase of cancer as well as inhibit progression of tumorigenesis. PEITC inhibits proliferation, progression and metastasis by directly blocking various proteins involved in these pathways. Pre-clinical studies have shown that combination of PEITC with conventional

anti-cancer agents exhibits additive effects in reducing the tumor burden. Based on accumulating evidence, PEITC appears to be a promising agent for cancer therapy and is already under clinical trials for leukemia and lung cancer (Gupta P et al., 2014).

1.2 Conclusion

Although significant advances are made during the last two decades in cancer therapy they failed to make significant impact in improving overall survival rates for cancer. Heterogeneity and presence of multiple driver genes poses biggest hurdle in designing therapies for cancer treatment. Recently, the idea of targeting bioenergetics for cancer therapy has attracted a lot of interest. All tumors mainly metabolize fuels through glycolysis and oxidative phosphorylation. Cancer cells can metabolize an array of fuels using conventional metabolic pathways to produce energy, synthesize biosynthetic precursors, and maintain redox balance. Metabolic reprograming is the result of mutations in different oncogenes and tumor suppressor genes. Cancer cells use metabolic reprograming to survive, proliferate, migrate and metastasize. For a successful metabolism-based therapy it is paramount to identify a potential "oncometabolic" enzyme that is responsible for metabolic reprograming in cancer cells. A detailed understanding of complex network associated with mutated enzymes also plays a significant role in designing specific targeted therapies against different types of cancers. Various drugs targeting glycolysis and OXPHOS have shown very encouraging results but all them have certain limitations. Although roles of glycolysis and TCA cycle in tumorigenesis are clearly established, their involvement in cancer metabolism is still not completely understood. Additionally, the complex network of the glycolysis and TCA cycle other biochemical and cell signaling pathways in tumor

microenvironment is yet to be characterized in detail. The intensive research effort in the coming years will undoubtedly deepen our understanding of the role of these central metabolic pathways in tumorigenesis, reveal vulnerabilities for therapeutic intervention, but also discovery of novel therapeutic targets.

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CHAPTER 2

INCREASED HEME FLUX TRIGGERS MITOCHONDRIAL RESPIRATION AND TUMORIGENIC FUNCTIONS IN NON-SMALL CELL LUNG CANCER CELLS[1]

2.1 Introduction

Lung cancer is the leading cause of cancer related death in United States. Its death rate is more than that of three most common cancers combined namely: colon, breast, and pancreatic cancer. Although there are several chemotherapeutic and targeted agents approved for treatment of lung cancer, the 5-year survival rate is around 18% (Siegel RL et al., 2015).

There are two main subtypes of lung cancer, small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC), accounting for 15% and 85% of all lung cancer, respectively (Sher T et al., 2008). NSCLC is further classified into three types: squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma. Squamous-cell carcinoma comprises 25–30% of all lung cancer cases and it shows strong correlation with cigarette smoking (Kenfield SA et al., 2008). Adenocarcinoma is the most common type of lung cancer and it constitutes 40% of all lung cancer. Compared to other types of lung cancer, adenocarcinoma tends to grow slower and has a greater chance of being found before it has spread outside of the lungs. Large cell (undifferentiated) carcinoma accounts for 5–10% of lung cancers. This type of carcinoma shows no evidence of squamous or glandular maturation and as a result is often diagnosed by default through exclusion of other possibilities. Large cell carcinoma often begins in the central part of

[1] The contents of this chapter are taken from an article published in AACR Cancer Research: **Cancer Res; 79(10) May 15, 2019; 2511 – 2525; Sohoni et al.**

the lungs, sometimes into nearby lymph nodes and into the chest wall as well as distant organs (Brambilla E et al., 2004). Large cell carcinoma tumors are strongly associated with smoking (Muscat JE et al., 1997).

Tobacco prevention is a very ambitious goal for the global fight against cancer and requires a comprehensive approach. Although tobacco prevention is a crucial component of the fight against lung cancer, it is not enough to win the war. Increasingly sophisticated therapies are required to meaningfully improve clinical outcomes for patients. Over the past two decades a substantial progress is made regarding applications of different technologies for better diagnosis and therapy. Although substantial progress is made with the advent of various targeted therapies and effective application of immunotherapy, their effectiveness is limited because of presence of multiple driver genes and intra-tumoral genetic heterogeneity. Thus, alternative strategies are still needed for lung cancer treatment. Targeting bioenergetics of cancer cell is one of the important strategies that can be applied for variety of tumors including NSCLC tumors. Cancer cells can exploit option of using multiple fuels but like normal cells, they can metabolize these fuels only through two fundamental pathways, glycolysis and oxidative phosphorylation.

Recent studies carried out on metabolism of ^{13}C-glucose in lung cancer patients show that both glycolysis and oxidative phosphorylation occurs simultaneously in lung tumors (Fan TW et al., 2009; and Hensley CT et al., 2016). Contrary to Warburg's hypothesis many studies over the past two decades have sown importance of mitochondrial OXPHOS and TCA cycle in development of different types of cancers including non-small cell lung cancer. Although NSCLC tumors are metabolically heterogeneous, a common feature among NSCLCs is that pyruvate from elevated glycolysis enters and intensifies the TCA cycle (Hensley CT et al., 2016).

Intensified TCA cycle provides more TCA intermediates for biosynthesis and more NADH for ATP generation via OXPHOS. In a follow-up study using ^{13}C-lactate tracers, Faubert et al. showed that lactate also fuels the TCA cycle in molecularly heterogeneous tumors (Faubert B et al., 2017). A separate study using two genetically engineered mouse models for lung cancer carrying different genetic mutations (KrasLSL-G12D/+Trp53-/- and KrasG12D/+Stk11-/-) also showed that the contribution of lactate to the TCA cycle is higher than that of glucose (Hui S et al., 2017). Additionally, components of OXPHOS complexes and markers of mitochondrial biogenesis are found to be highly predictive of reduced overall survival in NSCLC patients (Sotgia F et al., 2017). Likewise, another study showed that the expression of OXPHOS genes is negatively correlated with the prognosis of lung adenocarcinoma and that inhibition of OXPHOS activity impedes the migration and invasion of cisplatin-resistant cells (Jeon JH et al., 2016). Strikingly, several recent studies showed that drug-resistant cells of acute and chronic myeloid leukemia, breast cancer, lung cancer, and melanoma depend on OXPHOS and that targeting oxidative metabolism and mitochondrial respiration overcomes drug resistance in these cells (Farge T et al., 2017; Kuntz EM et al., 2017; and Lee KM et al., 2017).

Oxidative metabolism involving TCA cycle and OXPHOS is sustained by multiple fuels and requires coordinated functions of many enzyme subunits. Evidently, despite high molecular and metabolic heterogeneity, NSCLC cells and other resistant cancer cells share the common feature of elevated oxidative metabolism (Farge T et al., 2017; Kuntz EM et al., 2017; Lee KM et al., 2017; and Hooda J et al., 2013). However, the mechanism by which diverse and heterogeneous cancer cells develop such a common feature, involving multiple fuels and numerous enzyme subunits, is not immediately evident. Intriguingly, we previously reported that several types of

human lung tumor xenografts display elevated levels of the rate-limiting heme biosynthetic enzyme ALAS1, heme transporters, and oxygen-utilizing hemoproteins (Hooda J et al., 2013). Heme is a central metabolic and signaling molecule which regulates diverse molecular and cellular processes relating to oxygen utilization and metabolism. Heme serves as a prosthetic group in proteins and enzymes involved in oxygen transport, utilization, and storage, such as globins and cytochromes (Ortiz de Montellano PR., 2009). One crucial function of mitochondria is to use oxygen to carry out OXPHOS for ATP generation. Thus, heme function and mitochondrial respiration are tightly linked. Heme synthesis occurs in mitochondria, and mitochondria cannot function without heme. Multiple subunits in OXPHOS complexes II-IV contain heme. Additionally, heme acts as a signaling molecule to coordinate the expression of genes encoding OXPHOS complexes and the assembly of these complexes (Padmanaban G et al., 1989 and Kim HJ et al., 2012). Heme binds to and directly regulates the activities of many proteins controlling processes ranging from tyrosine kinase signaling to microRNA processing (Yao X et al., 2010; Barr I et al., 2012; and Kuhl T et al., 2014). Clearly, heme possesses unique signaling and structural properties that enable it to coordinate elevated mitochondrial OXPHOS in diverse cancer cells.

Here, to ascertain whether heme underpins the common feature of elevated oxidative metabolism in diverse NSCLC cells, we characterized an array of molecular and cellular characteristics relating to heme in several types of NSCLC cells. We show that the levels of the rate-limiting heme biosynthetic enzyme and heme uptake proteins are highly elevated in several types of non-small cell lung cancer (NSCLC) cells. Intracellular heme levels in mitochondria are increased in NSCLC cells. The increased heme levels are accompanied by elevated levels of

oxygen-utilizing hemoproteins. Elevated levels of heme and hemoproteins correlate strongly with intensified oxygen consumption rates and ATP generation.

2.2 Results

NSCLC cell lines show high heme flux and mitochondrial heme levels

To understand the degree of elevation and heterogeneity of heme metabolism and flux in lung tumors, we measured heme biosynthesis, uptake, and degradation in several representative types of NSCLC cell lines. These include H1299 (with Nras Q61K p53 null), A549 (with Kras G12S, LKB1 Q37*), H460 (with Kras K61H LKB1 Q37*), Calu-3 (with Kras G13D p53 M237I mutations), and H1395 (with LKB1 deletion). We also used two pairs of cell lines representing normal lung epithelial cells (HBEC30KT and NL20) and tumorigenic cell lines (NSCLC line HCC4017 and NL20-TA) (Whitehurst AW et al., 2007). Clearly, heme biosynthesis (Fig. 2.1A) and uptake (Fig. 2.1B) were both increased in NSCLC cell lines and NL20-TA, although the increases varied considerably among different cell lines. When the folds of increase in heme biosynthesis and uptake were added for every cell line (Fig. 2.1C), they varied from 2- to 8-fold among different lung tumor cell lines. Increases in heme biosynthesis and uptake correlated with increases in the rate-limiting heme biosynthesis enzyme ALAS1 (Fig. 2.1E; r=0.90, p-value=0.0003) and the cell membrane heme uptake protein SLC46A1 (SLC46A1) (Fig. 2.1F; r=0.70, p-value=0.02), respectively.

Heme degradation was also elevated in NSCLC cell lines relative to non-tumorigenic cell lines, albeit to a varying degree (Fig. 2.1D). This increase correlated with the increase in HMOX1 enzyme (Fig. 2.1G; r=0.70; p-value=0.02). Iron is an essential nutrient and is closely linked to heme (Beutler E et al., 2009). Heme synthesis in non-erythroid cells is generally not affected by

iron (Anderson KE et al., 2009). Nonetheless, we detected the levels of transferrin receptor (TFRC), which is responsible for cellular iron uptake from the circulation (Kawabata H., 2018). We found that TFRC levels were increased in some, while unaffected or decreased in other NSCLC cell lines (Fig. 2.1H). This is consistent with the idea that iron availability is not a limiting factor in NSCLC cells.

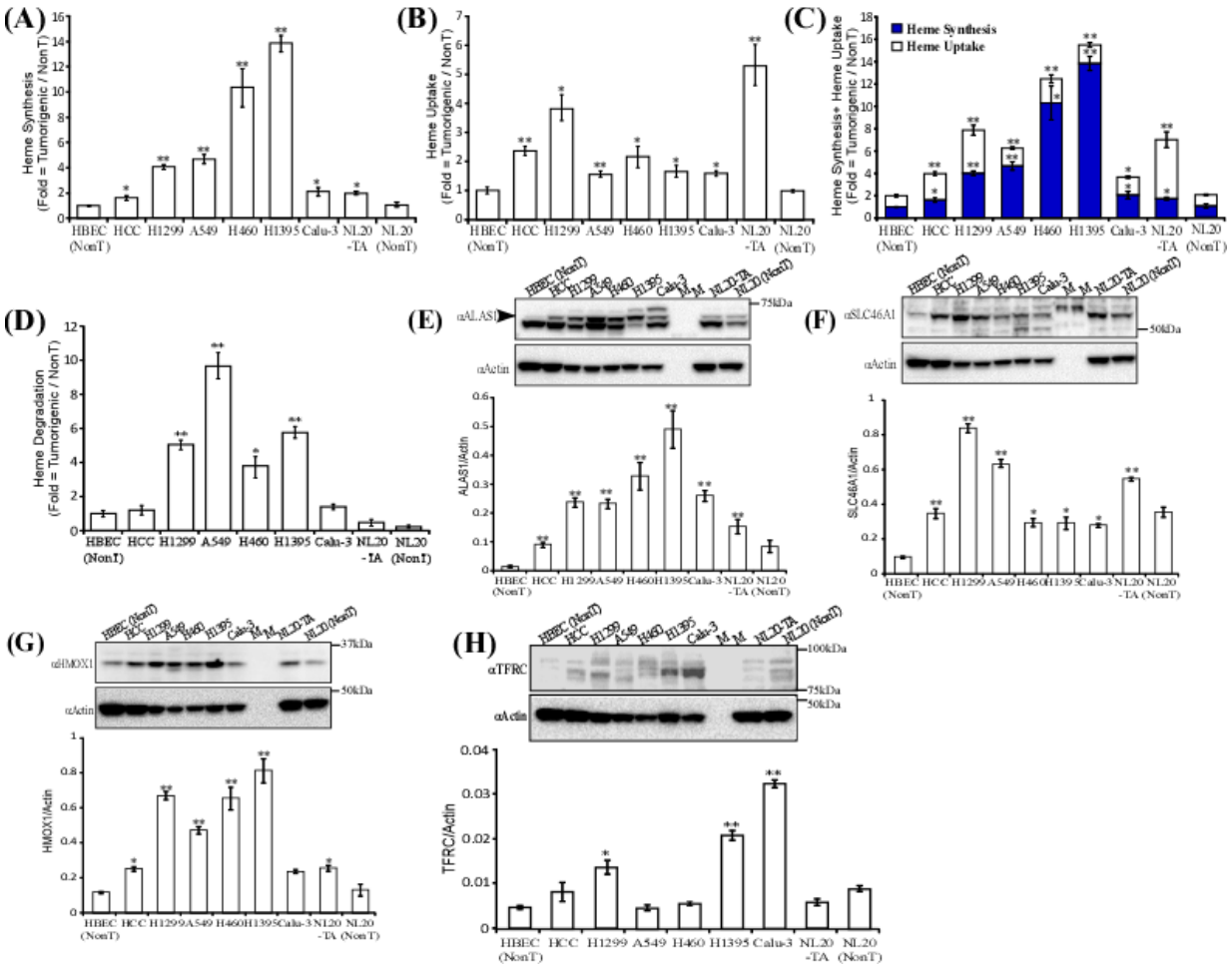

Figure 2.1: NSCLC cells exhibit high levels of heme flux. *(*A) Levels of heme synthesis (HS) (B) Heme uptake (HU) and (C) Total heme flux are elevated to varying degrees in NSCLC cells. (D) Levels of heme degradation are elevated in different NSCLC cells. (E) Levels of heme synthesis enzyme ALAS1 and (F) heme transporter SLC46A1 are elevated in NSCLC cells and correlate with HS and HU respectively. (G) levels of heme degradation enzyme HMOX-1 in different NSCLC cells (I) levels of iron transporter TFRC in different NSCLC cells. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells

were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

To determine how elevated heme metabolism in NSCLC cells affects subcellular heme levels, we used a series of previously developed subcellular peroxidase reporters designed to detect subcellular heme levels in mitochondria, cytosol, nuclei, and other organelles (Yuan X et al., 2016). All lung cell lines were efficiently transfected with the reporter plasmids, except for Calu-3, which did not allow efficient transfection of reporter plasmids. Clearly, the mitochondrial heme levels in NSCLC cell lines and the tumorigenic NL20-TA cell line were elevated relative to non-tumorigenic cell lines (Fig. 2.2A). The increase in heme synthesis and uptake was correlated with the increase in intracellular mitochondrial heme levels: r=0.68, p-value=0.03. Heme levels in other organelles were also increased in some tumor cell lines, but increases were not uniform (2.3A-2.3E). The increase in heme synthesis and uptake was not correlated significantly with heme levels in other organelles. Mitochondrial heme is crucial for OXPHOS formation and function. Our data suggest that increased heme synthesis and uptake in NSCLC cells leads to elevated mitochondrial heme levels.

Elevated levels of mitochondrial heme triggers respiration and ATP generation in NSCLCs

Next, we measured a series of bioenergetic and tumorigenic parameters. The rates of oxygen consumption (Fig. 2.2B) and levels of intracellular ATP (Fig. 2.2 C) were elevated in tumorigenic cell lines, except for Calu-3, relative to non-tumorigenic cell lines. Elevated oxygen consumption should be accompanied by increased levels of mitochondrial OXPHOS enzymes. Increased expression of mitochondrial proteins should be facilitated by regulators promoting mitochondrial biogenesis, such as NRF1 and TFAM (36).

Indeed, the levels of cytochrome c (CYCS) and COX4I1 (subunits of OXPHOS complexes), as well as the hemoprotein PTGS2, were elevated in NSCLC cell lines relative to non-tumorigenic cell lines (Figs. 2.2D-2.2F). Notably, two important regulators promoting mitochondrial biogenesis, NRF1 and TFAM (Figs. 2.2G & 2.2H), were also upregulated in tumorigenic cell lines relative to non-tumorigenic cells lines. Thus, these observations are consistent with increased oxygen consumption rates and ATP levels in tumorigenic cell lines.

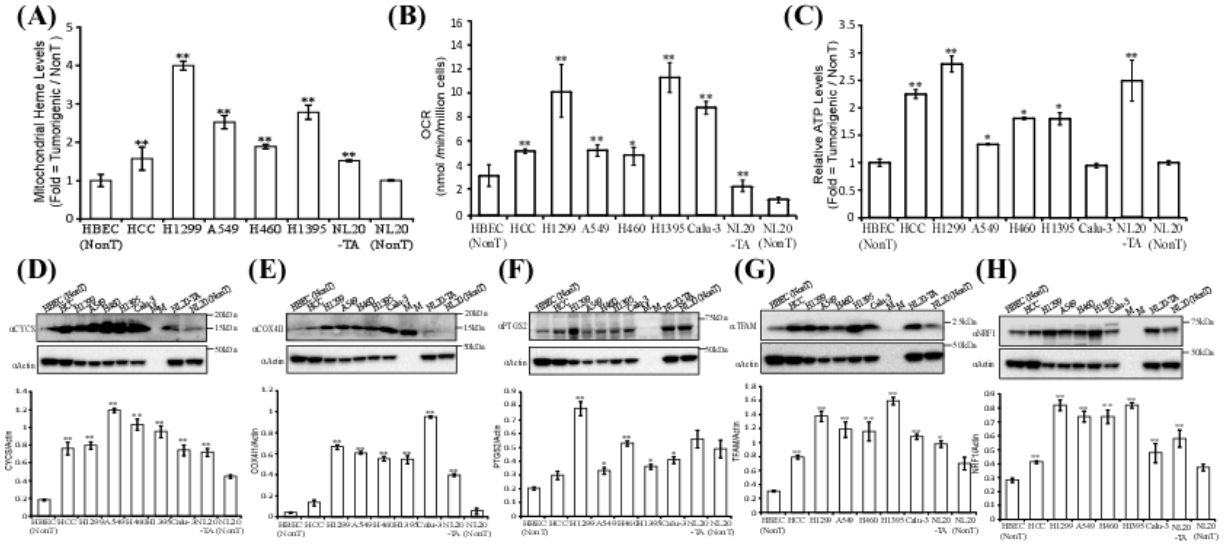

Figure 2.2: High heme flux in NSCLC cells triggers mitochondrial respiration. *(*A) Mitochondrial heme levels in different NSCLC cell lines measured using heme reporter plasmid (B) Oxygen consumption rate (OCR) in different NSCLC cells and (C) Total ATP levels are elevated NSCLC cells. Levels of different OXPHOS complexes and mitochondrial proteins are elevated in different NSCLC cell lines (D) Cytochrome C (CYCS). (E) COX 4I1 (F) COX2 (PTGS2) (G) TFAM and (H) NRF1. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

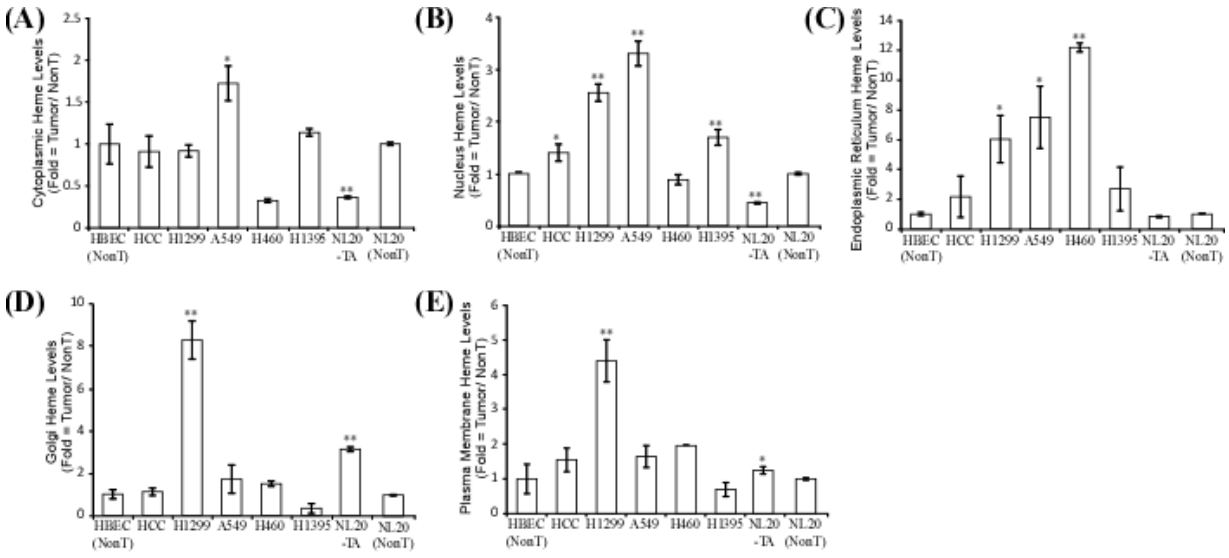

Figure 2.3: Heme levels in different cellular compartments of NSCLC cell lines. (A) Cytoplasm (B) Nucleus (C) Endoplasmic reticulum (D) Golgi and (E) Plasma membrane. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

Different NSCLC cell lines exhibit differences in their tumorigenic properties

Measurements of migration (Fig. 2.4A) and invasion (Fig. 2.4B) in NSCLC cell lines showed that they exhibit varying degrees of tumorigenicity. Interestingly, we found that the invasive capabilities of NSCLC cell lines, oxygen consumption rates, and intracellular ATP levels were well correlated with mitochondrial heme levels. The correlation coefficients are as follows: mitochondrial heme and oxygen consumption rates: Pearson r=0.72, p-value=0.02; mitochondrial heme and ATP levels: r=0.78, p-value=0.01; and mitochondrial heme and invasion: r=0.71, p-value=0.05. Together, these results strongly suggest that elevated heme biosynthesis and uptake in NSCLC cell lines lead to elevated levels of mitochondrial heme and OXPHOS subunits, which cause intensified oxygen consumption, ATP generation, and tumorigenic capabilities in NSCLC cells.

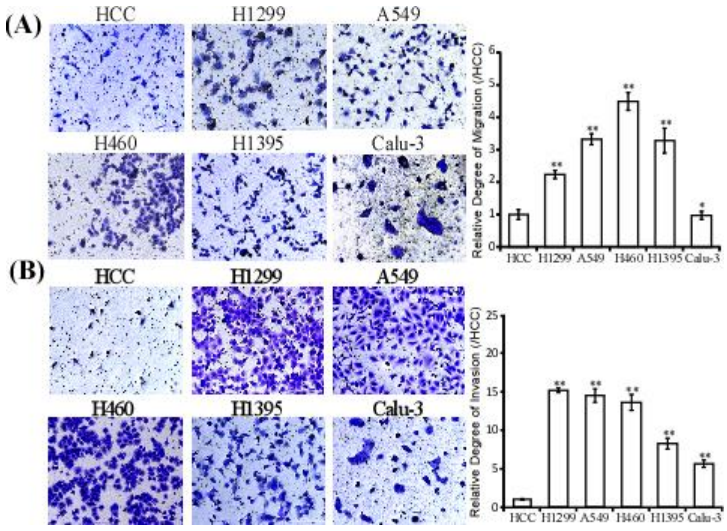

Figure 2.4: Tumorigenic properties of different NSCLC cell lines. (A) Migration and (B) Invasion. Data compared with fold change of non-tumorigenic cancer line HCC4017. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

Proteins and enzymes relating to heme and mitochondrial functions are upregulated in human NSCLC tissues relative to control normal tissues

We further confirmed the importance of enhanced levels of proteins/enzymes relating to heme function and mitochondrial respiration in lung cancer. Figs. 2.5A & 2.5B show that the levels of the rate-limiting heme synthetic enzyme ALAS1 and the heme transporter SLC48A1 (SLC48A1) were both significantly enhanced in human NSCLC tissues relative to normal tissues. In the same vein, the heme-containing cytochrome c (Fig. 2.5C) and cyclooxygenase-2 (PTGS2) (Fig. 2.5D) were enhanced in human NSCLC tissues relative to normal tissues. Both cytochrome c (CYCS) and PTGS2 levels have previously been shown to be elevated in NSCLC cell lines and xenograft tumors (16). Notably, the levels of the mitochondrial biogenesis regulator TFAM were also enhanced in human NSCLC tissues (Fig. 2.5E), as is the case in NSCLC cell lines (Fig. 2.3G). Together, data from human NSCLC tissues, NSCLC cell lines, and xenograft tumors show that

proteins/enzymes relating to heme function and mitochondrial respiration are upregulated in NSCLC cells and tumors.

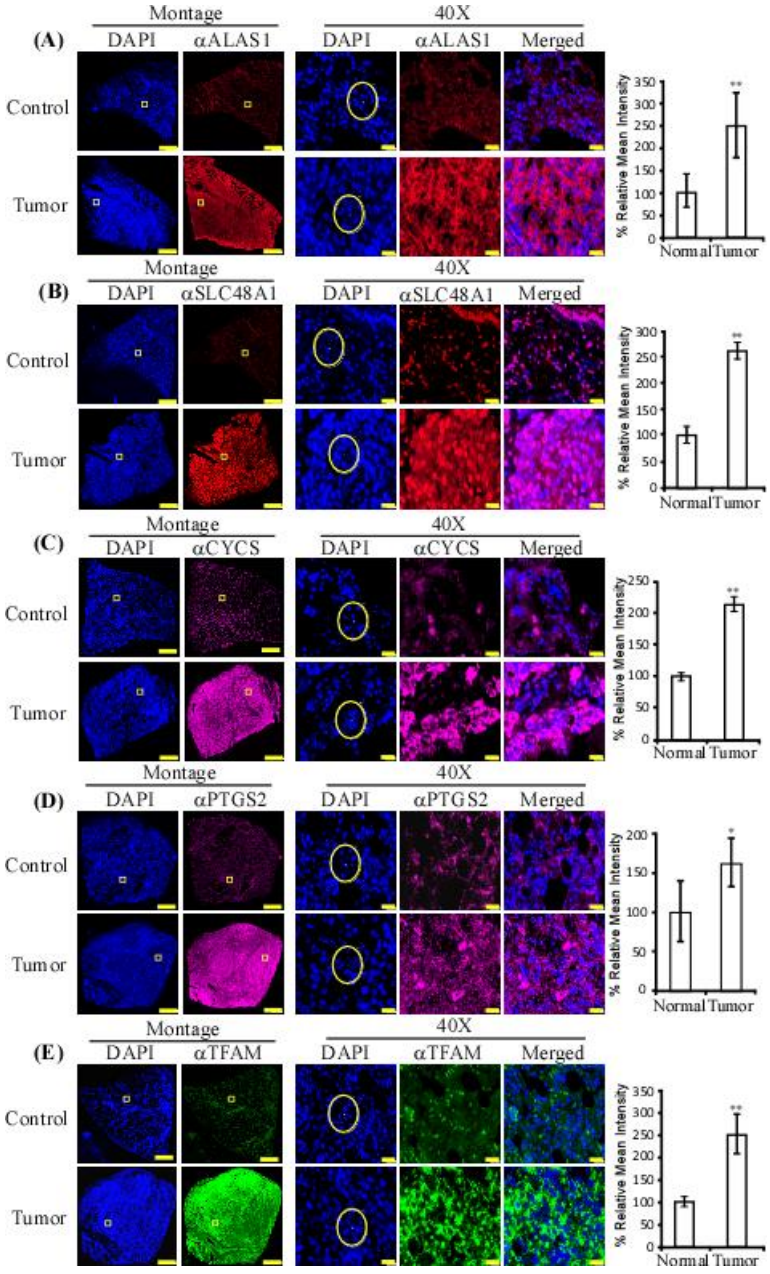

Figure 2.5: Levels of proteins related to heme flux and mitochondrial function are elevated in human NSCLC tissue samples compared to benign lung. (A) ALAS1 (B) SLC48A1 (C) CYCS (D) PTGS2 and (E) TFAM. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

Overexpression of ALAS1 or SLC48A1 promotes oxygen consumption, ATP generation, tumorigenic functions of NSCLC cells and tumor growth

To further ascertain the importance of heme in promoting NSCLC tumors, we generated NSCLC cell lines that overexpress the rate-limiting heme synthesis enzyme ALAS1 or the heme uptake protein/transporter SLC48A1 (Fig. 2.6A and 2.6B). We confirmed that relative to control cells, cells overexpressing ALAS1 exhibited elevated heme synthesis (Figs. 2.6C & 2.6D) while cells overexpressing SLC48A1 exhibited elevated heme uptake (Figs. 2.6E & 2.6F). These cells also showed elevated oxygen consumption (Fig. 2.6H).

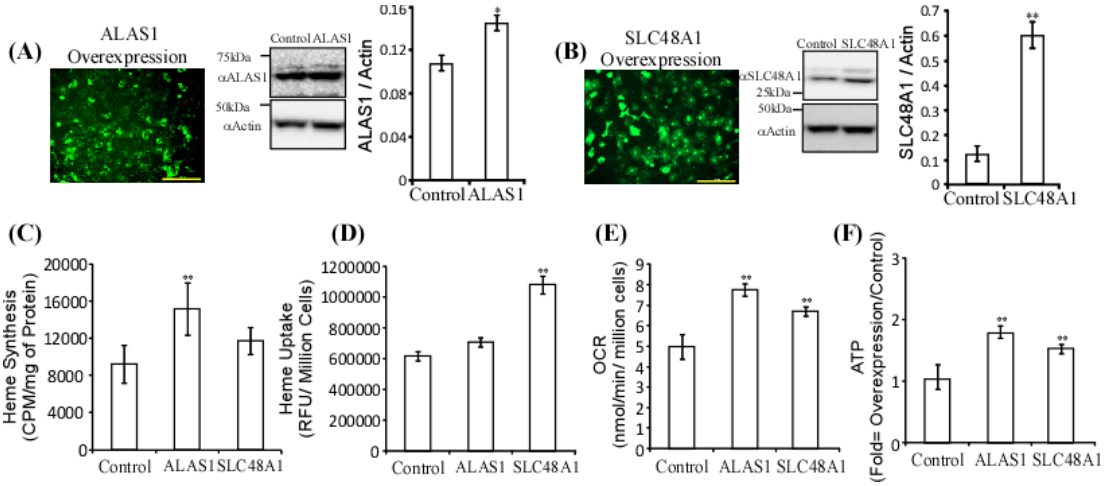

Figure 2.6: Overexpression of ALAS1 and SLC48A1 contributes in increased oxygen consumption and ATP generation. (A) Western blots and fluorescence imaging confirming stable overexpression of ALAS1 and (B) SLC48A1 (C) H1299 cell line overexpressing ALAS1 showed increase in heme synthesis (D) H1299 cell line overexpressing SLC48A1 showed increase in heme uptake (E) Increase in heme flux in overexpression lines leads to increase in OCR and (F) Total ATP. Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

Importantly, these cells overexpressing ALAS1 or SLC48A1 exhibited enhanced migration (Fig. 2.7A), invasion (Fig. 2.7B), and colony formation (Fig. 2.7C). When these cells were implanted subcutaneously in NOD/SCID mice, they form bigger tumors than control cells (Figs. 2.7D &

2.7E). Furthermore, tumors overexpressing ALAS1 or SLC48A1 exhibited elevated levels of oxygen consumption (Fig. 2.8F) and ATP generation (Fig. 2.8G). Taken together, these results strongly support the idea that increased heme availability resulting from elevated heme synthesis or uptake leads to higher oxygen consumption and ATP generation, which in turn fuels NSCLC cell tumorigenic functions and tumor growth.

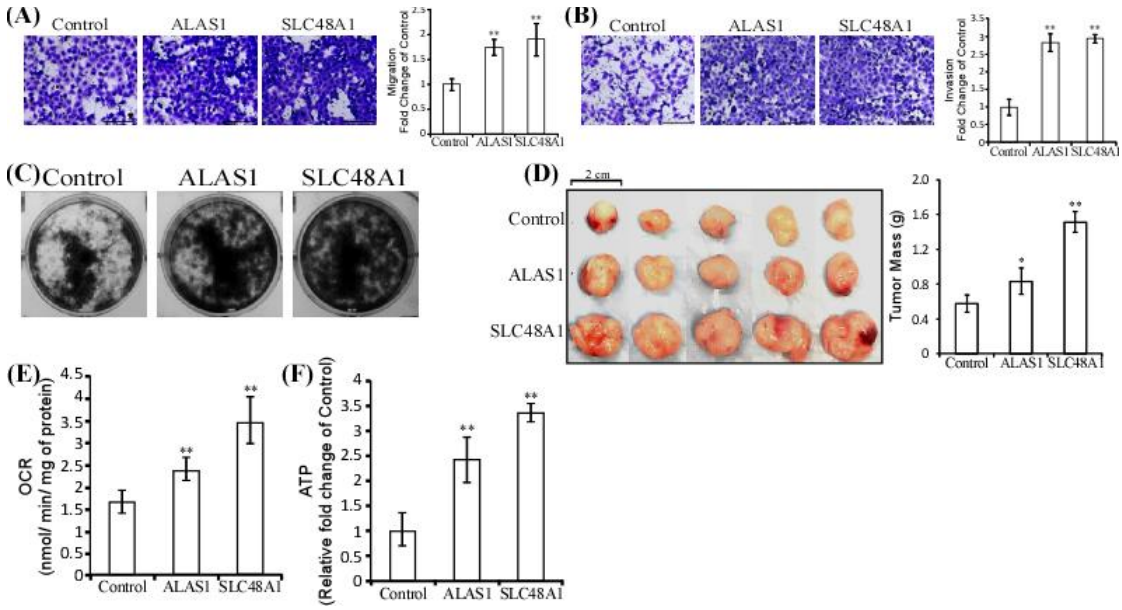

Figure 2.7: Increased heme flux and mitochondrial respiration triggers tumorigenic functions in NSCLC cells. (A) H1299 cells with overexpression of ALAS1 and SLC48A1 show elevated levels of migration (B) Invasion and (C) colony formation (D) Subcutaneous implantation of H1299 cells overexpression cell lines show increase in tumor mass (E) OCR and ATP (n=5/group). Data are plotted as mean ± SD. For statistical analysis, the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

2.3 Discussion

According to Warburg's hypothesis, tumor cells metabolize glucose and generate lactate at higher levels than normal cells despite the presence of ample oxygen, a phenomenon called the Warburg effect (Warburg O., 1930). However, elevated glucose consumption and glycolysis in

cancer cells do not lead to decreased OXPHOS, as tumor cells are capable of metabolizing variety of fuels (Hensley CT et al., 2016 and Faubert B et al., 2017). Numerous previous studies have shown that mitochondrial oxidative phosphorylation is intact in most tumors and high glycolytic rates occur concomitantly with oxidative phosphorylation (OXPHOS) in cells of most tumors (Alam MM et al., 2016). More recent studies have demonstrated the importance of mitochondrial OXPHOS in the growth and progression of several types of tumors (Viale A et al., 2014; LeBleu VS et al., 2014; and Tan AS et al., 2015). Further, several studies demonstrated that oxidative metabolism and OXPHOS are essential factors that contribute in developing drug resistance in most cancer cells and cancer stem cells. Farge et al. showed that OXPHOS contributes to acute myeloid leukemia resistance to cytarabine (Farge T et al., 2017). Kuntz et al. showed that targeting mitochondrial OXPHOS eradicates drug-resistant CML stem cells (Kuntz et al., 2017). Lee et al. showed that MYC and MCL1 confer chemotherapy resistance by increasing mitochondrial OXPHOS in cancer stem cells in triple negative breast cancer (Lee KM et al., 2017).

Heme is a central molecule in mitochondrial OXPHOS and in virtually all processes relating to oxygen transport, storage, detoxification, and utilization (Ortiz de Montellano PR 2009 and Padmanaban G et al., 1989). Heme serves as an essential prosthetic group or cofactor for many proteins and enzymes that bind and use oxygen, such as cytochrome P450 and nitric oxide synthases (NOSs), and that detoxify ROSs, such as catalase and peroxidases. Heme is extremely important for the function of three OXPHOS complexes, II, III, and IV. Multiple subunits in complexes III and IV require heme as a prosthetic group, and different forms of heme are present (Alam et al., 2016)). Furthermore, heme serves as a signaling molecule that directly regulates

diverse processes, including the expression and assembly of OXPHOS complexes (Padmanaban G et al., 1989 and Kim HJ et al., 2012). Conversely, heme synthesis occurs in mitochondria and requires oxygen (Anderson KE., 2009). Thus, heme and mitochondrial biogenesis are linked and are inter-dependent. Previously, our studies showed that the levels of the rate-limiting heme biosynthetic enzyme ALAS1, heme uptake and transport proteins SLC48A1 and SLC46A1, and oxygen-utilizing hemoproteins, including CYP1B1 and PTGS2, are highly elevated in NSCLC tumors (Hooda J et al., 2013 and Hooda J et al., 2014). Other studies also showed that the expression of proteins involved in mitochondrial respiration and heme function are elevated in the tumor tissues of NSCLC patients (Sotgia F et al., 2017 and Lam TK et al., 2014). Additionally, epidemiological studies indicated a positive association between intake of heme from meat and lung cancer (Tasevska N et al., 2009).

Here, we show that NSCLC cells exhibit elevated levels of heme biosynthesis and uptake, along with increase in rate-limiting heme biosynthetic enzymes and heme transporters, relative to non-tumorigenic lung cells (Fig. 2.1). Increased heme biosynthesis and uptake in turn lead to elevated mitochondrial heme levels (Fig. 2.2A). Based on the levels of heme synthesis in normal medium and medium with heme depleted, we estimate that NSCLC cells obtain about 2/3 of heme via de novo synthesis and about 1/3 via uptake from the medium. NSCLC cells are known to require serum for growth in culture while normal lung epithelial cells grow better in the absence of serum (Whitehurst AW et al., 2007 and Sato M et al., 2013). Fetal bovine serum used to culture NSCLC cells, like human blood, contains approximately 20 μM cell – free heme (Oh JY et al., 2016 and Muller-Eberhard U et al., 1968). Thus, both in vitro in culture and in vivo in mice and humans, tumor cells have ample supply of heme from the medium or circulation. Heme

degradation is also elevated in some NSCLC cells (Fig. 2.1D), and the inhibition of heme degradation by SnPP reduced colony formation in NSCLC cells. SnPP has been shown to be a strong inhibitor for the activities of heme oxygenases (Wong RJ et al., 2011). We observed SnPP treatment reduces colony formation in representative NSCLC cells H1299 and A549. This result is consistent with other studies indicating a role of heme degradation in promoting tumorigenesis (Frezza C et al., 2011 and Nowis D et al., 2008). For example, a previous study showed that inhibition of heme degradation is lethal to hereditary leiomyomatosis and renal-cell cancer cells when fumarate hydratase is deficient (Frezza C et al., 2011). Very likely, elevated heme degradation in cancer cells promotes tumorigenic functions by increasing the production of potent antioxidants bilirubin and beliverdin, as well as iron.

Elevated mitochondrial heme levels can potentially influence mitochondrial OXPHOS in two ways: (1) by increasing the pool of heme which is incorporated into OXPHOS complexes and other hemoproteins, and (2) by upregulating the translocation and assembly of OXPHOS complexes and other enzymes. Therefore, the rates of oxygen consumption and ATP levels are both elevated in NSCLC cells relative to non-tumorigenic cells (Figs. 2.2B & 2.2C). Two proteins important for mitochondrial biogenesis, TFAM and NRF1, are increased in NSCLC cells relative to non-tumorigenic cells (Figs. 2.2G & 2.2H). This is consistent with a previous study showing that loss of TFAM reduces tumorigenesis in an oncogenic Kras-driven mouse model of lung cancer (Weinberg F et al., 2010). Elevated heme biosynthesis and uptake ultimately lead to enhanced tumorigenic capabilities in NSCLC cells (Fig. 2.4). Therefore, our data presented here and previous studies all support the idea that heme is a pro-tumorigenic metabolic and signaling molecule. Hemoproteins and enzymes that are required for OXPHOS

are also pro-tumorigenic. Interestingly, a recent study from the authors' lab showed that viable NSCLC tumor cells resistant to the vascular disrupting agent combretastatin A-4 phosphate exhibit further elevated levels of hemoproteins and proteins and enzymes involved in heme metabolism (Dey S et al., 2018).

Further, to confirm the importance of heme flux in tumorigenicity of NSCLC, we overexpressed rate limiting heme synthesis enzyme ALAS-1 and heme transporter protein SLC48A1 in representative NSCLC cell lines. Our data show that increased levels of ALAS1 or SLC48A1 cause increased heme synthesis (Fig. 2.7C) or uptake (Fig. 2.7D), respectively. This increase causes elevated oxygen consumption (Fig. 2.7E) and ATP generation (Fig. 2.7F) in NSCLC cells. Increased heme flux in these overexpression lines showed increased tumorigenicity in vitro confirmed by migration, invasion, and colony formation assays (Fig. 2.7 A-C). When NSCLC cells overexpressing ALAS1 or SLC48A1 are subcutaneously implanted in mice; it was found that overexpressions of both ALAS1 and SLC48A1 contributes to tumorigenicity in vivo, as shown by increased tumor sizes and masses (Fig. 2.7 D). In order to confirm the mechanism of increased heme function in mitochondrial function in vivo, we prepared single cell suspension from these tumors and performed oxygen consumption and ATP assays. We observed tumors with overexpression lines showed increased oxygen consumption and ATP generation. Thus, these experiments prove our hypothesis that, NSCLC cells exhibit elevated heme flux that is responsible to drive mitochondrial respiration and energy generation which eventually contribute to the tumorigenicity. Thus, we expect that inhibitors of heme synthesis and uptake should suppress tumorigenesis and may overcome drug resistance in many tumors.

2.4 Materials and Methods

Reagents

Succinyl acetone was purchased from Sigma-Aldrich (Catalog # D1415-1G). Heme was purchased from Frontier Scientific Inc. (Catalog # H651-9). Zinc (II) protoporphyrin IX (Catalog # Zn 625-9) was purchased from Frontier Scientific Inc. [4-^{14}C]-5-aminolevulinic acid was custom synthesized by PerkinElmer. Antibodies were purchased from Santa Cruz Biotechnology, Cell Signaling Technology, Novus Biologicals, and abcam.

Measurement of heme synthesis and uptake

Measurement of heme synthesis in cells was carried out exactly as described (Sinclair PR et al., 1999 and Zhu Y et al., 2002). Briefly, 0.3 μC [4-^{14}C]-5-aminolevulinic acid (ALA) was added to each culture plate for 15 hours. Heme was subsequently extracted, and radiolabeled heme was quantified as described (Hooda J et al., 2015). For measuring heme uptake, a fluorescent analog of heme, zinc protoporphyrin IX (ZnPP), was used, as described previously (Bailao EF et al., 2014 and Yuan X et al., 2012). Briefly, 10,000 NSCLC cells were seeded in 96-well plates. Cells were incubated for 3 hours with 60 μM ZnPP in the presence or the absence of 40 μM HSPs. Fluorescence intensity was measured with a Biotek Cytation 5 plate reader. Experiments were conducted in triplicates, and ZnPP uptake was normalized with total cellular proteins. For measuring heme levels in various organelles, we used peroxidase-based reporters which express peroxidase activity along with a fluorescent marker like mCherry or eGFP in each organelle (Yuan X et al., 2016). Heme levels were measured exactly as described, and normalized with the fluorescent signals to correct for variations, such as that in transfection efficiency. Only Calu-3 did not show sufficient fluorescent signals to allow proper measurements (Yuan X et al., 2016).

Cell culture and analyses of tumorigenic functions

HBEC30KT and HCC4017 cell lines representing normal, non-tumorigenic and NSCLC cells from the same patient (Whitehurst AW et al., 2007 and Ramirez RD et al., 2004), respectively, were provided by Dr. John Minna's lab (UTSW) as a gift. They were developed from the same patient and were maintained in ACL4 medium supplemented with 2% heat-inactivated, fetal bovine serum (Ramirez RD et al., 2004). A pair of bronchial epithelial cell lines consisting of normal, non-tumorigenic cell line NL20 (ATCC Cat# CRL-2503) and tumorigenic cell line NL20-TA (ATCC Cat# CRL-2504), was purchased from American Type Culture Collection (ATCC). NL20 and NL20-TA cell lines were maintained in Ham's F12 medium with 1.5 g/L sodium bicarbonate, 2.7 g/L glucose, 2.0 mM L-glutamine, 0.1 mM nonessential amino acids, 0.005 mg/ml insulin, 10 ng/ml epidermal growth factor, 0.001 mg/ml transferrin, 500 ng/ml hydrocortisone and 4% fetal bovine serum. All other NSCLC cell lines, H1299 (ATCC Cat# CRL-5803), A549 (ATCC Cat# CRM-CCL-185), H460 (ATCC Cat# HTB-177), Calu-3 (ATCC Cat# HTB-55), and H1395 (ATCC Cat# CRL-5868) were purchased from ATCC, maintained in RPMI medium, and supplemented with 5% heat-inactivated, fetal bovine serum. All experiments using cells were conducted between passages 3-5 from revival of the initial frozen stocks. Cell lines were authenticated by Genetica and were found to be 96% identical to the standard (authentication requires >80%). Cell lines were tested for mycoplasma using a MycoFluor™ Mycoplasma Detection Kit (Molecular Probes), and the results were negative.

For generation of stable overexpression lines overexpressing ALAS1 or SLC48A1, lentiviral vectors expressing ALAS1, SLC48A1, and eGFP (control vector) were purchased from Genecoepia. The expression vectors for ALAS1 and SLC48A1 also express eGFP, making them

comparable with the control and easy for verification of positive clones. All vectors carry the neomycin selectable marker. Lentivirus particles were generated by co-transfecting 293T cells with packaging plasmids pMD2.G (addgene plasmid #12259) and psPAX2 (addgene plasmid # 12260) and vector for ALAS1 or SLC48A1 using Lipofectamine 3000. pMD2.G and psPAX2 were gifts from Didier Trono. For generating stable overexpression cell lines, H1299 and A549 cells (70-80% confluent) were transduced with virus particles ($2.8x10^4$ units/ well) in 48-well tissue culture plates. After series of passes and antibiotic selection stable clones were selected and verified for overexpression by Western blotting.

Cell migration and invasion assays were carried out with BD Falcon cell culture inserts (Corning Life Sciences) and the manufacturer's cell migration, chemotaxis, and invasion assay protocols. For the colony formation assay, 5000 NSCLC cells were seeded in every well in 6-well tissue culture plates in triplicates. Cells were treated with 0.5 mM succinyl acetone (Sigma Aldrich), Medium was changed every 3 days. After 6 days of treatment, cells were fixed with 70% ethanol and stained with 0.5% crystal violet. Images were acquired by using Carestream Gel Logic GL-112 imaging system.

Measurement of Oxygen Consumption and ATP Levels

Oxygen consumption was measured, as described previously (Hooda J et al., 2016). Briefly, 10^6 cells (in 350 µl) were introduced into the chamber of an Oxygraph system (Hansatech Instruments), with a Clark-type electrode placed at the bottom of the respiratory chamber. During measurements, the chamber was thermostated at 37°C by a circulating water bath. An electromagnetic stir bar was used to mix the contents of the chamber.

Total ATP was measured with the ATP-determination kit (Molecular Probes) following the manufacturer's protocol. Briefly, cultured cells were collected and immediately placed in ice-cold lysis buffer (Cell Signaling) with protease and phosphatase inhibitors. Cell lysates were then centrifuged at 10,000 g for 10 min. 10 µl of lysates or 10 µl of ATP standard solution was added to 90 µl of reaction buffer in each well of a 96-well plate. Luminescence was measured using a Biotek Cytation 5 plate reader. All experiments were carried out in triplicate, and the background luminescence was subtracted from the measurement. ATP concentrations were calculated from the ATP standard curve and normalized with the numbers of cells used. To measure oxygen consumption rates and ATP levels from freshly isolated tumors, subcutaneous tumors were surgically resected from mice and cut into small pieces. Tumors were weighed and homogenized immediately using mechanical homogenizer to gain a homogenous cell suspension. Tissue debris was removed by gentle centrifugation. Cells were suspended in 400ul of complete medium, and OCR was measured using a Clark-type electrode. ATP levels were measured with an ATP determination kit (Molecular probes). Both Oxygen consumption rates and ATP levels were normalized with protein amounts.

Preparation of protein extracts and Western blotting

Lung non-tumorigenic and tumorigenic cells were maintained (passage 3-5), collected, and lysed by using the RIPA buffer (Cell Signaling Technology) containing the protease inhibitor cocktail. Protein concentrations were determined by using the BCA assay kit (Thermo Scientific). 50 µg of proteins from each treatment condition were electrophoresed on 10% SDS–polyacrylamide gels, and then transferred onto the Immuno-Blot PVDF Membrane (Bio-Rad). The membranes were probed with antibodies, followed by detection with a chemiluminescence Western blotting

kit (Roche Diagnostics). The signals were detected by using a Carestream image station 4000MM Pro, and quantitation was performed by using the Carestream molecular imaging software version 5.0.5.30 (Carestream Health, Inc.). Antibodies used include those to the following proteins: ALAS1 (1:1000, Novus Cat# NBP1-91656), SLC46A1 (1:1000, Santa Cruz Biotechnology Cat# sc-134997), SLC48A1 (1:1000, Santa Cruz Biotechnology Cat# sc-101957), CYCS (1:1000, Santa Cruz Biotechnology Cat# sc-7159), COX4 (1:1000, Santa Cruz Biotechnology Cat# sc-292052), NRF1 (1:1000 Cell Signaling Technology Cat# 46743), TFAM (1:1000, Cell Signaling Technology Cat# 8076S), TFRC (1:1000, Novus Cat# NB100-92243), and β-actin (1:1000, Cell Signaling Technology Cat# 4967).

Immunohistochemistry (IHC)

IHC was carried out exactly as described (Dey S et al., 2018). Paraffin-embedded tumor tissues from mice described above were used. Six independent sets of human NSCLC grade 2 & 3 tissues and six independent sets of normal human lung tissues in paraffin slides were purchased from US Biomax, Inc. (Rockville, MD). Slides were deparaffinized, hydrated, and washed. After antigen retrieval, slides were blocked with 1XTBS/10% goat serum (16210-072, Gibco). Primary antibodies were diluted in 1XTBS/1%BSA/10% goat serum. The dilutions were 1:200 for ALAS1 (Santa Cruz Biotechnology Cat# sc-50531), 1:200 for SLC48A1 (Santa Cruz Biotechnology Cat# sc-101957); 1:200 for CYCS (Santa Cruz Biotechnology Cat# sc-7159), 1:200 for PTGS2 (Santa Cruz Biotechnology Cat# sc-7951); 1:200 for TFAM (Cell Signaling Technology Cat# 8076S). Sections were incubated with primary antibodies overnight at 4°C and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific Cat# 31460, RRID: AB_228341) at a dilution of 1:200 in 1X TBS/1%BSA for

45 mins at room temperature (RT). Slides were stained with tyramide signal amplification (TSA)-conjugated fluorophores, which were diluted 1:100 in 1X Plus Amplification Diluent (NEL810001KT, PerkinElmer). TSA-conjugated fluorophores were aspirated and slides were then washed. DAPI, diluted in TBST, was added to slides and incubated for 5 min at RT. Coverslips were mounted over the slides using VECTASHIELD mounting medium (Vector Laboratories), sealed, and stored in darkness at -20°C.

Slides were scanned at a 40X resolution with an Olympus VS120 slide scanner and quantified using cellSens software from Olympus. DAPI was used to visualize nuclei. Multiple regions of interest (ROIs) of equal area were drawn over tumor regions. ROIs were selected, so that equal numbers of cells (identified via nuclei) were included in each ROI. The ROIs were positioned evenly throughout tumor regions. ROIs were retested under three different filters—FITC, Cy3, and Cy5—to ensure that no artifacts were present. ROIs were re-positioned if artifacts were present under one or more filters. Minimum and maximum thresholds were set to avoid any background signal. Mean signal intensity from all ROIs were averaged, and the corresponding negative control average was subtracted to yield the signal intensity for each antigen.

Statistical analyses of data

Data from different treatment groups of cells, mice, and tissues were compared, and statistical analysis was performed with a Welch 2-sample t-test. For calculating correlation coefficients, we used the Pearson formula for calculating correlation coefficient r and p-value.

$$r\,(X,Y) = \frac{\sum(x-\overline{x})(y-\overline{y})}{\sqrt{\sum(x-\overline{x})^2\sum(y-\overline{y})^2}}$$

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

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CHAPTER 3

TARGETING HEME FLUX IS AN EFFECTIVE STRATEGY AGAINST NON-SMALL CELL LUNG CANCER[2]

3.1 Introduction

Lung cancer is the leading cause of cancer-related death in the US (Siegel R., 2014). It is mainly divided in two types: small cell lung cancer and nonsmall cell lung cancer. Non-small cell lung cancer (NSCLC) constitutes about 85% of the lung cancer cases (DeSantis CE et al., 2014). There are various targeted therapies that have recently been approved to treat lung cancer. For example, in 2013 the FDA approved erlotinib and Iressa as the first-line treatment of patients with metastatic NSCLC with epidermal growth factor receptor (EGFR) mutations. Unfortunately, only ten percent of NSCLC cases have EGFR gene mutations. In 2006 the FDA approved the labeling extension for bevacizumab in combination with paclitaxel and carboplatin for the treatment of locally advanced, recurrent, or metastatic, non-squamous, non-small cell lung cancer. Likewise, three PD-1/PD-L1 checkpoint inhibitors—nivolumab, pembrolizumab, and atezolizumab— have shown great promise as they helped extend median overall survival by about 3 months for second-line treatment of advanced NSCLC, compared to docetaxel alone (Brahmer J et al., 2015; Rittmeyer A et al., and Garon EB et al., 2015). Thus, spanning from 1990 to 2017, the various innovations in treatments have helped increase the survival of patients with advanced NSCLC, but this increase has only shifted average

[2] The contents of this chapter are taken from an article published in AACR Cancer Research: **Cancer Res; 79(10) May 15, 2019; 2511 – 2525; Sohoni et al.**

survival time from 7.1 to 11.4 months (Roth JA et al., 2015). Thus, alternative therapeutic strategies are still needed for lung cancer treatment. Thus, targeting bioenergetics of lung cancer cells has attracted a lot of interest from number of researchers around the globe.

Although, a number of tumors exhibit "Warburg effect", recent studies have demonstrated that cancer cells are much more efficient than initially thought and they toggle between glycolysis and OXPHOS depending on availability of oxygen and nutrients (Reitzer et al., 1978 and Chen et al, 2012; and Pavlova et al., 2016). Recent studies carried out on metabolism of ^{13}C-glucose in lung cancer patients show that both glycolysis and oxidative phosphorylation occurs simultaneously in lung tumors (Fan TW et al., 2009; and Hensley CT et al., 2016). Thus, contrary to Warburg's hypothesis many studies over the past two decades have sown importance of mitochondrial OXPHOS and TCA cycle in development of different types of cancers including non-small cell lung cancer. Although NSCLC tumors are metabolically heterogeneous, a common feature among NSCLCs is that pyruvate from elevated glycolysis enters and intensifies the TCA cycle (Hensley CT et al., 2016). Hence, It is imperative to know that mitochondrial function and oxidative phosphorylation (OXPHOS) play critical roles in pathophysiology of cancer. Mitochondria are essential intracellular organelles that regulate energy metabolism, cell death, and signaling pathways that are important for cell proliferation and differentiation. Therefore, mitochondria are fundamentally implicated in cancer biology, including initiation, growth, metastasis, relapse, and acquired drug resistance. Based on these implications, mitochondria have been proposed as a major therapeutic target for cancer treatment.

Heme is a central metabolic and signaling molecule which regulates diverse molecular and cellular processes relating to oxygen utilization and metabolism. Heme serves as a prosthetic

group in proteins and enzymes involved in oxygen transport, utilization, and storage, such as globins and cytochromes (Ortiz de Montellano PR., 2009). One crucial function of mitochondria is to use oxygen to carry out OXPHOS for ATP generation. Thus, heme function and mitochondrial respiration are tightly linked. Heme synthesis occurs in mitochondria, and mitochondria cannot function without heme. Multiple subunits in OXPHOS complexes II-IV contain heme. Additionally, heme acts as a signaling molecule to coordinate the expression of genes encoding OXPHOS complexes and the assembly of these complexes (Padmanaban G et al.,1989 and Kim HJ et al., 2012). Heme binds to and directly regulates the activities of many proteins controlling processes ranging from tyrosine kinase signaling to microRNA processing (Yao X et al., 2010; Barr I et al., 2012; and Kuhl T et al., 2014). Clearly, heme possesses unique signaling and structural properties that enable it to coordinate elevated mitochondrial OXPHOS in diverse cancer cells. Importantly, it has been shown that heme levels directly affect a number of physiological and disease processes in humans. A decrease in levels of heme is associated with porphyrias, anemia, and neurological disorders like Alzheimer's disease (Zhang L., 2011). On the other hand, various epidemiological and experimental studies directly relate high heme intake to risk of heart disease, diabetes, and cancer (Hooda J et al., 2014).

Intriguingly, we previously reported that several types of human lung tumor xenografts and NSCLC cells show elevated levels of heme flux and mitochondrial function (Hooda J et al., 2013 and Sohoni et al.,2019). The elevated levels of heme triggers mitochondrial function and ATP generation contributing to their tumorigenicity (Sohoni et al., 2019).

Here, to ascertain whether inhibiting heme availability can affect mitochondrial function and in turn alter proliferation and tumorigenicity of NSCLC cells, we took advantage of a bacterial

hemophore, HasA produced by a bacteria, *Yersinia pestis* (HSPs). Using site directed mutagenesis, we mutated couple of residues in HasA sequence to obtain heme sequestering proteins (HSPs) which exhibit higher binding affinity than parent protein (HasA). We observed that HSPs alter heme availability by sequestering extracellular heme. Treatment with HSPs severely affected rates of proliferation of different NSCLCs and their tumorigenic properties in vitro and in vivo. These results suggest targeting heme and mitochondrial function is an effective strategy to combat NSCLC.

3.2 Results

Engineered heme-sequestering peptides (HSPs) can inhibit heme uptake in NSCLC cell lines

As shown previously elevated heme flux is crucial for tumorigenicity of different NSCLCs, limiting heme availability may be effective for suppressing lung tumor growth and progression. Previous studies have identified succinyl acetone as an effective inhibitor of heme biosynthesis, as it inhibits the rate-limiting heme synthesis enzyme 5-aminolevulic synthase (ALAS1) in non-erythroid cells (De Matteis F et al., 1983). However, succinyl acetone is not very effective in suppressing lung tumors in mice (Fig. 3.1A). Therefore, we tried to lower heme availability by taking advantage of bacterial hemophores. We took advantage of the well-characterized hemophore HasA from *Yersinia pestis* (Kumar R et al., 2013). We identified specific amino acid residues whose mutations may improve heme binding without disrupting protein structure with the help of computational algorithm based on coevolution (Morcos F et al., 2011). Using this method, we designed several heme-sequestering peptides (HSPs), including HasA Q32H (HSP1) and HasA Q32H Y75M (HSP2) (Fig. 3.1B). Both, HSP1 and HSP2 bind to heme strongly (Fig.

3.1C), like the wild type HasA (Kumar R et al., 2013). The changed amino acids in HSP1 and HSP2 are known to coordinate heme well. Thus, the changes are not expected to reduce heme binding. As a bacterial hemophore, HasA is not internalized by human host cells. Thus, HSPs are not expected to be internalized by NSCLC cells. Indeed, HSP2 remained in the medium even after prolonged incubation with NSCLC cells (Figs. 3.1 D & 3.1 E).

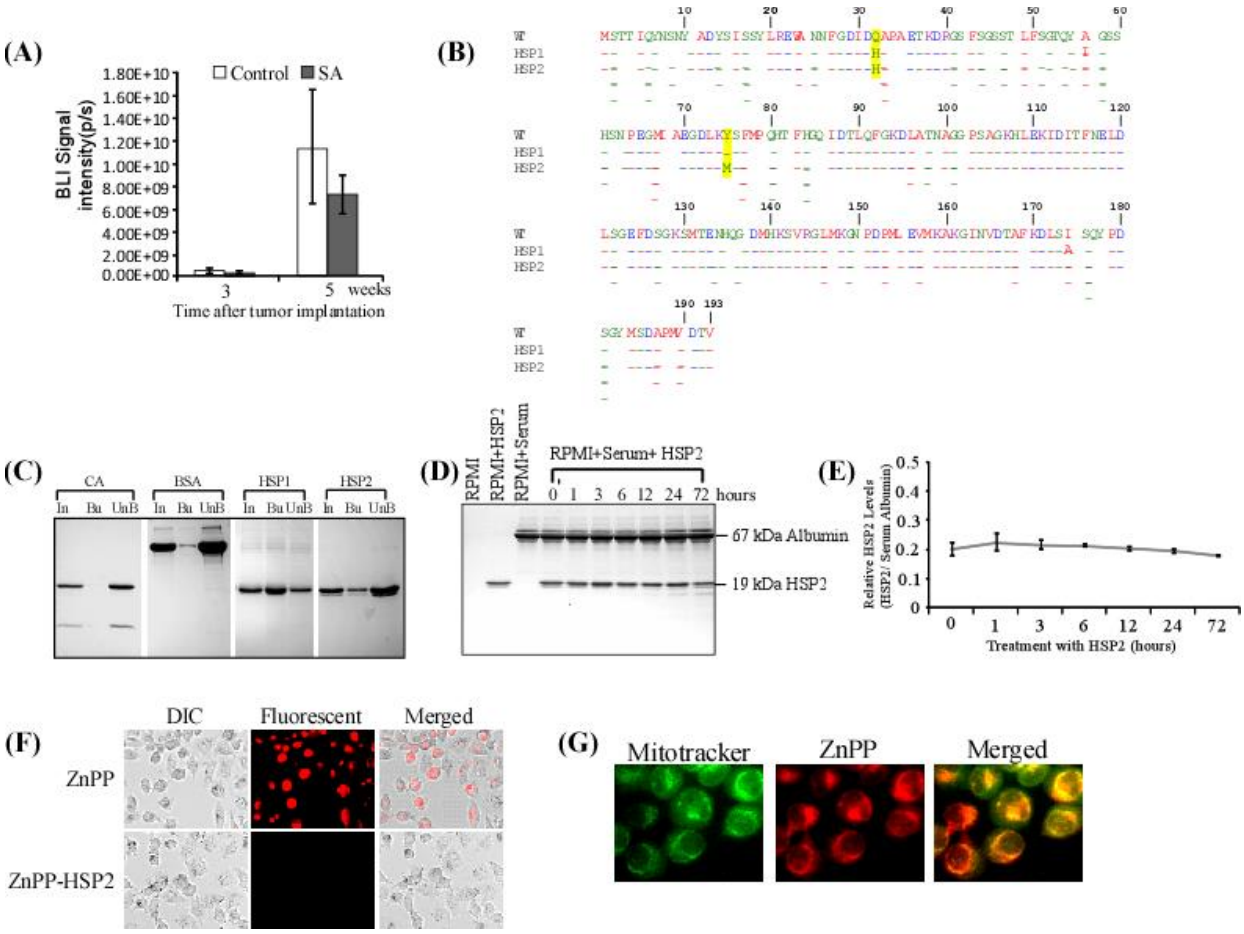

Figure 3.1: Mutations in HSPs bind and sequester extracellular heme. (A) Succinyl acetone is not efficient in suprresing lung tumors in mice (B) Aligned sequences of HSPs; Mutations at Q32H (HSP1) and Q32H, Y75M HSP2 (C) HSP2 binds heme stronger (D) HSP2 is not internalized by mammalian cells even after 72 hrs of incubation (E) Relative HSP2 levels in media after 72hrs incubation with mammalian cells (F) Fluorescence image showing HSP2-ZnPP complex is not internalized by mammalian cells (G) ZnPP colocalizes in mitochondria. Data are plotted as mean ± SD. For statistical analysis, the levels in

tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

Interestingly, HSP1 and HSP2 have enhanced capabilities to inhibit heme uptake in NSCLC cells (Fig. 3.2A). HSP2 is the most potent in inhibiting heme uptake by NSCLC cell lines, reducing heme uptake by 5-fold in some cell lines (Fig. 3.2A). Furthermore, the effect of HSPs on heme uptake was reversed if more ZnPP (a heme analogue used for measuring heme uptake) was included (compare 1xZnPP, 2xZnPP, and 2xZnPP + HSP2 in Fig. 3.2A), indicating that HSP2 does not reduce heme uptake by causing other toxicities. Note that 1xZnPP likely saturated the capabilities of cells to uptake heme/ZnPP so that 2xZnPP did not cause more

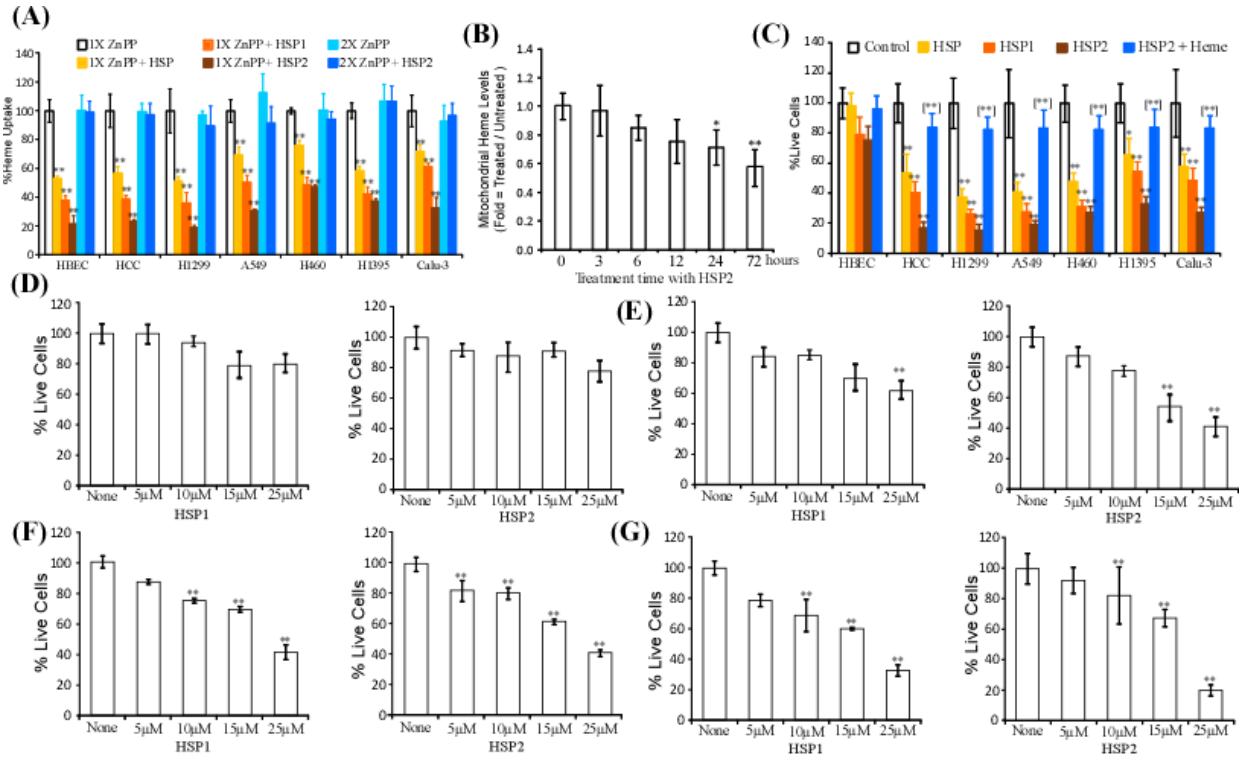

Figure 3.2: Engineered heme sequestering peptides (HSPs) alter heme availability and proliferation in NSCLC cells. (A) HSPs inhibit heme uptake in NSCLC cell lines in reversible manner (B) HSPs affect mitochondrial heme levels within 72hrs of exposure (C) HSPs affect proliferation rates of different NSCLC cell lines (D) HSPs did not affect proliferation of HBEC in dose-dependent manner unlike other tumorigenic lines (E) HCC4017 (F) H1299 and (G) A549. Data are plotted as mean ± SD. For statistical analysis,

the levels in tumorigenic cells were compared to the levels in non-tumorigenic cells with a Welch 2-sample t-test. *, p-value, 0.05, **, p-value < 0.005.

uptake. We further confirmed these results using fluorescent images of NSCLC cells incubated with ZnPP and ZnPP-HSP2 complex. It was found that ZnPP-HSP2 did not enter cells (Fig. 3.1F), while ZnPP in the medium without HSP2 entered cells and co-localized with mitotracker (Fig. 3.1G). Furthermore, we detected the effect of HSP2 treatment on mitochondrial heme levels in NSCLC cells, because mitochondrial heme levels are correlated with heme synthesis and uptake, as well as invasion (see above results). Fig. 3.2 B shows that mitochondrial heme levels gradually decreased as the treatment time with HSP2 increased. Together, these results strongly suggest that HSP2 acts on NSCLC cells by lowering heme uptake and mitochondrial heme levels.

HSPs effectively suppress NSCLC cell proliferation and tumorigenic functions

We further checked the effects of HSP1 and HSP2 on proliferation in different NSCLC cell lines. As expected, both HSP1 and HSP2 inhibited NSCLC cell proliferation in various NSCLC cell lines (Fig. 3.2 C) and in a dose-dependent manner (Fig. 3.2 D-G). The effects of HSPs on the proliferation of the HBEC30KT cell line representing normal lung epithelial cells were much less severe relative to NSCLC cell lines (Figs. 3.2C and 3.2D). This is consistent with the idea that normal cells do not need as much heme as NSCLC cells need. We also tested and compared the efficacies of HSP1, HSP2, and succinyl acetone at inhibition of tumorigenic functions in NSCLC cells. Evidently, HSP2 was more effective than succinyl acetone and HSP1 at inhibiting migration of H1299 and A549 cells (Fig. 3.3A). Likewise, HSP2 was more effective than succinyl acetone and HSP1 at inhibiting invasion by H1299 and A549 cells (Fig. 3.3B). Notably, addition of heme largely reversed the effects of HSP1 and HSP2, like succinyl acetone (SA), on

reducing proliferation, migration and invasion of NSCLC cells (Figs. 3.2C, 3.3A , 3.3B). The reversal of HSP1 and HSP2 effects by heme addition supports the idea that the effects of HSP1 and HSP2, like SA, on migration and invasion are attributable to their effect on heme uptake. We also found that SA, HSP1, and HSP2 strongly suppressed colony formation in H1299 and A549 (Figs. 3.3C) cells. Inhibition of heme degradation by SnPP appeared to reduce colony formation in NSCLC H1299 and A549 cells (Figs. 3.3C). Addition of heme to cells treated with SA, HSP1, or HSP2 largely reversed the effects of these agents on colony formation (Figs. 3.3C), indicating that their effects are attributable to lack of heme. As expected, iron chelator deferoxamine (DFX) also reduced colony formation in NSCLC cells, and addition of iron largely reversed the effect of DFX. Addition of heme to DFX-treated cells partially reversed the effect on colony formation, but addition of iron to SA-, HSP1-, or HSP2-treated cells did not reverse the effects on colony formation (Figs. 3.3C). This is consistent with the fact that iron can be obtained via heme. However, extra iron cannot overcome the effect on heme synthesis or uptake, likely because iron is not a limiting factor in NSCLC cells, as in most non-erythroid cells (34).

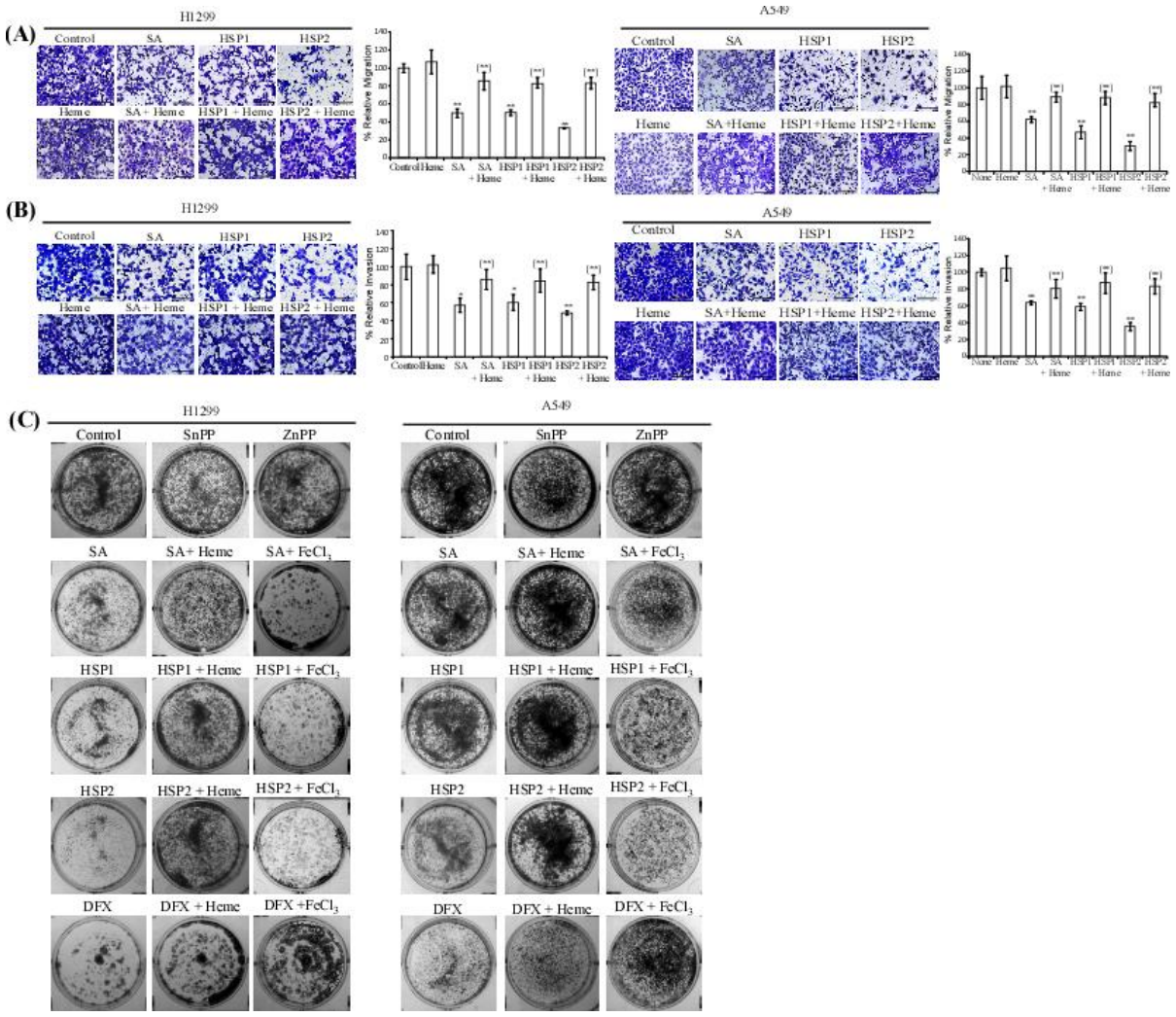

Figure 3.3: HSP2 inhibits tumorigenic functions of NSCLC cell lines by sequestering extracellular heme. HSP2 is more effective than SA and HSP1 in inhibiting (A) migration (B) Invasion and (C) Colony formation in H1299 and A549 Cells. Cells were treated with 10µM HSP1 or HSP2, 0.5mM SA and 10 µM was used for add-back experiments. SnPP and ZnPP are used for inhibition of heme oxygenase while DFX was used to characterize effect of iron on growth of these cell lines. Data are plotted as mean ± SD. For statistical analysis, the levels in treated cells were compared to the levels in untreated cells with a Welch 2-sample t-test. *, p-value ,0.05, **, p-value < 0.005. For heme add-back experiments, the levels in cells treated with heme and HSP2 were compared to the levels in cells treated with only HSP2. [**], p-value < 0.005.

HSP2 effectively suppresses the growth of H1299 NSCLC tumor xenografts in mice

To further assess the anti-tumor activity of HSP2 in vivo, we examined the effects of administering HSP2 on the growth of human xenograft tumors in the lungs of NOD/SCID mice (Fig. 3.4A). To further ascertain the effect of HSP2 on oxygen consumption, we decided to directly detect oxygen consumption and ATP generation in lung tumors in mice. We used subcutaneously implanted NSCLC tumors. HSP2 was very effective in suppressing subcutaneously implanted NSCLC tumors (Figs. 3.4B & 3.4C). Notably, the oxygen consumption rates and ATP levels in HSP2-treated tumors were both significantly reduced relative to untreated tumors (populations of cells isolated quickly from tumors) (Figs. 3.4D & 3.4E).

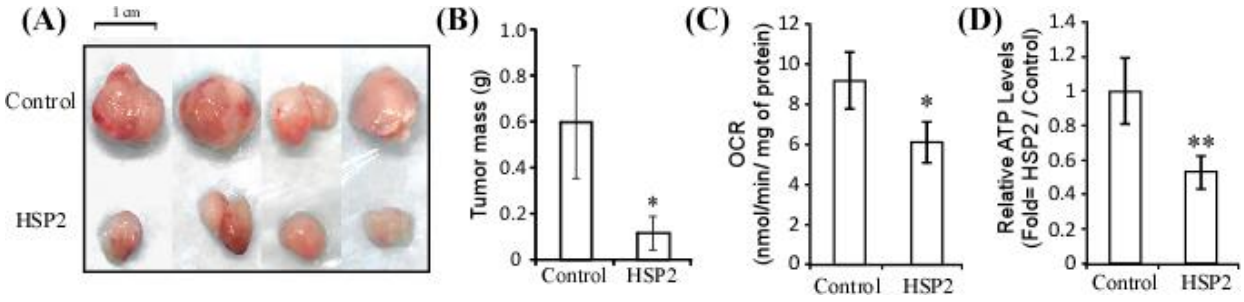

Figure 3.4: HSP2 significantly alters growth of H1299 lung tumor xenografts. (A) HSP2 treatment significantly reduced tumor burden in mice bearing H1299 lung tumor xenograft (B) quantification of total tumor mass control vs HSP2 (C) Tumors isolated from HSP2 treated mice showed significant reduction in OCR and (D) ATP generation (n=6/group). Data are plotted as mean ± SD. For statistical analysis, the levels in treated tumors were compared to the levels in untreated tumors with a Welch 2-sample t-test. *, p-value < 0.05.

HSP2 has low toxicity in mice

As HSP2 binds and sequesters extracellular heme we checked toxicity of HSP2 in mice and its effect of red blood cells. HSP2 did not significantly change the body masses of animals after 4

weeks of treatment (Fig. 3.5A). HSP2 did not significantly affect red cell counts, morphology, (Fig. 3.5B) and hemoglobin levels (Fig. 3.5C) in the blood. In order to test liver toxicity we measured serum Alanine transaminase (ALT) activity in mice treated with HSP2 (Fig. 3.5D). These mice did not show any sign of liver toxicity as serum ALT activity was not different between control and HSP2 treated groups.

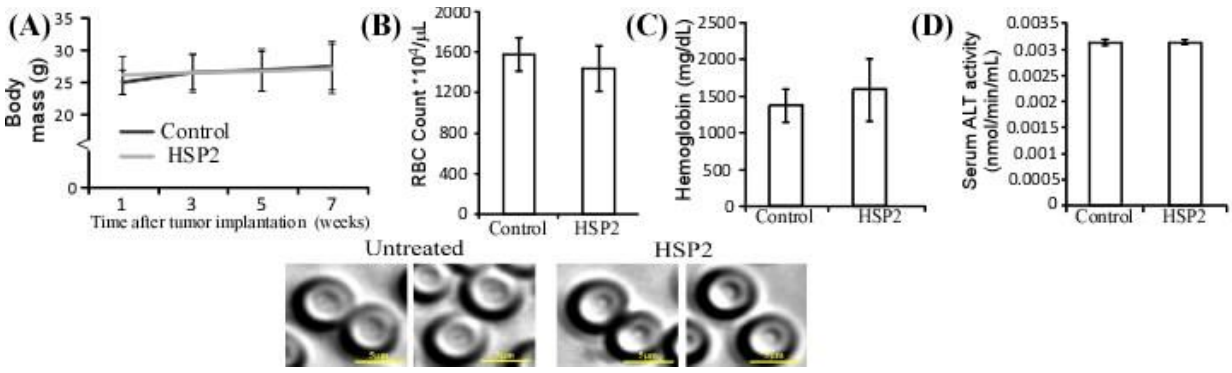

Figure 3.5: HSP2 has low toxicity in mice. (A) Prolonged HSP2 treatment did not affect body mass in mice (B) HSP2 did not show any signs of blood toxicity measured by total RBC count, morphology and (C) Serum hemoglobin levels (D) HSP did not show any signs of liver toxicity measured by ALT activity assay.

3.3 Discussion

Heme is a central molecule in mitochondrial OXPHOS and its principal function is related to oxygen transport, storage, detoxification, and utilization (Ortiz de Montellano PR 2009 and Padmanaban G et al., 1989). Heme serves as an essential prosthetic group or cofactor for many proteins and enzymes that bind and use oxygen, such as cytochrome P450 and nitric oxide synthases (NOSs), and that detoxify ROSs, such as catalase and peroxidases. Heme regulates function of three OXPHOS complexes, II, III, and IV. Multiple subunits in complexes III and IV require heme as a prosthetic group, and different forms of heme are present (Alam et al., 2016). Heme also regulates expression and assembly of OXPHOS complexes (Padmanaban G et al.,

1989 and Kim HJ et al., 2012). Heme synthesis occurs in mitochondria and requires oxygen (Anderson KE., 2009). Thus, heme and mitochondrial biogenesis are linked and are inter-dependent.

We have shown previously that NSCLC cells exhibit high heme flux through increased rates of heme biosynthesis and uptake which eventually leads to increase in mitochondrial heme levels. (Fig. 2.1 & 2.2A). Elevated mitochondrial heme levels can potentially influence mitochondrial OXPHOS in two ways: (1) by increasing the pool of heme which is incorporated into OXPHOS complexes and other hemoproteins, and (2) by upregulating the translocation and assembly of OXPHOS complexes and other enzymes. Therefore, the rates of oxygen consumption and ATP levels are both elevated in NSCLC cells relative to non-tumorigenic cells (Figs. 2.2B & 2.2C). Elevated heme biosynthesis and uptake ultimately lead to enhanced tumorigenic capabilities in NSCLC cells (Figs. 2.4 & Figs. 2.7). Therefore, our data presented here and previous studies all support the idea that heme is a pro-tumorigenic metabolic and signaling molecule. Hemoproteins and enzymes that are required for OXPHOS are also pro-tumorigenic. Interestingly, a recent study from the authors' lab showed that viable NSCLC tumor cells resistant to the vascular disrupting agent combretastatin A-4 phosphate exhibit further elevated levels of hemoproteins and proteins and enzymes involved in heme metabolism (Dey et al., 2018).

Thus, we expected that inhibitors of heme synthesis and uptake should suppress tumorigenesis and may overcome drug resistance. Indeed, our data presented here show that inhibition of heme synthesis by succinyl acetone (SA) or inhibition of heme uptake by HSPs reduces tumorigenic functions of NSCLC cells (Fig.3.3). HSP2, which inhibits heme uptake

more strongly than HSP1, diminishes tumorigenic functions of NSCLC cells most strongly. This raises the possibility that HSP2 can be a more effective agent against NSCLC cells than succinyl acetone. We observed anti-tumorigenic effects of HSP2 both in vitro and in vivo. (Figs. 3.3& 3.4A). Notably, addition of heme largely reverses the effects of SA and HSPs on proliferation, migration, invasion, and colony formation suggesting anti-tumorigenic effects of SA and HSP2 are specifically through heme sequestration (Figs.3.3).

Succinyl acetone has low toxicity to animals (Raff RF et al., 1992 and Bourque SL et al., 2010). Likewise, our data from mouse studies suggest that HSP2 is not highly toxic to mice (Fig.3.5). HSP2 did not affect red cell counts hemoglobin levels, and ALT activity indicative of liver function (Fig. 3.5D). Moreover, HSP2 did not significantly affect the proliferation of HBEC30KT cell line representing normal lung epithelial cells in the concentration range that affected the proliferation of NSCLC cells (Figs.3.2 C, E, F and G). Bacterial hemophore is not internalized by host mammalian cells. Thus, it is expected that HSP2 does not get into NSCLC cells, as indicated in Fig 3.1 D&E. Notably, our data clearly showed that HSP2 inhibits heme uptake and reduces mitochondrial heme levels in NSCLC cells (Fig.3.2). The lack of strong blood toxicity of HSP2 is likely attributable to the lack of the need for heme uptake in normal cells. It is important to note that serum is the main source of heme for cells in vitro. Normal lung cells can grow in serum deficient media, whereas NSCLC cells need serum for proliferation and tumorigenicity (Sato M et al., 2013). Our data presented here show that the levels of heme synthesis, uptake, oxygen consumption, and ATP are significantly lower in non-tumorigenic lung cells relative to NSCLC cells (Figs. 2.1 & 2.2). Notably, during erythropoiesis, heme synthesis is induced prior to and is essential for globin synthesis (Anderson KE., 2009 and Dailey

HA et al., 2013). Erythroid heme synthesis is very high and excessive. Previous experimental data suggested that erythrocytes produce excess heme for export and transport to other organs (Dailey HA et al., 2013, Khan AA et al., 2011). Thus, heme uptake is not needed for erythropoiesis, and erythrocytes can provide heme for other cells and tissues, including tumors. Thus, it is not surprising that heme sequestration by HSP2 does not cause erythroid toxicity during the treatment periods in mice (Figs.3.5 A-C). It is also worth noting that suppression of tumor growth should also lower the demand for iron for tumor growth, thereby alleviating potential blood toxicity posed by HSP2.

3.4 Materials and Methods

Reagents

Succinyl acetone was purchased from Sigma-Aldrich (Catalog # D1415-1G). Heme was purchased from Frontier Scientific Inc. (Catalog # H651-9). Zinc (II) protoporphyrin IX (Catalog # Zn 625-9) was purchased from Frontier Scientific Inc. Tin (IV) protoporphyrin was purchased from Porphyrin Products Inc (Catalog # Sn749-9). Deferoxamine mesylate was purchased from Sigma-Aldrich (Cat# D9533-1G). Ferric chloride was purchased from Sigma-Aldrich (Catalog # 157740-100G). The pET11a expression vector for *Y. pestis* HasA residues 1-193 was kindly provided by Dr. Mario Rivera (University of Kansas) (Kumar R et al., 2013). HSP1 contains the Q32H mutation, and HSP2 contains Q32H Y75M double mutations. The mutations were generated with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). The double mutations were generated with the expression vector for the Q32H mutation. All DNA clones were confirmed by sequencing (Eurofins Genomics LLC). HSP1 and

HSP2 were purified with a Q-Sepharose Fast Flow column (GE Healthcare), followed by size exclusion chromatography, as described (Kumar R et al., 2013). The reporter plasmids for measuring subcellular heme levels were kindly provided by Dr. Iqbal Hamza (Yuan X et al., 2016).

Cell culture and analyses of tumorigenic functions

HBEC30KT and HCC4017 cell lines representing normal, non-tumorigenic and NSCLC cells from the same patient (Whitehurst AW et al., 2007 and Ramirez RD et al., 2004), respectively, were provided by Dr. John Minna's lab (UTSW) as a gift. They were developed from the same patient and were maintained in ACL4 medium supplemented with 2% heat-inactivated, fetal bovine serum (Whitehurst AW et al., 2007). A pair of bronchial epithelial cell lines consisting of normal, non-tumorigenic cell line NL20 (ATCC Cat# CRL-2503) and tumorigenic cell line NL20-TA (ATCC Cat# CRL-2504) was purchased from American Type Culture Collection (ATCC). NL20 and NL20-TA cell lines were maintained in Ham's F12 medium with 1.5 g/L sodium bicarbonate, 2.7 g/L glucose, 2.0 mM L-glutamine, 0.1 mM nonessential amino acids, 0.005 mg/ml insulin, 10 ng/ml epidermal growth factor, 0.001 mg/ml transferrin, 500 ng/ml hydrocortisone and 4% fetal bovine serum. All other NSCLC cell lines, H1299 (ATCC Cat# CRL-5803), A549 (ATCC Cat# CRM-CCL-185), H460 (ATCC Cat# HTB-177), Calu-3 (ATCC Cat# HTB-55), and H1395 (ATCC Cat# CRL-5868) were purchased from ATCC, maintained in RPMI medium, and supplemented with 5% heat-inactivated, fetal bovine serum. All experiments using cells were conducted between passages 3-5 from revival of the initial frozen stocks. Cell lines were authenticated by Genetica and were found to be 96% identical to the standard

(authentication requires >80%). Cell lines were tested for mycoplasma using a MycoFluor™ Mycoplasma Detection Kit (Molecular Probes), and the results were negative.

Cell proliferation was measured by detecting the luciferase activity in live cells and by using a hemocytometer. Cell migration and invasion assays were carried out with BD Falcon cell culture inserts (Corning Life Sciences) and the manufacturer's cell migration, chemotaxis, and invasion assay protocols. For the colony formation assay, 5000 NSCLC cells were seeded in every well in 6-well tissue culture plates in triplicates. Cells were treated with 0.5 mM succinyl acetone (Sigma Aldrich), 10 μM HSPs, 50 μM deferoxamine mesylate (DFX), 10 μM zinc (II) protoporphyrin IX, 10 μM tin (IV) protoporphyrin IX, or 50 μM ferric chloride for 6 days. For heme add-back experiments, 10 μM heme was included. Medium was changed every 3 days. After 6 days of treatment, cells were fixed with 70% ethanol and stained with 0.5% crystal violet. Images were acquired by using Carestream Gel Logic GL-112 imaging system.

Measuring Heme uptake

For measuring heme uptake, a fluorescent analog of heme, zinc protoporphyrin IX (ZnPP), was used, as described previously (Bailao EF et al., 2014 and Yuan X et al., 2012). Briefly, 10,000 NSCLC cells were seeded in 96-well plates. Cells were incubated for 3 hours with 60 μM ZnPP in the presence or the absence of 40 μM HSPs. Fluorescence intensity was measured with a Biotek Cytation 5 plate reader. Experiments were conducted in triplicates, and ZnPP uptake was normalized with total cellular proteins. For measuring heme levels in various organelles, we used peroxidase-based reporters which express peroxidase activity along with a fluorescent marker like mCherry or eGFP in each organelle. Heme levels were measured exactly as described and normalized with the fluorescent signals to correct for variations, such as that in transfection

efficiency. Only Calu-3 did not show sufficient fluorescent signals to allow proper measurements (Yuan X et al., 2016).

Oxygen consumption was measured, as described previously (Hooda J et al., 2013). Briefly, 10^6 cells (in 350 µl) were introduced into the chamber of an Oxygraph system (Hansatech Instruments), with a Clark-type electrode placed at the bottom of the respiratory chamber. During measurements, the chamber was thermostated at 37°C by a circulating water bath. An electromagnetic stir bar was used to mix the contents of the chamber.

Total ATP was measured with the ATP-determination kit (Molecular Probes) following the manufacturer's protocol. Briefly, cultured cells were collected and immediately placed in ice-cold lysis buffer (Cell Signaling) with protease and phosphatase inhibitors. Cell lysates were then centrifuged at 10,000 g for 10 min. 10 µl of lysates or 10 µl of ATP standard solution was added to 90 µl of reaction buffer in each well of a 96-well plate. Luminescence was measured using a Biotek Cytation 5 plate reader. All experiments were carried out in triplicate, and the background luminescence was subtracted from the measurement. ATP concentrations were calculated from the ATP standard curve and normalized with the numbers of cells used. To measure oxygen consumption rates and ATP levels from freshly isolated tumors, subcutaneous tumors were surgically resected from mice and cut into small pieces. Tumors were weighed and homogenized immediately using mechanical homogenizer to gain a homogenous cell suspension. Tissue debris was removed by gentle centrifugation. Cells were suspended in 400ul of complete medium, and OCR was measured using a Clark-type electrode. ATP levels were measured with an ATP determination kit (Molecular probes). Both Oxygen consumption rates and ATP levels were normalized with protein amounts.

Treatment of human xenograft lung tumors in NOD/SCID mice

For subcutaneous tumor models, 2×10^6 H1299-luc cells in serum-free medium containing 50% Matrigel were injected subcutaneously into the left flank region of 4-6 weeks old female NOD/SCID mice. Mice were randomized into treatment groups that received saline (for control) and HSP2 (I.V. 25 mg/kg every 3 days), respectively. Body masses were recorded once every week. Treatments were started only after BLI detected authentic tumor signals and tumors were visible to ensure the proper implantation of tumors. When the tumors in the control group reached 1 cm^3, mice were euthanized by cervical dislocation. Tumors were resected and weighed.

Toxicity assays

Blood samples are drawn from control and treated mice through retro-orbital vein. RBCs were counted using hemocytometer. Serum hemoglobin and ALT activity assay were performed following manufacturer's protocols.

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CHAPTER 4

SUMMARY OF THE STUDY AND FUTURE DIRECTIONS

4.1 Summary of the Study

Lung cancer is a leading cancer killer in United States (Siegel RL et al., 2015). Over the past two decades a number of targeted therapies have been developed to treat lung cancer. Unfortunately, however statistical data over the past two decades suggest only a slight improvement in a patient's survival rate after diagnosis. Clonal evolution and tumor heterogeneity are the major obstacles in designing effective targeted treatments against lung cancer. To create more comprehensive treatments, emerging therapies target bioenergetic pathways of cancer cells. Like normal cells, cancer cells can generate energy only through glycolysis and oxidative phosphorylation. Notably, a number of studies have shown that many types of cancer cells rely heavily on mitochondrial respiration. Metabolic studies carried out in lung cancer patients using ^{13}C- glucose showed that lung cancer cells metabolize glucose using both glycolysis and oxidative phosphorylation (Fan TW et al., 2009 and Hensley CT et al., 2016). Oxidative phosphorylation in lung tumors can be sustained by number of fuels such as glucose, glutamine, and fatty acids and tumor cells have to coordinate between different OXPHOS complexes and TCA cycle enzymes. Heme an iron protoporphyrin IX is a central molecule that performs diverse functions in cells related to oxygen utilization and metabolism (Ortiz de Montellano PR., 2009). One crucial function of mitochondria is to carry oxygen for OXPHOS and ATP generation. Thus, mitochondrial respiration and heme function are tightly linked. Heme serves as a prosthetic group or cofactor for a number of mitochondrial complexes mainly II, III, and IV and regulates their function and folding (Alam et al., 2016; Padmanaban G et al., 1989 and Kim HJ et al.,

2012). Previous studies from our lab have outlined fundamental role of heme in non-small cell lung cancer. Several types of human lung tumor xenografts display elevated levels of the rate-limiting heme biosynthetic enzyme ALAS1, heme transporters, and oxygen-utilizing hemoproteins (Hooda J et al., 2014). Other studies also showed that the expression of proteins involved in mitochondrial respiration and heme function are elevated in the tumor tissues of NSCLC patients (Sotgia F et al., 2017 and Lam TK et al. 2014). Additionally, epidemiological studies indicated a positive association between intake of heme from red meat and lung cancer (Tasevska N et al., 2009).

Here, we carried out a systematic study using an array of non-small cell lung cancer cell lines to further investigate and prove role of heme in tumorigenicity of NSCLCs. Here we show that NSCLCs exhibit elevated expressions of heme synthesis enzyme ALAS1, heme transporters, different hemoproteins, and OXPHOS complexes compared to non-tumorigenic lung lines. These increased expressions of various hemoproteins and OXPHOS complexes correlate with elevated levels of heme synthesis, heme uptake, and most importantly mitochondrial heme levels. Elevated mitochondrial heme levels can influence mitochondrial OXPHOS in two ways (1) by increasing the pool of heme which is incorporated into OXPHOS complexes and other hemoproteins, and (2) by upregulating the translocation and assembly of OXPHOS complexes and other enzymes. Hence, the rates of mitochondrial respiration and ATP levels were both elevated in NSCLC cells compared to non-tumorigenic cells. Elevated heme biosynthesis and uptake ultimately lead to enhanced tumorigenic capabilities in NSCLC cells. Therefore, our data presented here and previous studies all support the idea that heme is a pro-tumorigenic metabolic

and signaling molecule. Hemoproteins and enzymes that are required for OXPHOS are also pro-tumorigenic.

In order to further prove the importance of heme in tumorigenicity of NSCLC cells, we generated stable NSCLC cell lines overexpressing heme synthesis enzyme ALAS1 or heme transporter SLC48A1 using a lentivirus carrying respective plasmids. Overexpression lines showed significantly higher heme flux and mitochondrial respiration with high ATP generation. This elevated levels of heme flux and mitochondrial function also aided the tumorigenicity of the overexpression cell lines compared to control cell line both in vitro and in vivo. Thus, these experiments help establish a strong link between heme availability and tumorigenesis in non-small cell lung cancer.

Next, we expected inhibitors of heme synthesis and uptake should suppress tumorigenesis and drug resistance. Indeed, our data presented here show that inhibition of heme synthesis by succinyl acetone (SA) or inhibition of heme uptake by HSPs reduces tumorigenic functions of NSCLC cells. HSP2, which inhibits heme uptake more strongly than HSP1, diminishes tumorigenic functions of NSCLC cells most strongly. Notably, addition of heme largely reverses the effects of SA and HSPs on proliferation, migration, invasion, and colony formation. These results strongly support the idea that the effects of SA and HSPs on NSCLC cell tumorigenic functions are attributable to their effects on heme synthesis and uptake, respectively. This raises the possibility that HSP2 can be a more effective agent against NSCLC cells than succinyl acetone. Indeed, HSP2 strongly suppressed the growth of both orthotopically implanted NSCLC tumors and subcutaneously implanted tumors. Most importantly, our data show that HSP2 is not highly toxic in mice. It did not affect hemoglobin or serum ALT activity. The blood toxicity

results from the animals which undergone HSP2 treatments were also normal. It was also important to note that HSP2 did not affect proliferation of non-tumorigenic lung cell line HBEC30KT in the concentration range that affected other tumorigenic cell lines. This could be mostly because of the fact that normal cells require much less amounts of heme compared to tumor cells.

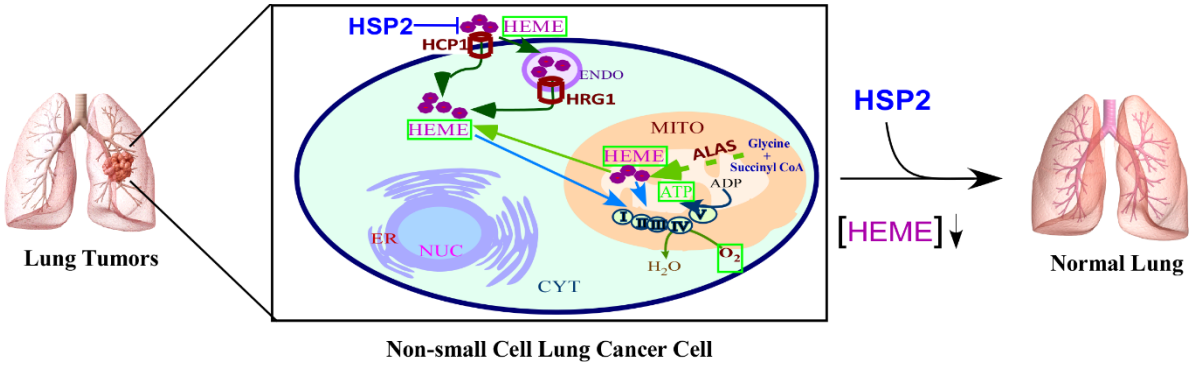

Figure 4.1: Summary figure. Lung cancer cells exhibit elevated heme synthesis and uptake, which promotes tumorigenic functions. Heme-sequestering peptide 2 (HSP2) inhibits heme uptake and reduces tumor burden.

4.2 Future Directions

Over the last two decades a lot of effort has been put in to develop targeted therapies against lung cancer without significantly impacting overall survival rate of lung cancer after diagnosis. This failure can be largely attributable to the heterogeneity of lung cancer tumors. A solution to this problem can be found by targeting bioenergetics in tumor cells. The rationale behind targeting bioenergetics of tumor cells is that all tumor cells fundamentally metabolize diverse fuels mainly by glycolysis or oxidative phosphorylation depending on availability of oxygen and nutrients. Importantly, OXPHOS has been shown as a key determinant in the development of NSCLC and drug resistance in breast cancer, melanoma, and leukemia. Here we have outlined

an importance of heme in tumorigenicity of NSCLC. Drugs targeting OXPHOS and glycolysis have shown some promise in recent clinical trials but they come with a huge baggage of off target effects. Here we have shown that heme is a potential oncometabolite. Non-small cell lung cancer cells are addicted to heme and exhibit high levels of heme flux and mitochondrial respiration compared to non-tumorigenic cells. Also, inhibiting heme availability severely impacts proliferation and tumorigenic function of NSCLC cells in vitro and in vivo. Thus, this study lays a foundation and opens a novel avenue of targeting heme and mitochondrial function for treatment of non-small cell lung cancer and possibly other types of cancers (Sohoni et al., 2019).

It will be crucial to investigate the molecular mechanisms underlying heme function in cancer cells in more detail using proteomic and metabolomic studies. These studies will not only help establish the importance of heme in pathogenicity of cancer but can also reveal novel therapeutic targets for cancer treatments.

The concept of limiting heme availability using HSPs is not restricted to only cancer therapy but can be applied in other diseases where heme metabolism is severely implicated such as Type II diabetes, Alzheimer's disease and myocardial infections.

4.3 References

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BIOGRAPHICAL SKETCH

Sagar Sohoni was born in Pune, India on October 16th, 1986. He received his master's in Microbiology from University of Pune, India in 2009. He joined the Department of Molecular and Cell Biology at The University of Texas at Dallas as a graduate student in August 2011. During the master's program, he joined the laboratory of Dr. Chanrashekhar Pasare in UT Southwestern Medical Center to study role of B-cell adapter proteins for PI3K in immunoglobulin production in response to *Salmonella* infections. He completed his master's with thesis in Molecular and Cell Biology in August 2013. To continue his studies, he enrolled in the doctorate program in Molecular and Cell Biology in August 2013. He joined Dr. Li Zhang's laboratory in January 2014 to complete his doctoral dissertation.

CURRICULUM VITAE

SAGAR SOHONI

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SUMMARY

Cell Biologist with more than 8 years of experience in handling multiple research projects in following areas:

- 5 years of experience in heme biology and mitochondrial functions in Non-small cell lung cancer cells • 4 years of teaching experience in Immunology, Cell biology, Cancer biology, and Biochemistry.
- 1.5 years of experience in Toll-like receptor signaling and immunoglobulin production
- 1.5 years of Industrial experience in bioethanol production

EDUCATION

PhD Molecular and Cell Biology The University of Texas at Dallas, USA
Expected: Aug, 2019

MS Molecular and Cell Biology The University of Texas at Dallas, , USA
Aug, 2013

MSc Microbiology University of Pune, Maharashtra, India
May 2009

RESEARCH AND PROFESSIONAL EXPERIENCE

Department of Biological Sciences, University of Texas at Dallas; Graduate Research Assistant Mentor: *Dr. Li Zhang, PhD* **2014 - Present**

- **Project:** Investigate the role of heme and oxidative metabolism in tumorigenicity of non-small cell lung cancer and to design therapeutic heme sequestering agents to alter heme homeostasis in non-small cell lung cancer.

- **Responsibilities:** Responsible for concept and research design; Implementation and management of research project; Development of methodology; Construction and

purification of heme sequestering agents; Analysis and interpretation of data; Writing and reviewing manuscript. concurrently advise three graduate students.

- **Methodologies:** Handling of non-small cell lung cancer cell lines; Essential *in vitro* techniques such as western blotting, Immonoprecipitation; PCR, Mitochondrial OCR and ECAR using Seahorse XF analyzer; Protein design; Protein purification using Ion-exchange chromatography and size exclusion chromatography; Invasion and migration assays; Breeding and Handling mice; Generating NSCLCs tumor xenograft models; Confocal microscopy, Epifluorescence microscopy, Bioluminescence imaging, Immunohistochemistry.

- **Other Projects:**
- Investigate role of heme in mitochondrial biogenesis,
- Analyze effect heme and mitochondrial targeting agents on tumorigenicity of different cancer lines.
- Study effect of heme in differentiation of hematopoetic stem cells.

Department of Immunology, UT Southwestern Medical Center **Masters Thesis**
Mentor: *Dr Chandrashekhar Pasare, PhD* **Aug, 2012- Sep,2013**

- **Project:** Role of B-cell adapter protein for PI3K (BCAP) in immunoglobin production in steady state and in response to *Salmonella typhimurium* infections in mice.
- **Responsibilities:** Design and execution of experiments; Analysis and interpretation of data; writing and reviewing thesis.
- **Methodologies:** Handling and breeding mice; genotyping, PCR, Establish *Salmonella typhimurium* infections in mice by oral gavage, ELISA; dissection of lymph nodes, spleen, bone marrow and gut from mice; FACS, MACS.

Praj Industries Ltd., Pirangut, Pune, India **Research Associate**
Nov, 2009- Nov, 2010

- **Project:** To develop ethanol and inhibitor tolerant yeast strains by carrying out physical and chemical mutagenesis. Construct yeast clones for high yield ethanol from cellulose.

- **Responsibilities:** To lead a project of strain improvement and maintenance; Generate a yeast strain that can withstand different inhibitors produced during fermentation; Develop and implement new research strategies to maximize the yield of

bioethanol. Record and store high bioethanol yield strains; Literature survey for inhibitors present in lignocellulosic hydrolysates.

- **Methodologies:** Microbiology; Cell culture and maintenance; PCR; Mutagenesis; Pilot Fermentation; Distillation; measuring ethanol titer.

- **Achievement:** Strain developed by the team is still being used for production of bioethanol from lignocellulosic hydrolysates.

TEACHING EXPERIENCES

Teaching Assistant University of Texas at Dallas **Dec, 2012 – July, 2016**

Molecular biology of HIV/ AIDS	Dr. John Burr	May, 2016 – Aug, 2016
Cell and Molecular Biology	Dr. John Burr and Dr. Uma Srikanth	Jan, 2014 – May, 2016
Biochemistry	Dr. Stephen Spiro and Dr. Robert Marsh	Aug, 2013 – Dec, 2013
Immunology	Dr. John Burr	Dec, 2012 – May, 2013

PUBLICATIONS

- **Sohoni S**, Ghosh P, Wang T, Kalainayakan S, Vidal C, Dey S, Zhang L. (2019) Elevated heme synthesis and uptake underpin intensified oxidative metabolism and tumorigenic functions in non-small cell lung cancer cells. Cancer Research. 2019; 15;79(10):2511-2525.

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- Kalainayakan S. Ghosh P, Dey S, FitzGerald K, **Sohoni S**, Konduri P, Liu L, Zhang L. Targeting heme function and mitochondrial respiration effectively arrest lung tumor growth and progression. Scientific Reports. 2019; 9:1405

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PESENTATIONS

- **Sohoni S**, Ghosh P, Zhang L. Increased heme influx dictates tumorigenic functions of non-small cell lung cancer cells. AACR Annual Meeting 2019. Atlanta, GA.

- **Sohoni S**, Vidal C, Zhang L. The effect of heme on mitochondrial biogenesis and function of non-small cell lung cancer cells. AACR Annual Meeting 2018. Chicago, IL.

- **Sohoni S**, Vidal C, Zhang L. "Understanding molecular mechanisms underlying heme function in nonsmall cell lung cancer cells". AACR Annual Meeting 2017, Washington, DC.

- **Sohoni S**, Vidal C, Zhang L. "The effect of heme influx on initiation and tumorigenesis of non-small cell lung cancer cells. AACR Annual Meeting 2016. New Orleans, LA.