TARGETING HEME FUNCTION AND MITOCHONDRIAL RESPIRATION IN NON-SMALL CELL LUNG CANCER

by

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I dedicate this dissertation to my teachers and mentors whose dedication, support, and encouragement has constantly motivated me and has made this journey possible.

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by

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Each year lung cancer causes more morbidities than cancers of colon, breast, and prostate combined. The American Cancer Society estimates that lung cancer will claim about 150,000 lives in 2018. Conventional and targeted therapies are reported to have reached the plateau in effectively improving the survival of lung cancer patients. Despite the advent of advanced therapies like immunotherapy, 5-year survival rates remain abysmal at 30% and 10% for Stage III and Stage IV respectively. Therefore, it is imperative to explore different strategies to effectively treat lung cancer and improve survival outcomes. There are two major histological types of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for about 85% of the lung cancer cases. Several studies demonstrate that enhanced mitochondrial respiration or oxidative phosphorylation (OXPHOS) is a key feature of NSCLC. Therefore, targeting OXPHOS could be an effective strategy for intervention in NSCLC. Previous studies from our lab showed that hedgehog pathway antagonist, cyclopamine tartrate (CycT), significantly reduced OXPHOS and proliferation in NSCLC cell lines. However, *in vitro* models do not offer reliable evidence of therapeutic efficacy, thereby, necessitating studies on *in vivo* models. Previous studies from our lab

demonstrated intensified heme uptake and synthesis in NSCLC cell lines compared to normal cell line. Since heme is a central factor in oxygen consumption, this study also probes the effect of CycT on heme metabolism. The objective of this study is two-fold: (i) to test the efficacy of targeting OXPHOS in NSCLC *in vivo*, and (ii) to investigate the therapeutic efficacy and the mechanism of action of CycT in growth and progression of NSCLC *in vivo*. We utilized subcutaneous and lung orthotopic xenografts of NSCLC cell lines with luciferase to track the growth and progression of NSCLC in immunodeficient mouse model, NOD/SCID (Non-obese diabetic/ severe combined immunodeficiency), via bioluminescence imaging. The lung tissues of the mice were probed using immunohistochemistry to discern the mechanisms of action. We found that CycT effectively hampered the growth and progression of subcutaneous and lung orthotopic xenografts of NSCLC cell lines. CycT significantly reduced proteins involved in heme metabolism and OXPHOS in addition to other pro-oncogenic hemoproteins and regulators of OXPHOS. *In vitro* studies demonstrated that the effects of CycT on heme metabolism and OXPHOS are independent of its antagonist properties on the hedgehog pathway. The significance of this study is that it shows that CycT acts via diminishing heme metabolism, hemoproteins involved in oxygen consumption, and oxygen consumption in NSCLC. This is the first study to demonstrate the effect of CycT on heme metabolism and OXPOS *in vivo*. This novel mechanism of action of CycT is independent of its previously known antagonistic effect on hedgehog signaling. This study demonstrates that CycT has the potential to be an effective therapeutic agent in treating NSCLC. This study provides compelling evidence to further assess the feasibility of using CycT in treating NSCLC.

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CHAPTER 1

INTRODUCTION TO ESSENTIAL ROLES OF MITOCHONDRIAL AND HEME FUNCTION IN LUNG CANCER

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INTRODUCTION

Contrary to Warburg's hypothesis, mitochondrial oxidative phosphorylation (OXPHOS) contributes significantly to fueling cancer cells (Danhier et al., 2017). Several recent studies have demonstrated that radiotherapy-resistant and chemotherapy-resistant cancer cells depend on OXPHOS for survival and progression (Davidson et al., 2016; Lee et al., 2017). Several cancers exhibit an increased risk in association with heme intake (N. Bastide et al., 2016; N. M. Bastide et al., 2015; Gamage, Dissabandara, Lam, & Gopalan, 2018; Hooda et al., 2014; Lam et al., 2014). Mitochondria are widely known to carry out oxidative phosphorylation. In addition, mitochondria are also involved in heme synthesis (L. Zhang, 2011). Heme serves as a prosthetic group for several proteins that constitute the complexes of mitochondrial electron transport chain (L. Zhang, 2011; Alam, Lal, FitzGerald, & Zhang, 2016; Hooda, Alam, & Zhang, 2015). Therefore, heme plays a pivotal role in OXPHOS and oxygen consumption. Further, lung cancer cells exhibit heme accumulation and require heme for proliferation and invasion *in vitro* (Hooda et al., 2013). Abnormalities in mitochondrial biogenesis and mutations are implicated in cancer (LeBleu et al., 2014; Xu et al., 2016). This study explores the strategy of targeting mitochondrial OXPHOS and lesser explored area of heme metabolism to stall the progression of lung cancer.

Lung cancer tissue exhibits prominent genetic differences in comparison to healthy tissue

The value of genetic drivers in lung cancer cannot be overstated. It is believed that up to 60% of lung adenocarcinomas have driver mutations. These mutations most commonly occur in proto-oncogene B-Raf (BRAF), kirsten rat sarcoma viral oncogene (KRAS), anaplastic lymphoma kinase (ALK), and epidermal growth factor receptor (EGFR) (Xu et al., 2016). In lung cancer, the

EGFR pathway is the main signaling pathway. The mutation rate of genes in the EGFR pathway are as high as 70-80% in lung cancer tissues (Xu et al., 2016). The EGFR mutations frequently occur in 4 tyrosine kinase domains which are coded by exons 18-21. The mutations occur as point mutations, in-frame deletions/insertions/duplications (Mollberg et al., 2011). The most commonly occurring mutations are an in-frame deletion in exon 19 and the point mutation L858R in exon 21 (Mollberg et al., 2011). The enhanced kinase activity of EGFR cause amplification of downstream signals eventually resulting in increased proliferation, angiogenesis, metastasis, and decreased apoptosis (da Cunha Santos, Shepherd, & Tsao, 2011; Xu et al., 2016). KRAS mutations are responsible for around 30-35% of lung adenocarcinoma genetic variation, 97% of which occur on codon 12 or 13 (Xu et al., 2016).

Ras mutations frequently occur in human cancers (Minamoto, Mai, & Ronai, 2000). Insensitivity to GTPase-activating proteins is caused by activating mutations in Ras guanosine nucleotide-binding proteins (Telang et al., 2012). Glucose uptake and flux, important for the survival and proliferation of lung cancer, are partially promoted by activated GTP-bound Ras family members. Oxygen consumption and tricarboxylic acid cycle activity are also increased by activated Ras, further fueling the metastatic capacity of cancer cells. Ras activates cytochrome c oxidase, a vital component of complex IV of the electron transport chain containing 10 genomic DNA-encoded subunits and 3 mitochondrial DNA-encoded subunits (Dejean, Beauvoit, Bunoust, Guerin, & Rigoulet, 2002; Kadenbach, Huttemann, Arnold, Lee, & Bender, 2000). The levels of cytochrome c oxidase are elevated in some forms of cancer (Kadenbach et al., 2000; Wang et al., 2014). A549, a lung adenocarcinoma cell line, is unable to grow without activated cytochrome c

oxidase (Telang et al., 2012). Ras mutations are far from the only nuclear genes with frequent mutations in lung cancer. Genes for isocitrate dehydrogenase, including IDH1 and IDH2, and succinate dehydrogenase, including SDHB, SDHC, and SDHD, code for mitochondrial components (Sequist et al., 2011). As structure determines function, the deformities caused by these mutations affect mitochondrial function. This altered mitochondrial function is either causal to or associated with lung cancer.

In addition to genomic differences, lung adenocarcinoma exhibits proteomic differences. ATP synthase subunit d (ATP5D) are expressed in much higher levels in cancerous tissues than in healthy tissues (G. Chen et al., 2002). Similarly, two important enzymes associated with aerobic glycolysis and fatty acid synthesis, malic enzyme and ATP-citrate lyase, are highly associated with non-small cell lung cancer (NSCLC) cells. Malic enzyme correlates with metastases to mediastinal lymph nodes and ATP-citrate lyase correlates with local tumor stage. Interestingly, upregulation of these two enzymes is associated with increased survival rates in young patients and decreased survival rates in elderly patients (Csanadi et al., 2015). Malic enzyme levels are also increased in the lung tissues of smokers compared to non-smokers (Csanadi et al., 2015). Nonsmokers, however, are believed to have adenocarcinomas that evolve locally. Non-smokers with adenocarcinoma displayed four times as many differentially expressed genes than smokers with adenocarcinoma did (Powell et al., 2003). Lung adenocarcinomas are the most common form of NSCLCs found in non-smokers. Interestingly, non-smokers and smokers appear to follow very different modes of tumorigenesis. It is believed that in smokers with adenocarcinoma, a small area within the lung becomes genetically altered. From there, it evolves into cancer. Fascinatingly, the

level of upregulation of some genes in smokers is correlated with the frequency that the smoker smoked (Powell et al., 2003).

From the enormous body of research on the genetic basis of lung cancer, it is obvious that lung cancer is highly genetically heterogeneous. Therefore, it is unsurprising that chemo-resistance is a major problem for lung cancer patients. Any resistant cells will replicate after the cancer is subjected to the first treatment, and therefore the drug will quickly lose efficacy in a heterogeneous tumor as the tumor acquires genetic and epigenetic changes (Nowell, 1976). Understanding both inter-tumoral and intra-tumoral heterogeneity is consequently of great value when seeking combination therapies for lung cancer.

Elevated heme uptake and synthesis are hallmarks of NSCLC cells

Iron protoporphyrin IX, also known as heme, is linked with oxygen utilization, transport, and storage. Heme enters cells through two pathways. Most mammalian cells possess machineries allowing *de novo* synthesis of heme. In addition, most cells possess heme transporters, such as HCP1 (heme carrier protein 1) and HRG1 (heme related gene 1), for heme uptake. Heme serves as a prosthetic group for hemoglobin, myoglobin, catalases, peroxidases, cytochromes, and several mitochondrial respiratory complexes (Figure 1.1) (Alam, Lal, FitzGerald, & Zhang, 2016; Hooda, Alam, & Zhang, 2015).

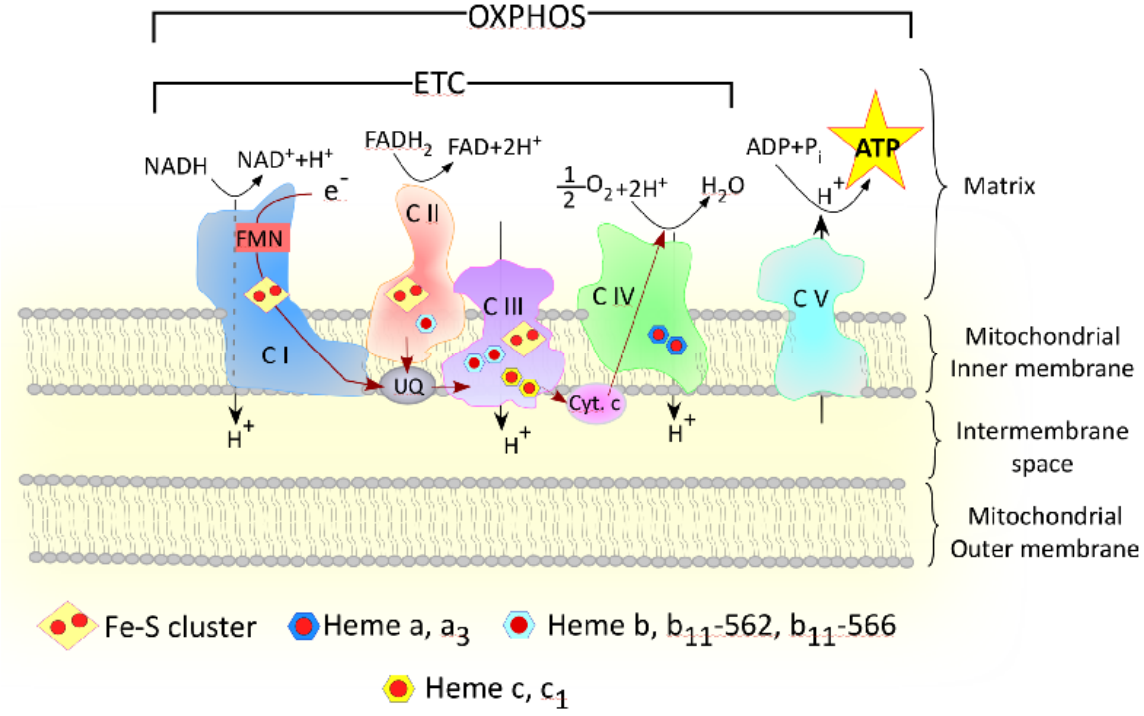

Figure 1.1. Multiple forms of heme are required for proper functioning of mitochondrial OXPHOS complexes. Heme serves as the prosthetic group for many mitochondrial respiratory complexes. This cartoon demonstrates the different types of heme that serve as prosthetic groups for mitochondrial complexes- complex II, complex III, and complex IV. Hence, heme serves a pivotal role in mitochondrial oxidative phosphorylation. Indicated in red is the direction of electron transport through a series of transporters embedded in the mitochondrial inner membrane that shuttles electrons from NADH and $FADH_2$ to molecular oxygen.

Heme also has a plethora of biological functions, and is important for circadian rhythm, pancreatic development, neurogenesis, and erythroid biogenesis (L. Zhang, 2011). Heme directly regulates many vital cellular processes that include cell cycle, cell death, transcription, and translation (J. J. Chen, 2007; Hooda, Shah, & Zhang, 2014; Yao, Balamurugan, Arvey, Leslie, & Zhang, 2010; Ye & Zhang, 2004; Zhu, Hon, & Zhang, 1999). Abnormal levels of heme are associated with diverse disease states, including anemia; porphyria; neurodegenerative disorders;

Type-II diabetes; coronary heart disease; and lung, pancreatic, and colorectal cancers (Hooda et al., 2013; Hooda et al., 2014). Therefore, heme levels are tightly regulated.

There have been a plethora of epidemiological studies linking dietary heme or red-meat intake with increased risks of several cancers (N. Bastide et al., 2016; N. M. Bastide et al., 2015; Gamage, Dissabandara, Lam, & Gopalan, 2018; Hooda et al., 2014; Lam et al., 2014). A meta-analysis by Gnagnarella et. al. showed that high consumption of red meat is associated a statistically significant 24% increased risk of lung cancer (Gnagnarella, Caini, Maisonneuve, & Gandini, 2018). A previous review article extensively discussed epidemiological studies linking dietary heme intake with increased risks of various cancers (Hooda et al., 2014). Thus, this article will focus mainly on the molecular and cellular actions of heme germane to lung cancer. Studies from the author's lab implicated heme in lung cancer development (Hooda et al., 2013; Hooda et al., 2014). We have shown that NSCLC cell lines exhibited elevated levels of heme synthesis to the normal cell line HBEC30KT. Inhibition of the rate-limiting heme synthetic enzyme 5-aminolevulic acid synthase (ALAS1) resulted in inhibition of cell proliferation and migration of NSCLC cell lines (Hooda et al., 2013). This suggests that heme is crucial for progression and metastasis of NSCLC cell lines, albeit *in vitro*. NSCLC cells also exhibited elevated OXPHOS and elevated expression of oxygen-utilizing hemoproteins, such as CYP1B1 (Cytochrome P450 family 1 subfamily B member 1) and cytoglobin. Hemoproteins constitute the mitochondrial electron transport chain complexes that carry out mitochondrial OXPHOS for energy production (Hooda et al., 2013). Notably, one enzyme in glycolysis, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), has been shown to have a function in heme delivery (Chakravarti, Aulak, Fox, &

Stuehr, 2010) while multiple subunits require heme (Figure 1.1). These provide intrinsic links between heme and OXPHOS and cellular bioenergetics.

Mitochondrial DNA mutations and changes in copy number are associated with lung cancer

Somatic and germline mitochondrial mutations may both be carcinogenic. However, while most DNA within the body is protected by histones and introns, mitochondrial DNA lacks these important safeguards (Hosgood et al., 2010). Mitochondria also lack DNA-repair machineries present in the nucleus (Wallace, 1994). Therefore, mitochondrial DNA is especially prone to mutations when exposed to reactive oxygen species (ROS). The resulting instability causes both changes in copy number and mutations (van Gisbergen et al., 2015).

According to the multiple hit hypothesis, mitochondrial DNA may function as a complementary gene mutation or as a driver, allowing cancer cells to possess increased clonogenic and/or mutagenic capabilities (van Gisbergen et al., 2015). Mitochondria increase their copy number substantially to avoid these mutations. The copy number may be changing during the epithelial-to-mesenchymal transition, which is thought to be the most important stage for metastatic potential (Xie et al., 2012). For example, in A549 cells, copy number increased from 1700 to 2800 during the epithelial-to-mesenchymal transition. According to some studies, carcinogenicity is correlated with the copy number of mitochondrial DNA, an association drawn especially in lung adenocarcinoma (Akgul, Kurt, Gulcan Kurt, & Cayci, 2013; Mi et al., 2015; Reznik et al., 2016). The sets of genes associated with the tricarboxylic acid cycle and the respiratory electron transport have the strongest correlation. However, this may be skewed by

diseases that cause both cancer and increased mitochondrial copy number. To ascertain whether this relationship holds true outside of latent diseased states, peripheral white blood cells were tested for increased mitochondrial DNA copy number. These studies indicated that even after correcting for latent disease, mitochondrial copy number is still associated with increased risk of lung cancer (Hosgood et al., 2010). Lung adenocarcinoma is noted to exhibit increased copy number. However, this is not true for all forms of cancer, as is evidenced by some cancers that are associated with decreased copy numbers (Mi et al., 2015; Reznik et al., 2016).

Another factor affecting mitochondrial copy number is the expression of transcription factor A, mitochondria, also known as TFAM (Reznik et al., 2016). Because TFAM binds to mitochondrial DNA, it helps control mitochondrial gene expression. TFAM expression is positively correlated with mitochondrial DNA copy number (Lin et al., 2016; Reznik et al., 2016).

Genes associated with mitochondrial respiration are also differentially expressed in lung tumor cells. In lung adenocarcinoma, 1000 differentially expressed genes, including 535 upregulated genes and 465 down regulated genes, were found utilizing matched tissues from the same patient. Genes associated with the mitochondrial oxidative phosphorylation and the electron transport chain constitutes one class of differentially upregulated genes. UQCRC2, UQCR11, NDUFA1, NDUFA2, NDUFA7, NDUFB1, NDUFB8, NDUFV1, NDUFV2, NDUFS3, NDUFS7, and ATP5D are related to bioenergetic pathways, including the electron transport chain and mitochondrial ATP synthesis coupled electron transport (Xu et al., 2016). Genes specific to mitochondrial biogenesis are also upregulated in circulating lung cancer cells and seem to be

imperative for metastatic potential. For example, peroxisome proliferator-activated receptor gamma coactivator 1 alpha, also known as PGC-1α, was found to increase oxygen consumption, mitochondrial biogenesis, and oxidative phosphorylation, thereby fueling metastases. Interestingly, LeBleu et al. found that PGC-1α only seemed to affect metastases, and had no demonstrated effect on cancer cell proliferation, epithelial-to-mesenchymal transfer, or primary tumor growth (LeBleu et al., 2014).

Inhibition of mitochondrial function inhibits lung tumor progression

Oxidative phosphorylation is crucial for anchorage-independent cancer cell proliferation (Viale, Corti, & Draetta, 2015). Suppression of oxidative phosphorylation substantially limits the tumorigenic capacity of cancer cells. Targeting mitochondria, which are necessary for the electron transport chain and oxidative phosphorylation, starves cancer cells of ATP and limits growth and metastasis of tumors. Studies have shown intensified oxygen consumption in NSCLC cell lines HCC4017 when compared to the normal HBEC30KT cells isolated from the same patient. Hypoglycemia also correlates with higher oxygen consumption and encourage glutamine consumption in lung cancer cells. Conversely, low levels of glutamine correlate with low oxygen consumption and encourage glucose utilization (Kuhn, Muscaritoli, Wischmeyer, & Stehle, 2010). Therefore, when mitochondria are targeted, lung tumor cells become more susceptible to cytotoxic drugs. Several treatments have been shown to interfere with normal mitochondrial function in lung tumor cells, including metformin, BAY87-2243, a lead structure; and microRNA-126 (Alam, Sohoni, Kalainayakan, Garrossian, & Zhang, 2016; Ellinghaus et al., 2013; Jara & Lopez-Munoz, 2015; Tomasetti et al., 2014). These treatments target different aspects of mitochondrial function.

Cyclopamine, a known inhibitor of Hedgehog signaling pathway, has been shown to exhibit anti-carcinogenic properties. Cyclopamine tartrate, 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. This agent also generates ROS, which perturb tumor cell mitochondria. Cyclopamine tartrate can induce mitochondrial fission and fragmentation in some NSCLC cell lines, including A549, H1299, and H460 cells, impeding mitochondrial respiration (Alam, Sohoni, et al., 2016).

Metformin, which has been historically used to alleviate Type II diabetes, was noted in 2001 to have anticancer properties in mammals (Schneider et al., 2001). Patients taking metformin also had fewer instances of cancer than individuals who did not (H. J. Kim et al., 2018; Li, Yeung, Hassan, Konopleva, & Abbruzzese, 2009; Libby et al., 2009; Noto, Goto, Tsujimoto, & Noda, 2012; Tseng, 2014). Metformin reduces oxygen consumption in the presence of pyruvate and malate, starving mitochondrial Complex I of its substrate: NADH. Metformin may also disrupt the lipid metabolism, glucose metabolism, tricarboxylic acid cycle, the methionine cycle, the folate cycle, and nucleotide synthesis (Andrzejewski, Gravel, Pollak, & St-Pierre, 2014; Janzer et al., 2014; Liu, Romero, Litchfield, Lengyel, & Locasale, 2016; Schneider et al., 2001).

Although most cells have mitochondria, not all cells are strongly affected by mitochondrial inhibitors. Healthy cells likely have lower energy requirements than tumor cells and consequently

are not as strongly affected my mitochondrial inhibitors. Healthy cells can maintain functionality even in the presence of mitochondrial inhibitors, making these agents viable treatment options.

Mitochondria and OXPHOS play a pivotal role in drug resistance

Drug resistance is one of the major hurdles of an effective cancer therapy (Longley & Johnston, 2005). Numerous studies have shown that tumors that regress considerably in response to targeted therapy relapse as a more aggressive drug resistant form. Some populations of the cells remain dormant during therapy and subsequently become resistant. The resistant cells continue to establish their clonal populations, metastasize, and result in poor survival outcomes (Longley & Johnston, 2005). Therefore, tackling drug resistance is very vital to improve the treatment outcome.

Mitochondria have long been associated with ATP generation and ROS production in cancer. In this section we are delving into much lesser known roles of mitochondria in cancer cell survival and drug resistance. Recent studies have shown that several cancers have adapted and rewired their cells to rely more on mitochondrial OXPHOS for drug resistance in addition to their energy needs (Bosc, Selak, & Sarry, 2017; Datta et al., 2017; Davidson et al., 2016; Lee et al., 2017; Navarro et al., 2016; G. Zhang et al., 2016). Cancer cells have shifted gears to elevated OXPHOS through several different mechanisms ranging from gene upregulations to ectopic protein expressions (Lee et al., 2017; Yang et al., 2016).

Cancer stem-like cells (CSCs) are associated with metastasis and resistance to adjuvant chemotherapy and radiotherapy (Lee et al., 2017). Triple negative breast cancer (TNBC) exhibiting markers of CSCs are associated with poor outcomes. A recent study has shown that proto-

oncogene MYC (a human gene over-expressed in various cancers that is homologous to an oncogene carried by the avian myelocytomatosis virus) and MCL1 (myeloid cell leukemia-1) protein (stimulates mitochondrial respiration when localized in mitochondrial matrix) are enhanced in CSCs of TNBC (Lee et al., 2017). Both MYC and MCL1 promote OXPHOS. Elevated OXPHOS induced ROS which in turn activated HIF-1α, thereby conferring resistance to adjuvant chemotherapy (Davidson et al., 2016; Lee et al., 2017). Similar observation was made in Small Cell Lung Cancer (SCLC) where CSCs exhibit elevated OXPHOS and preferential dependence on OXPHOS over glycolysis for energy. Oligomycin, an inhibitor of OXPHOS, abolished the tumor initiating abilities of CSCs, thereby implicating the role of OXPHOS in initiation of SCLC (Gao, Shen, Jin, Miao, & Qiu, 2016). Similarly, NSCLC cells that are resistant to EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, were shown to exhibit elevated OXPHOS accompanied by elevated glycolysis and activity in TCA cycle (Yang et al., 2016). This metabolic shift to increased OXPHOS was found to be a result of MET (mesenchymal-epithelial transition factor) proto-oncogene expression in the mitochondrial membrane in addition to plasma membrane. Pharmacological inhibition of MET resulted in cytotoxicity and apoptosis (Yang et al., 2016). Interestingly, cancer cells rewire the metabolism by altering the localization of proteins and expressing them ectopically in mitochondria (Lee et al., 2017; Yang et al., 2016). 3-Oxoacid CoA-Transferase 1 (OXAT1) and Acetyl-CoA Acetyltransferase 1 (ACAT1) are proteins localized in mitochondria and are involved in utilization of ketone bodies to aid in tumor growth and metastasis. Epithelial cancers like breast cancer over express these mitochondrial proteins to utilize ketone bodies like 3-hydroxybutyrate and aceto-acetate for fuel to promote tumor progression and metastasis to lungs (Lee et al., 2017; Yang et al., 2016). It has been shown by *in silico* drug

designing and mammosphere assay that targeting the mitochondrial proteins – OXAT1 and ACAT1 – can effectively inhibit activity and propagation of CSCs in breast cancer (Ozsvari et al., 2017).

Activating mutations in KRAS oncogene is prevalent in lung, colon, and pancreatic cancers. A recent study that performed a CRISPR/Cas9 screening and identified several mitochondrial genes involved in ribosomes and translation shared lethal interactions with K-ras gene (Weinberg et al., 2010). In renal cell carcinoma (RCC) an NADPH oxidase isoform, NOX4, localized to the inner mitochondria membrane and that subcellular redistribution of ATP levels from the mitochondria activated NOX4. NOX4-derived ROS inhibited P300/CBP-associated factor (PCAF)-dependent acetylation and lysosomal degradation of the pyruvate kinase-M2 isoform (PKM2). Silencing NOX4 sensitized cultured and *ex vivo* freshly isolated RCC cells to etoposide-induced cell death in xenograft models and *ex vivo* cultures by acting via PKM2 (Shanmugasundaram et al., 2017).

A recent study showed that cisplatin resistant lung adenocarcinoma cells exhibit higher mitochondrial membrane potential (MMP) and intracellular ATP levels than the non-resistant cells which confer migratory and invasive abilities to these cells (Jeon et al., 2016). Inhibition of mitochondrial complex I abolished the ability of the resistant cells to invade (Jeon et al., 2016) suggesting the pivotal role of mitochondria in metastasis of resistant cells. Another recent study showed that mitochondrial oxygen consumption is a vital source of energy in paclitaxel resistant lung adenocarcinoma cells (Datta et al., 2017). In lung adenocarcinoma cell line A549,

mitochondrial membrane depolarization increased during the initial phase where cells died in response to paclitaxel. However, as the cells gained resistance, normal mitochondrial membrane were restored by increased activity of catalase and glutathione peroxidase which counteracted the effect of increased ROS produced as result of paclitaxel treatment. Further, paclitaxel-resistant cells exhibited elevated extracellular acidification rates and oxygen consumption (Datta et al., 2017). Several studies have shown that many cancer cells which are resistant, rely on mitochondrial oxidative phosphorylation for energy (Bosc et al., 2017; Navarro et al., 2016; G. Zhang et al., 2016). Recently, cyatarabine-resistant cells from PDX models of leukemia exhibited higher mitochondrial mass and consequently higher OXPHOS and ATP production (Farge et al., 2017).

Besides elevation of oxidative phosphorylation and oxygen consumption, several recent studies have shown that genes involved in mitochondrial biogenesis, mitochondrial electron transport chain, mitochondrial transcription factors as well as mitochondrial fission and fusion mediators are upregulated in resistant cancer cells (Bosc et al., 2017; Navarro et al., 2016; C. Zhang et al., 2017; G. Zhang et al., 2016). There is evidence that signaling axis involving TFAM - a transcriptional factor involved in mitochondrial biogenesis-is involved in conferring resistance to MAP Kinase inhibitors in melanoma. Additionally, cancer stem cells that are implicated in tumor progression and drug-resistance, also exhibit elevated OXPHOS and depend on OXPHOS for energy. These studies show that regardless of the type of cancer or method of interception, mitochondria play indispensable and multi-faceted roles in resistant cancer cells. Hence, targeting mitochondria could be an effective strategy to target drug-resistant cancer cells. Recent studies

show that targeted drug therapies such as MAPK inhibitors and first-line chemotherapeutic agents, such as platinum-based drugs, increase mitochondrial activity and OXPHOS (Datta et al., 2017; C. Zhang et al., 2017). These studies show that a combination of first-line therapeutic agents and mitochondrial targeting agents would plausibly serve as an effective strategy to curtail tumor progression.

Further, *in-silico* analyses have shown that mitochondrial biogenesis could be used as a reliable factor for predicting outcomes in lung cancer patients (Sotgia & Lisanti, 2017). In another study, about 33 mitochondrial-related genes correlated with NSCLC patient survival (Sotgia et al., 2012). Another recent study showed difference in mitochondrial phenotypes between tumors that depend on glycolysis versus tumors that depend on OXPHOS (Giedt et al., 2016). Studies are underway to utilize mitochondrial imaging techniques to assess possible modes of prognosis and outcome for a patient (Giedt et al., 2016). Mitochondria can be specifically targeted via mitochondrial sirtuins that play an important role in cellular homeostasis and is a major regulator of metabolism in cancer cells. Therefore, it is a potential drug target and further studies are required to assess the ability of targeting mitochondrial sirtuins to specifically target cancer cells (George & Ahmad, 2016). Apart from drugs that particularly target mitochondria, there are several vehicles like PEG coated CNTABT737 nanoparticles that are internalized into early endosomes via micropinocytosis and clathrin-mediated endocytosis and subsequently delivered into mitochondria (S. W. Kim, Lee, Lee, Hong, & Khang, 2017).

Importantly, a recent study in our lab (Dey et al., 2018) indicated a link between heme and drug resistance in NSCLC tumor cells. Subcutaneous xenografts of NSCLC cells treated with a vascular disrupting agent, combretastatin A4-phosphate (currently in Phase II clinical trials for non-squamous NSCLC), resulted in initial tumor regression followed by relapse. The treatment resulted in central necrosis; however, cells that constituted the rim of the tumor proliferated to repopulate the tumor. These resistant cells exhibited increased levels of protein and enzymes involved heme synthesis and heme uptake, as well as elevated levels of oxygen-utilizing hemoproteins and mitochondrial respiratory chain complex subunits (Dey et al., 2018).

CONCLUSIONS

Clearly, many experimental evidences have convincingly demonstrated the importance of mitochondrial oxidative phosphorylation. As a key signaling and structural molecule for processes involved in oxygen utilization and oxidative metabolism, heme can impact lung tumorigenesis in a multi-faceted manner. It is worth noting that the K_m of heme synthetic enzymes and cytochrome c oxidase for oxygen is very low (less than 1 μM or ~0.1%) (Andrew, Riley, & Dailey, 1990; Chance, 1965; Chandel, Budinger, Choe, & Schumacker, 1997; Labbe-Bois & Labbe, 1990; Solaini, Baracca, Lenaz, & Sgarbi, 2010; Tormos & Chandel, 2010). This is below oxygen levels experienced by human cancer cells under hypoxia (0.3-4.2% oxygen saturation) (McKeown, 2014). Thus, both heme synthesis and mitochondrial respiration can be maintained under clinically defined tumor hypoxia. Therefore, the reliance of aggressive or drug-resistant tumor cells on OXPHOS does not conflict with the fact that aggressive and drug-resistant tumors are hypoxic.

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CHAPTER 2

TARGETING HEME FUNCTION AND MITOCHONDRIAL RESPIRATION WITH CYCLOPAMINE TARTRATE EFFECTIVELY SUPPRESSES LUNG CANCER

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ABSTRACT

Lung cancer is the leading cause of cancer-related death in the US. Despite advances in targeted therapies and immunotherapies, positive outcomes in lung cancer remain hard to achieve. Several studies demonstrate that enhanced mitochondrial respiration or oxidative phosphorylation (OXPHOS) is a key feature of non-small cell lung cancer (NSCLC) and drug-resistance. This suggests that targeting OXPHOS could be an effective strategy for intervention in NSCLC. Here, we demonstrate that cyclopamine tartrate (CycT) significantly suppresses the growth and progression of orthotopic NSCLC xenograft tumors in mice. CycT decreases oxygen consumption and proteins involved in OXPHOS in addition to regulators that promote OXPHOS: MYC and MCL1. Several oxygen-utilizing proteins and mitochondrial respiratory complex proteins require heme as the prosthetic group. CycT reduces the levels of oxygen-utilizing hemoproteins as well as proteins and enzymes involved in heme uptake, synthesis, and degradation. Further, CycT diminishes heme synthesis and degradation in NSCLC cell lines. CycT is a well-known inhibitor of hedgehog signaling. SANT-1, another inhibitor of hedgehog signaling, does not affect heme synthesis or degradation suggesting that the effect of CycT on heme metabolism is independent of its action on hedgehog signaling. In addition, CycT treatment downregulates proteins involved in OXPHOS and heme metabolism, which are not known targets of hedgehog signaling. To conclude, CycT inhibits heme metabolism and OXPHOS to mediate anti-tumorigenic effect in mouse models of NSCLC. This study highlights that the novel mechanism of anti-tumorigenic effect of CycT is independent of hedgehog signaling. CycT exerts pleiotropic effects and suggests a new strategy for designing cancer therapy. Therefore, CycT is a novel OXPHOS and heme targeting agent with a high potency to treat NSCLC and drug-resistant tumors.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the US (Siegel, Miller, & Jemal, 2016). About 85-90% of cases are classified as non-small cell lung cancer (NSCLC) (Riaz et al., 2012). Despite the advent of targeted therapies and immunotherapies, an effective treatment or cure for lung cancer remains an unlikely outcome for most patients. The five-year survival rate remains 10-20%, lower than many other cancers, such as breast (90%) and prostate (99%) cancers (Allemani et al., 2015). Further, even for early stage patients typically treated with surgical or radiological procedures, the five-year survival rate is less than 60%, as compared to greater than 95% in the cases of early stage prostate and breast cancers (Demicheli et al., 2012). Targeted therapies are limited by two factors (Santarpia, Karachaliou, & Rosell, 2017): Firstly, patients with targetable genomic alterations represent a relatively small percentage of all NSCLC cases. Secondly, resistance to molecularly targeted agents inevitably develops in tumor cells under chronic drug exposure, as further mutations in many potential oncogenic drivers develop. A 2016 study of 17664 patients with NSCLC (Barlesi et al., 2016) showed that the presence of a targetable genetic alteration vs. none was associated with moderately improved first-line progression-free survival (10·0 months vs. 7·1 months; $p<0·0001$) and overall survival (16·5 months vs. 11·8 months; $p<0·0001$). Recently, immunotherapies have attracted intense interest (Herzberg, Campo, & Gainor, 2017). Since 2015, the FDA has approved 3 PD-1/PD-L1 checkpoint inhibitors—nivolumab, pembrolizumab, and atezolizumab—for treatment of advanced NSCLC. These inhibitors, compared to docetaxel, generally extend the median overall survival by about 3.0 months. In the front-line setting, the median progression-free survival extended from 6.0 months with platinum-doublet chemotherapy to 10.3 months with pembrolizumab in patients with

untreated NSCLC with a high level of PD-L1 expression (Reck et al., 2016). As such, for the overwhelming majority of NSCLC patients, immunotherapies and targeted therapies extend survival for only several months (Barlesi et al., 2016; Reck et al., 2016). Therefore, there is still an urgent need to develop novel therapeutic strategies, by targeting previously under-tested cellular functions and pathways, to substantially improve lung cancer patient survival rates.

Notably, several recent studies showed that drug-resistant cells of acute and chronic myeloid leukemia, breast cancer, and melanoma depend on OXPHOS and that targeting oxidative metabolism and mitochondrial respiration overcomes their drug resistance (Farge et al., 2017; Kuntz et al., 2017; K. M. Lee et al., 2017; Navarro et al., 2016; G. Zhang et al., 2016). Although NSCLC tumors are metabolically heterogeneous, stable isotope resolved-metabolomics for pathway tracing identified a common feature of human NSCLC tumors: pyruvate from elevated glycolysis enters and intensifies the TCA cycle (Hensley et al., 2016). An intensified TCA cycle should provide more TCA intermediates for biosynthesis and more NADH for ATP generation via OXPHOS. Further, it was shown that lactate also fuels the TCA cycle in molecularly heterogeneous tumors (Faubert et al., 2017). A separate study using two genetically engineered mouse models for lung cancer carrying different genetic mutations ($Kras^{LSL\text{-}G12D/+}Trp53^{-/-}$ and $Kras^{LSL\text{-}G12D/+}Stk11^{-/-}$) showed that the contribution of lactate to the TCA cycle is higher than that of glucose (Hui 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, Fiorillo, & Lisanti, 2017).

Recent work in the author's lab indicated that cyclopamine tartrate (CycT) inhibits mitochondrial respiration in NSCLC cell lines (Alam, Sohoni, Kalainayakan, Garrossian, &

Zhang, 2016), but it is unknown whether it can suppress lung tumors *in vivo*. Here, we show that CycT is highly effective at suppressing NSCLC cell tumorigenic functions and NSCLC xenograft tumors in NOD/SCID mice. Furthermore, we show that the efficacy of CycT at suppressing lung tumors is not attributable to its function as a Hh signaling inhibitor. Rather, CycT effectively diminishes the levels of proteins involved in heme biosynthesis, uptake, and transport. Heme is a central metabolic and signaling molecule in oxygen utilization and metabolism (L. Zhang, 2011). 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, 2009). Multiple subunits in OXPHOS complexes II-IV contain heme. We found that CycT promptly inhibits OXPHOS in NSCLC cells and diminishes the levels of heme- and non-heme-containing subunits of OXPHOS complexes in lung xenograft tumors. Further, we show that CycT effectively suppresses the levels of two regulators promoting OXPHOS, MYC and MCL1, and the levels of proteins/enzymes involved in glucose consumption/glycolysis. CycT also effectively alleviates tumor hypoxia. Together, our data demonstrate that heme function and OXPHOS are crucial for lung tumor growth and progression and that targeting heme function and OXPHOS is an effective strategy to eradicate lung tumors. Very likely, CycT represents a unique class of pleiotropic anti-cancer agents that effectively suppresses OXPHOS and heme function.

MATERIALS AND METHODS

Reagents

Cyclopamine tartrate (>99% purity) was provided by Logan Natural Products. D-Luciferin and the Opal 4 color IHC kit were purchased from PerkinElmer (USA). Bevacizumab (17.26 mg/ml) was provided by Genentech, Inc. SANT-1 and Pimonidazole Hydrochloride were purchased from Santa Cruz Biotechnology and Hypoxyprobe, Inc., respectively. [4-^{14}C]-5-aminolevulinic acid was custom synthesized by PerkinElmer.

Cell culture

NSCLC cell lines H1299 (CRL-5803) and A549 (CRM-CCL-185) were purchased from American Type Culture Collection (ATCC). Cell lines expressing luciferase were generated by infection with lentiviral particles bearing the EF1a-Luciferase gene (AMSBIO) at passage 3. Cell lines were authenticated by short tandem repeat (STR) profiling (PowerPlex 16HS) (Genetica DNA Laboratories, Inc.) and were found to be 96% identical to the standard (authentication requires >80%).

Measurements of OCR and ECAR

Oxygen consumption was measured with a Clark-type electrode, as described previously [12]. To measure OCR and ECAR with a Seahorse Bioscience XF243 Extracellular Flux Analyzer, 2500 cells were seeded in the Seahorse XF Cell Culture Microplate for 3 days and then the Seahorse Bioscience XF Cell Mito Stress Test Assay Kit was used. Cell proliferation was measured by detecting luciferase activity. Cell migration and invasion assays were carried out with BD Falcon

cell culture inserts (Corning Life Sciences) following the manufacturer's protocols. For the colony formation assay, 5000 NSCLC cells were seeded per well in 6-well tissue culture plates in triplicates. Cells were treated with 0.5 mM succinyl acetone (Sigma Aldrich) or 25 µM CycT for 6 days. Cells were then fixed and stained with 0.5% crystal violet. Images were acquired by using the Carestream Gel Logic GL-112 imaging system.

Measurement of heme synthesis and degradation

Measurement of heme synthesis in cells at passage 3 was carried out in triplicates exactly as described (Hooda, Alam, & Zhang, 2015). Briefly, cells were treated with or without 25 µM CycT or 50 µM SANT-1 for 7 days. 0.3 µCi [4-^{14}C]-5-aminolevulinic acid (ALA) was the added to each well for 15 hours. Heme was subsequently extracted, and radiolabeled heme was quantified exactly as described (Hooda, Alam, & Zhang, 2015).

Animals

NOD/SCID (CRL:394) mice were purchased from Charles River, maintained in a pathogen-free facility in accordance with the Protocol # 13-05 approved by IACUC of UT Dallas.

Subcutaneous and orthotopic xenograft mouse models

For subcutaneous models, 2.5×10^6 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 (n=6 per group). Mice were randomized into two groups that received I.V. saline (for control) and cyclopamine tartrate (CycT, 7.5 mg/kg) every 3 days), respectively. Body masses

were recorded once every week. When the tumors reached 1 cm^3, mice were euthanized by cervical dislocation. Tumors were resected and weighed. This experiment was repeated two times.

For orthotopic models, $0.75x10^6$ H1299-luc cells (passages 3-5) in serum-free medium containing 50% Matrigel were implanted orthotopically in 6-8-week-old female NOD/SCID mice. Mice were anesthetized and placed in right lateral decubitus position. H1299-luc cells were injected about 1.5 cm above the lower left rib line through the intercostal region. Mice were observed until they revived from anesthesia. Mice were randomized into three groups (n=6 per group) that received I.V. saline (for control), CycT (7.5 mg/kg, I.V.), bevacizumab (5 mg/kg, I.P.), and SA (50 mg/kg, I.V.) every 3 days. Treatments started 4 days after cell implantation. This experiment was repeated three times.

In Vivo Bioluminescence Imaging (BLI)

Mice bearing subcutaneous or orthotopic tumor xenografts were imaged with an IVIS Lumina III In Vivo Imaging system. Mice were anesthetized with 2% isoflurane. Luciferin (80 µl of 40 mg/ml) was administered subcutaneously. A BLI time course was acquired over 30 mins (Exposure time: auto, F Stop: 1.2, Binning: medium)**.** The images were quantified using Living Image software version 4.5.2 (Perkin Elmer). Regions of interest (ROIs) were selected. Bioluminescence signals between 600 to 60000 counts were accepted as authentic signals. The total bioluminescent signals (photon/sec) from ROIs of mice were calculated according to the manufacturer's instructions. Analyses of BLI data were done by personnel who were blinded to the objectives of the study. BLI data of the mice with tumors outside the lungs were excluded.

Tissue processing and hematoxylin and eosin (H&E) staining

60 mg/kg pimonidazole-HCl was administered to mice via tail vein 90 minutes before sacrifice. Lungs were removed and fixed in 4% formalin. Paraffin embedding was performed at the histopathology core at UTSW Medical Center. The paraffin blocks were sectioned into 5 µm sections which were utilized for H&E staining and immunohistochemical staining.

Immunohistochemistry (IHC)

IHC was carried out exactly as described (Dey et al., 2018).. Antibodies for ALAS1 (sc-50531), HCP1 (sc-134997), HRG1 (sc-101957), HO-1 (sc-10789), CYCS (sc-7159), PTGS2 (sc-7951), GLI1 (sc-20687), UQCRC2 (sc-390378), ATP5F1B (sc-33618), CYP1B1 (sc-32882), and MYC (sc-40) were purchased from Santa Cruz Biotechnology. Antibodies for PGRMC1 (#13856), GAPDH (#5174) were purchased from Cell Signaling Technology and for Pimonidazole Hydrochloride from Hydroxyprobe, Inc., respectively. Antibodies for NOX4 (ab133303), PDHA1 (ab92696), SLC2A1 (ab40084), and MCL1 (ab32087) were purchased from Abcam. Antibodies for CA9 (100-417), HRP-conjugated goat anti-mouse IgG (NB7539) and for Hexokinase II (PA5-29326), HRP-conjugated goat anti-rabbit IgG (#31460) were purchased from Novus and Thermo Fisher Scientific, respectively.

Imaging and data collection

Slides were scanned at 40X with an Olympus VS120 slide scanner and quantified using cellSens 1.16 software (Olympus), exactly as described (Dey et al., 2018). Briefly, multiple regions of interest (ROIs) of equal area were drawn over tumor regions. ROIs were positioned evenly

throughout tumor regions and were retested under three different filters—FITC, Cy3, and Cy5—to exclude any artifacts. 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 analysis

Data from different treatment groups of cells, mice, and tissues were compared, and statistical analyses were performed with a Welch 2-sample t-test. An n of 6 per group will provide enough statistical power to detect a 50% difference with a power of 95% and a p-value of 0.05.

RESULTS

CycT effectively inhibits NSCLC cell proliferation and tumorigenic functions of NSCLC cell lines

Although CycT inhibits oxygen consumption (Alam, Sohoni, Kalainayakan, Garrossian, & Zhang, 2016), it was not clear if it can suppress lung tumors *in vivo*. Other agents, such as succinyl acetone, can inhibit mitochondrial respiration, but has little anti-tumor activity *in vivo*. Here, we first examined and compared the effects of CycT and SA on oxygen consumption rate (OCR) after only 3 hours of treatment with a Clark-type electrode (Fig. 2.1A & 2.1B). SA doses that effectively inhibit heme synthesis have previously been found to be more than 0.1 mM (Hooda et al., 2013). Fig. 2.1A shows that CycT (25 μM) was somewhat more effective at inhibiting oxygen consumption than 0.5 mM SA. Using an Agilent XF24 extracellular flux analyzer, we measured the effects of CycT and SA on both OCR and extracellular acidification rate (ECAR) (Fig. 2.1B). Clearly, both methods detected the strong effect of CycT on OCR (Figs. 2.1A and 2.1B). Interestingly, CycT, not SA, reduced ECAR (Fig. 2.1B). The effect of SA on OCR detected by the analyzer (Fig. 2.1B) is different from that detected by the electrode (Fig. 2.1A), likely due to the different ways used to detect oxygen. The electrode does not involve the use of other agents (Alam, Sohoni, Kalainayakan, Garrossian, & Zhang, 2016), so it is likely more reflective of the effect of SA. The dose responses of H1299 proliferation to CycT and SA were as expected (Figs. 2.2A and 2.2B). CycT also strongly inhibited NSCLC A549 cells (Fig. 2.2C). We also characterized the effects of CycT on the tumorigenic functions of NSCLC cell lines. CycT effectively inhibited transwell migration (Fig. 2.1C), invasion (Fig. 2.1D), and colony formation (Fig. 2.2D) in H1299 cells. Likewise, the tumorigenic functions of A549 NSCLC cells were inhibited by CycT (Fig.

2.2E, 2.2F, and 2.2G). Together, the data show that CycT possesses strong anti-tumorigenic activities against NSCLC cells.

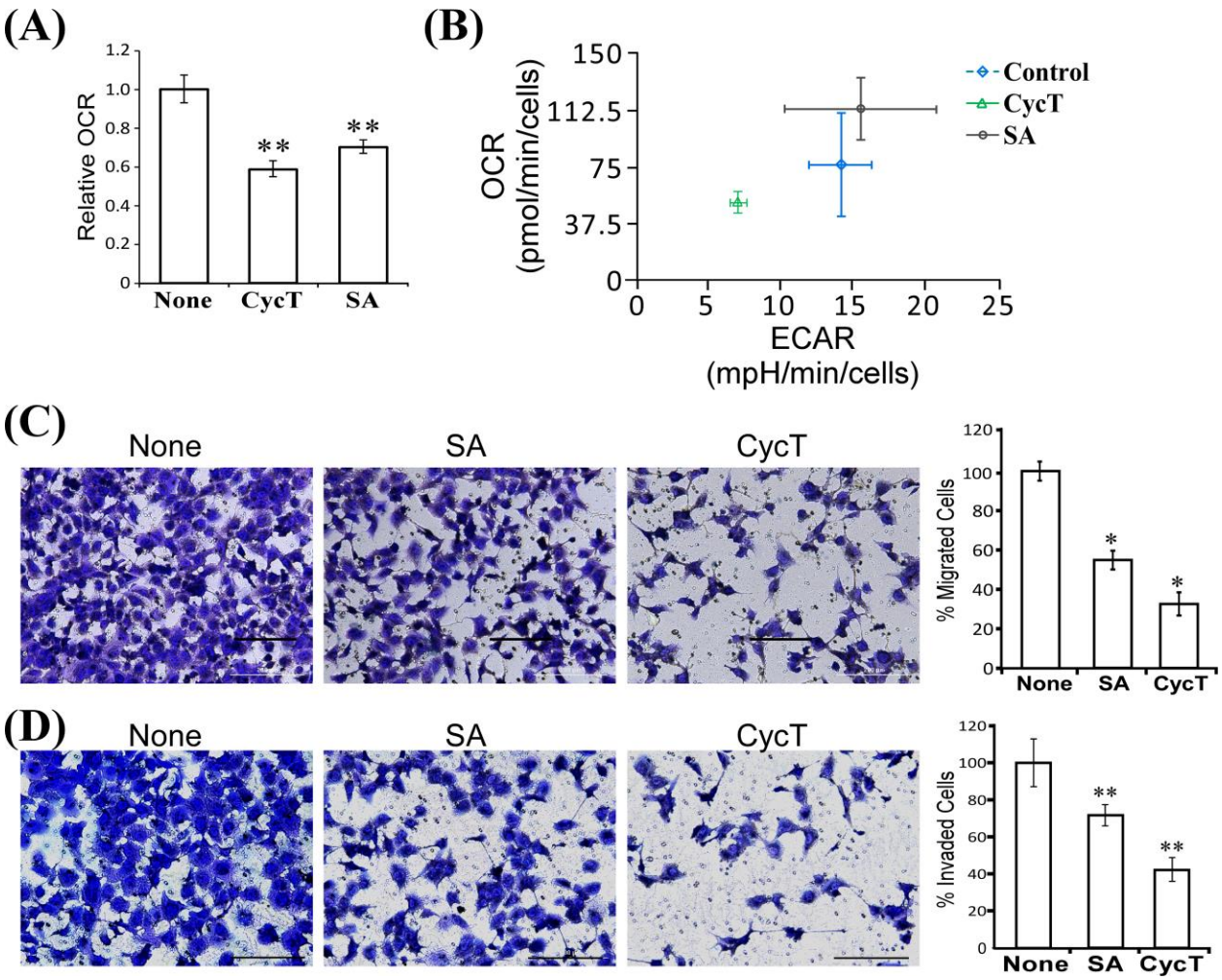

Figure 2.1. Cyclopamine tartrate (CycT) inhibits proliferation and oxygen consumption rates of NSCLC cell lines. (A) The effect of CycT and succinyl acetone (SA) on oxygen consumption rates (OCR) in H1299 cells. (B) The effects of CycT and SA on extracellular acidification rate (ECAR) in H1299 cells. CycT and SA were incubated with H1299 cells for 3 hours prior to measurements in both (A) and (B). (C) CycT inhibits transwell migration of H1299 cells. (D) CycT inhibits invasion of H1299 cells. The heme synthesis inhibitor SA is shown for comparison. CycT and SA were incubated with cells for 6 days in both (C) and (D). Data are plotted as mean ± standard deviation. Scale bar: 200 μm. 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; ***, p-value < 0.0005.

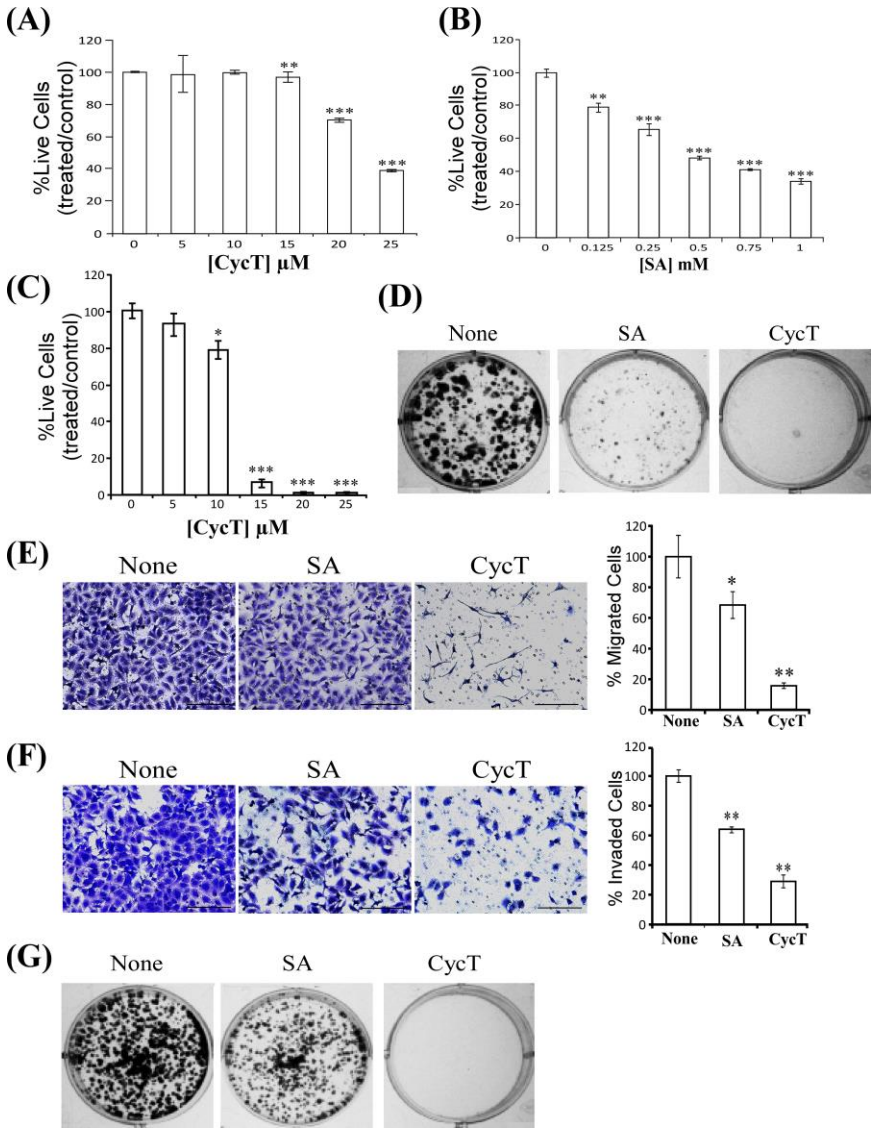

Figure 2.2. (A) Cyclopamine tartrate (CycT) inhibits tumorigenic functions of NSCLC cells. The dose response of H1299 NSCLC cell proliferation to CycT. (B) The dose response of H1299 NSCLC cell proliferation to SA. (C) The dose response of A549 NSCLC cell proliferation to CycT. (D) CycT inhibits colony formation by H1299 cells. (E) CycT effectively inhibits migration by A549 NSCLC cells. (F) CycT effectively inhibits invasion by A549 NSCLC cells. Scale bar: 200 □m. Data are plotted as mean ± standard deviation. (G) CycT effectively inhibits colony formation by A549 NSCLC cells. 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 < 0.005; ***, p-value < 0.0005.

CycT effectively suppresses the growth of subcutaneous NSCLC tumor xenografts

Next, we examined the anti-tumor efficacy of CycT using NOD/SCID mice bearing subcutaneously implanted NSCLC tumor xenografts. Bioluminescence imaging (BLI) showed that CycT significantly delayed tumor growth (Fig. 2.3A). When mice were sacrificed 6 weeks after initial tumor implantation, the masses of CycT-treated tumors were less than 50% of saline-treated (control) tumors (Fig. 2.3B). H&E staining also showed that tumor size was substantially reduced by CycT treatment (Fig. 2.3C). However, lungs are well-perfused by oxygen. Subcutaneously implanted tumors, which consist of densely packed tumor cells with few stromal cells, do not mimic the tumor microenvironment of human lung tumors.

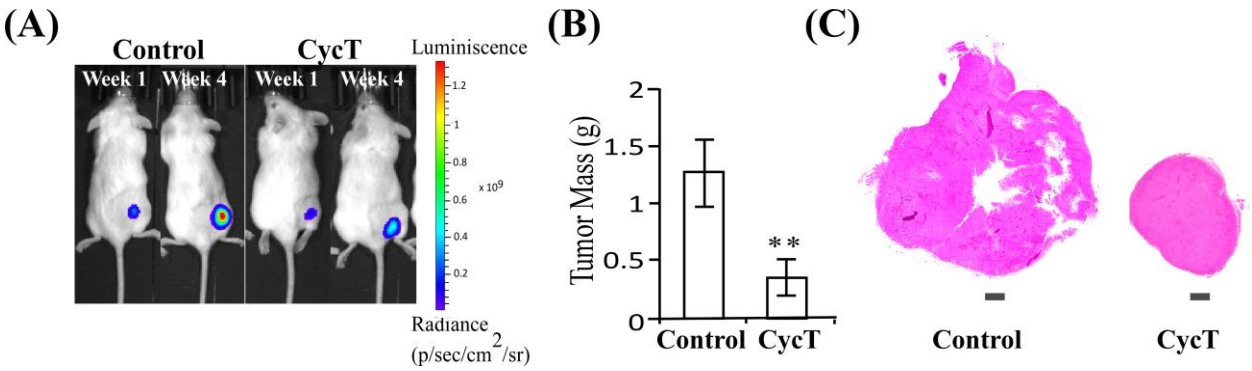

Figure 2.3. CycT suppresses the growth of subcutaneous NSCLC tumor xenografts. (A) Representative bioluminescence images of mice bearing subcutaneous H1299 tumor xenografts treated with CycT or saline (control). n = 6/group. (B) Average tumor mass after 4.5 weeks of CycT treatment. Data are plotted as mean ± standard deviation. For statistical analysis, the levels in CycT treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. (C) Representative H&E images of tumors with CycT or without CycT (control) treatment.

CycT is more effective than bevacizumab at suppressing the growth and progression of orthotopically implanted NSCLC tumor xenografts in mice

Orthotopic tumors have been shown to be more clinically relevant models of lung tumors than subcutaneous xenografts (Graves, Maity, & Le, 2010; Vilalta, Hughes, Von Eyben, Giaccia, & Graves, 2016). Thus, we decided to test the efficacy of CycT at suppressing orthotopically implanted NSCLC tumor xenografts. We also examined the effect of bevacizumab in comparison. Bevacizumab is an anti-VEGF antibody approved by FDA for treating lung cancer (Keating, 2014). Inhibition of angiogenesis and inhibition of OXPHOS should both lead to reduction in ATP production in tumor cells via decreasing oxygen supply and consumption, respectively. Thus, CycT and bevacizumab may share overlapping mechanisms in tumor suppression. BLI showed that CycT was very effective at suppressing lung tumor growth and progression and that CycT was significantly more effective than bevacizumab (Figs. 2.4A & 2.4B). Both CycT and bevacizumab were much more effective than SA. Histological analysis with H&E staining showed that CycT nearly eradicated lung tumors (Fig. 2.4C). The administration of CycT, bevacizumab, or SA at the indicated doses did not cause strong toxicity in mice, as expected (Fan et al., 2011). The body masses of treated mice appeared to be slightly higher than control mice, albeit not statistically significant (Fig. 2.4D). These results demonstrate that CycT has the potential to be a highly effective agent for the treatment of NSCLC.

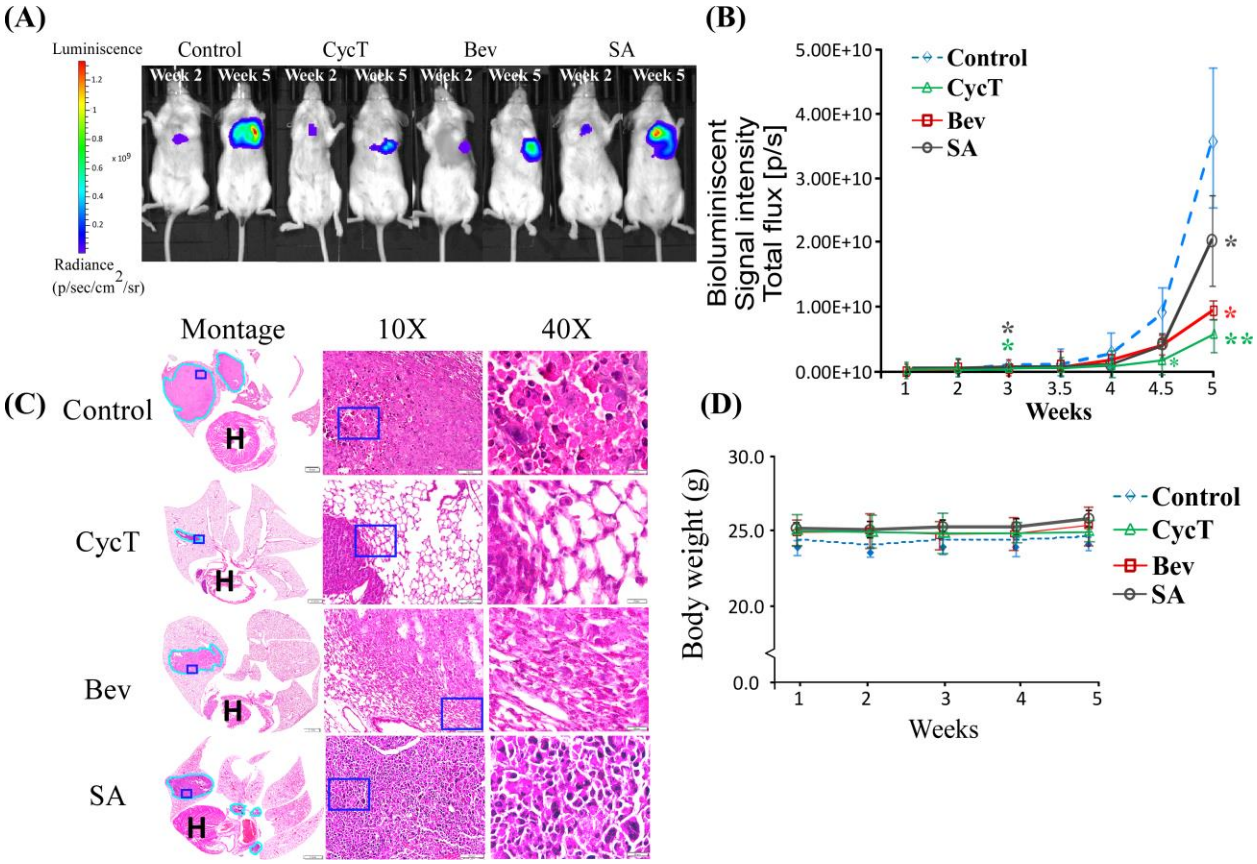

Figure 2.4. CycT suppresses the growth of NSCLC orthotopic tumor xenografts. (A) Representative bioluminescence images of mice bearing orthotopic H1299 tumor xenografts treated with saline (control), cyclopamine tartrate (CycT), bevacizumab (Bev), or succinyl acetone (SA). n = 6/group. (B) The quantified luminescence signals representing tumor volumes. Data are plotted as mean ± standard deviation. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. *, p-value < 0.05; **, p-value < 0.005. (C) Representative H&E images of control tumors and tumors treated with CycT, Bev or SA. Tumors are marked with light blue outlines. Montage (scale bar: 1 mm), 10X (scale bar: 100 μm), and 40X (scale bar: 20 μm) images of the H&E sections are shown from left to right. The light blue rectangles in Montage and 10X denote the regions shown in 10X and 40X, respectively. (D) The body masses of mice under every treatment condition.

CycT strongly decreases the levels of enzymes and proteins involved in heme biosynthesis, uptake, transport, and degradation

To gain insights into the molecular basis underlying the effectiveness of CycT at suppressing lung tumors, we decided to detect the levels of key proteins which CycT may affect. As expected,

CycT, not bevacizumab, dramatically decreased the protein levels of Gli1 (see Fig. 2.5 A), a target of SMO and a transcriptional regulator mediating hedgehog (Hh) signaling (J. K. Chen, Taipale, Cooper, & Beachy, 2002; Taipale et al., 2000). However, other studies have shown that CycT is not a simple SMO antagonist as it has other functions, including agonist functions (Sharpe, Wang, Hannoush, & de Sauvage, 2015; Teperino et al., 2012). Heme is a central molecule for oxidative metabolism and ATP generation via the TCA cycle and OXPHOS. Multiple subunits in OXPHOS complexes II-IV contain heme (Ortiz de Montellano, 2009). Heme also directly regulates many molecular and cellular processes involved in oxygen utilization (L. Zhang, 2011). Therefore, it would be insightful to examine the effects of CycT on the levels of proteins controlling the levels and flux of heme in tumor cells. However, it is difficult to quantify the levels of proteins in tumor cells of orthotopic lung tumor xenografts using Western blotting, particularly in treated tumors, which are small. Furthermore, changes in mitochondrial proteins, including many hemoproteins, are difficult to detect using proteomic methods, particularly in complex tumor tissues with stromal cells. Thus, we decided to carry out quantitative immunohistochemistry (IHC) analyses.

Fig. 2.6 A shows representative images from IHC analyses. The first two panels represent the montages of DAPI (staining nuclei) and fluorescent stains of the detected protein, i.e., the heme transporter HRG1 (SLC48A1). HRG1 is a major heme transporter and is located on endosomes, lysosomes, and cell membranes (O'Callaghan et al., 2010; Rajagopal et al., 2008). Notably, the montages show that HRG1 levels were generally much higher in tumor regions than in adjacent normal lung regions (Fig. 2.6A), indicating its pro-tumorigenic role in lung tumors. Clearly, HRG1 protein levels in NSCLC tumors were dramatically diminished by both CycT and bevacizumab.

Another heme transporter on the cell membrane, HCP1 (SLC46A1), was also significantly reduced by CycT in orthotopic tumors (Fig. 2.5B). The levels of the rate-limiting heme synthetic enzyme ALAS1 were reduced by CycT, but not by bevacizumab (Fig. 2.6B). We also detected the level of a putative heme sensor and heme chaperone necessary for maintenance of cellular heme and hemoprotein levels, PGRMC1 (Piel et al., 2016). The levels of PGRMC1 were greatly reduced by CycT, while bevacizumab reduced them to a lesser extent (Fig. 2.6C). Likewise, another putative heme sensor and heme chaperone protein, GAPDH (Chakravarti, Aulak, Fox, & Stuehr, 2010), was greatly reduced by CycT (Fig. 2.5C). Interestingly, the levels of the heme degradation enzyme HO-1 (HMOX1) were reduced by CycT, as well as bevacizumab (Fig. 2.7A). These results demonstrated that important proteins involved in heme uptake, biosynthesis, and maintenance are dramatically reduced by CycT, while bevacizumab has lesser effects.

To further ascertain whether the effects of CycT on heme-related enzymes are attributable to its SMO-antagonist activity, we compared the effects of CycT and the strong antagonist SANT-1 on heme synthesis and degradation. Fig. 2.8A shows that CycT reduced the levels of heme synthesis in H1299 cells more than 6-fold, while SANT-1 reduced it less than 2-fold. Likewise, CycT reduced the levels of heme degradation about 4-fold, while the effect of SANT-1 was not significant (Fig. 2.8B). Together, Figs. 2.6, 2.7, & 2.8 show that CycT strongly reduces heme synthesis, uptake, transport, and degradation and that this effect is not attributable to its SMO antagonist function.

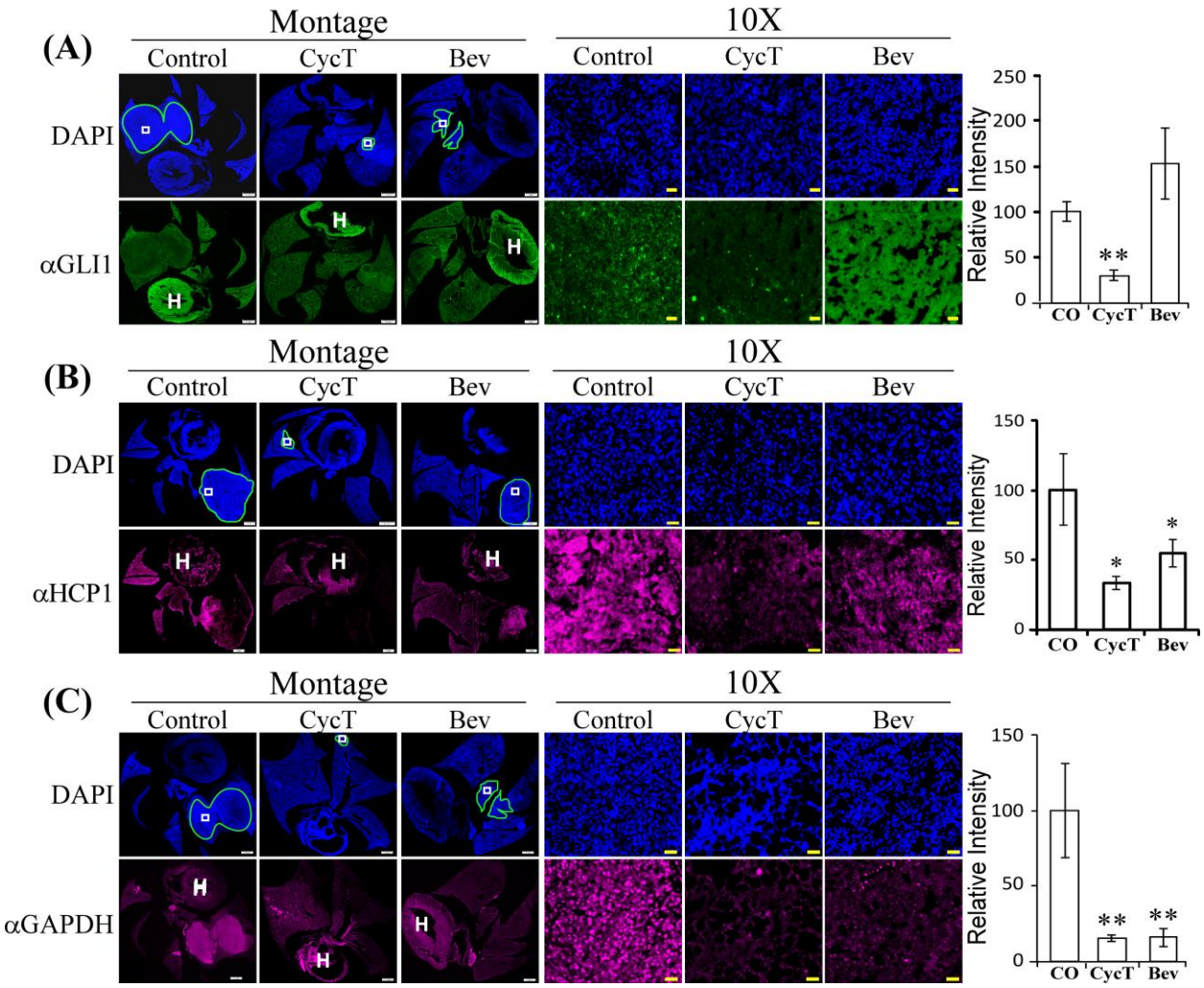

Figure 2.5. CycT inhibits Gli1, HCP-1, and GAPDH. (A) Effects of CycT and Bev on the levels of the transcriptional regulator GLI1 mediating Hh signaling in orthotopic tumor xenografts. (B) The effects of CycT and Bev on the levels of the heme transporter HCP1 in orthotopic tumor xenografts. (C) The effects of CycT and Bev on the levels of the putative heme-binding and chaperone protein GAPDH in orthotopic tumor xenografts. Scale bar: Montage, 1 mm; 10X, 20 μm. Data are plotted as mean ± SEM. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. IHC images are representative of 3 independent experiments.

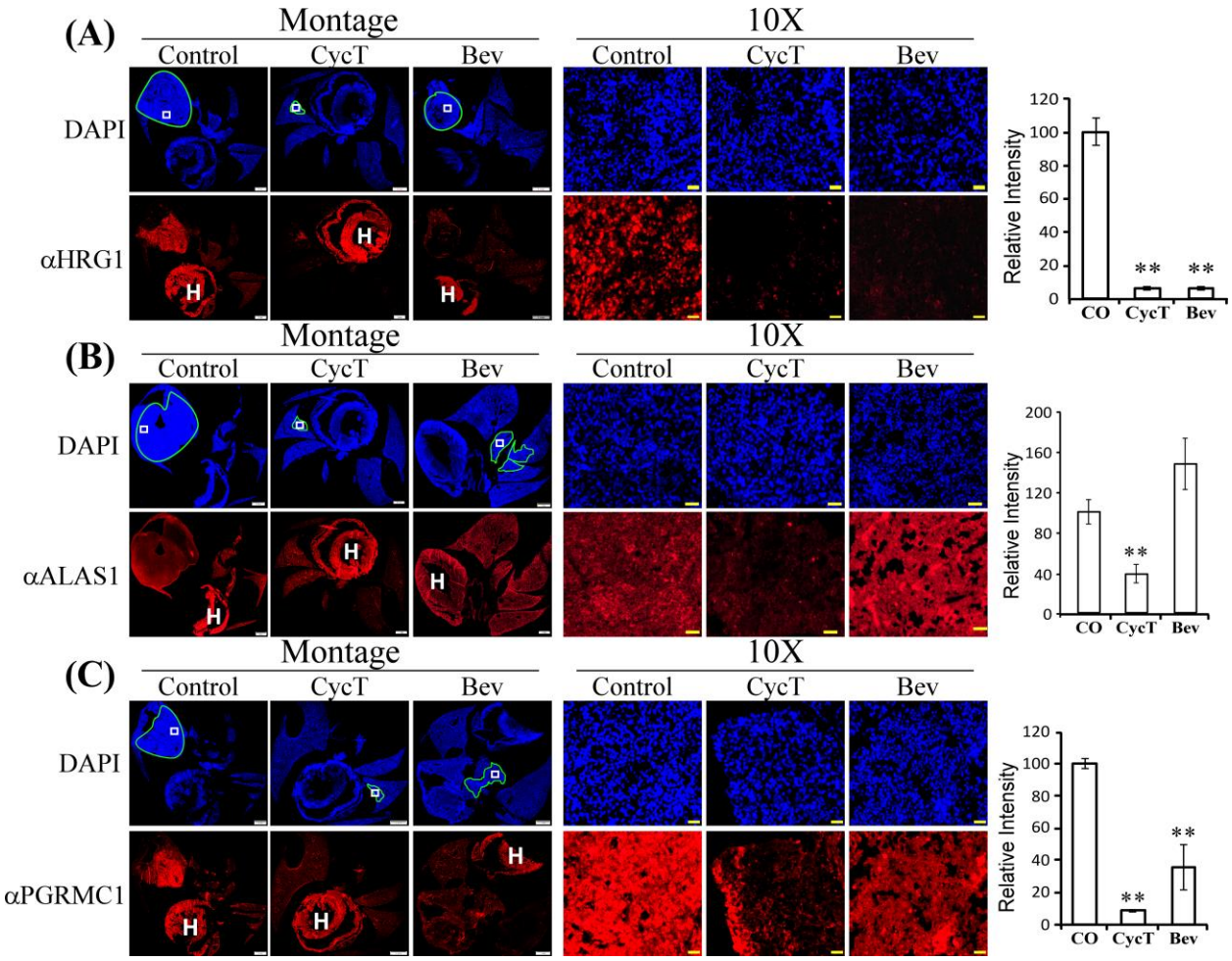

Figure 2.6. CycT effectively inhibits the levels of proteins and enzymes involved in heme metabolism and transport. (A) Representative IHC images of H1299 NSCLC tumor tissue sections and graph showing the levels of HRG1 in control and treated tumors. Shown are montages and 10X images of control, CycT-treated, and Bev-treated tumor tissue sections stained with DAPI or antibodies against the indicated protein HRG1. The light blue lines in DAPI images outline the tumors in the lung. The white rectangles in DAPI images denote the regions shown in 10X images. The lines and rectangles are not placed on IHC fluorescent images to avoid the obstruction of tumor images. The heart was often stained and is marked with "H". Scale bar: montage, 1 mm; 10X, 20 μm. Protein levels were quantified with cellSens dimension software (Olympus), as described in Methods. Data are plotted as mean ± SEM. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. (B) The effects of CycT and Bev on the levels of the rate-limiting heme biosynthetic enzyme ALAS1 in orthotopic tumor xenografts. (C) The effects of CycT and Bev on the levels of the heme chaperone and sensor protein PGRMC1 in orthotopic tumor xenografts. IHC images are representative of 3 independent experiments.

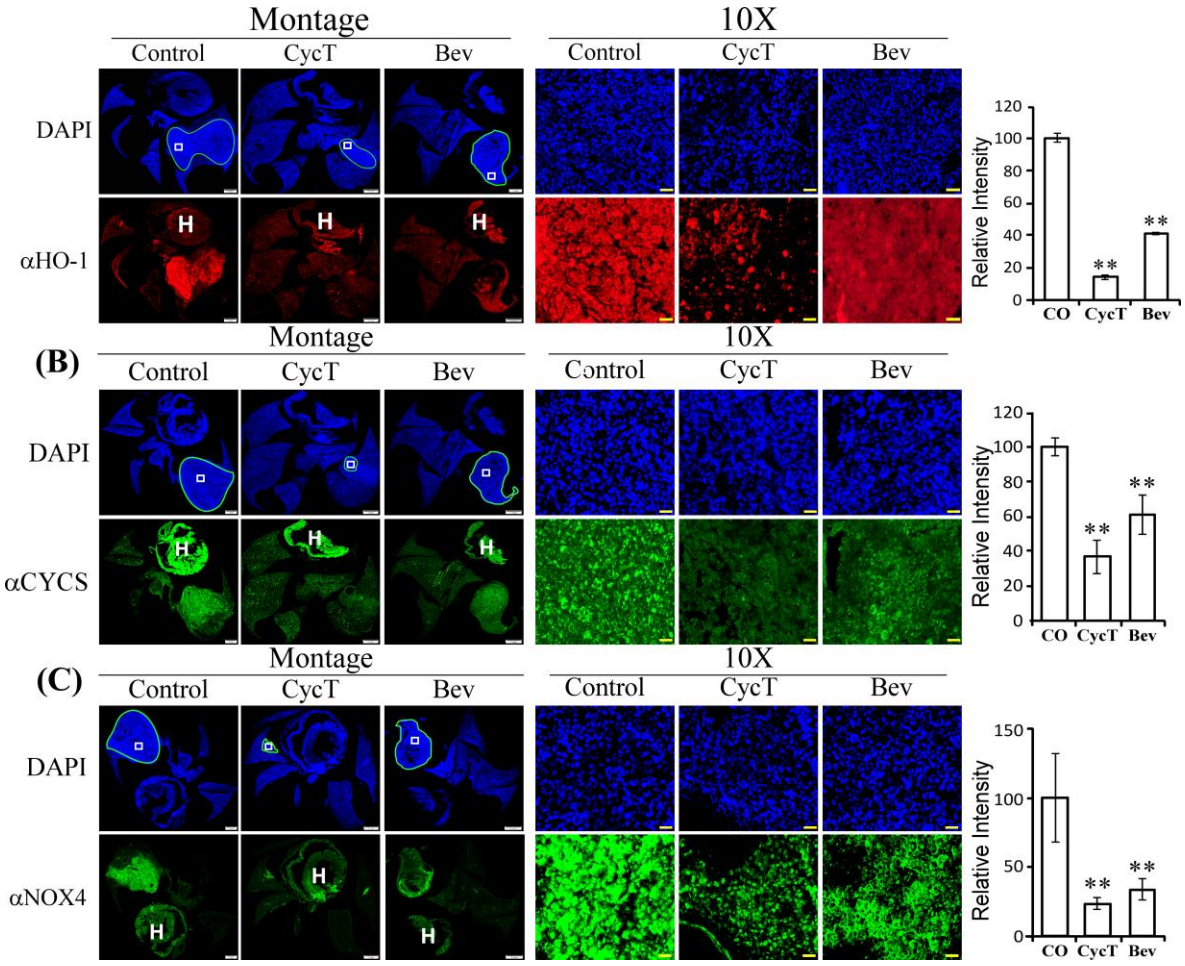

Figure 2.7. CycT effectively inhibits the levels of enzymes involved in heme degradation, OXPHOS, and pro-tumorigenic hemoproteins. (A) The effects of CycT and Bev on the levels of the heme degradation enzyme HO-1 in orthotopic tumor xenografts in control and treated tumors. Shown are montages and 10X images of control, CycT-treated, and Bev-treated tumor tissue sections stained with DAPI or antibodies against the indicated protein HO-1. The light blue lines in DAPI images outline the tumors in the lung. The white rectangles in DAPI images denote the regions shown in 10X images. The lines and rectangles are not placed on IHC fluorescent images to avoid the obstruction of tumor images. The heart was often stained and is marked with "H". Scale bar: montage, 1 mm; 10X, 20 μm. Protein levels were quantified with cellSens dimension software (Olympus), as described in Methods. Data are plotted as mean ± SEM. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. (B) The effects of CycT and Bev on the levels of cytochrome c (CYCS) in orthotopic tumor xenografts. (C) The effects of CycT and Bev on the levels of NOX4 in orthotopic tumor xenografts. IHC images are representative of 3 independent experiments.

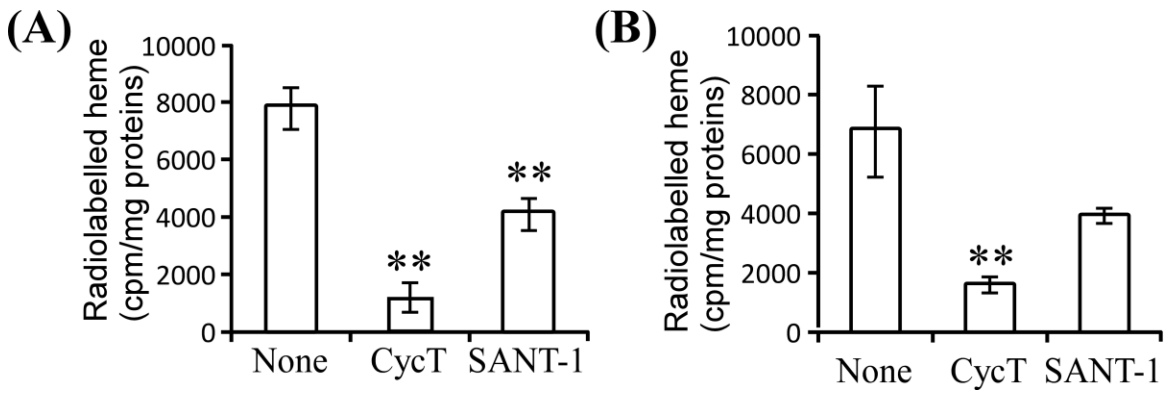

Figure 2.8. CycT diminishes heme biosynthesis and degradation. CycT, not the SMO antagonist SANT-1, strongly diminishes heme biosynthesis (A) and degradation (B) in H1299 NSCLC cells. Data are plotted as mean ± standard deviation. For statistical analysis, the levels in treated cells were compared to the levels in untreated cells with a Welch 2-sample t-test. **, p-value < 0.005.

CycT effectively reduces the levels of subunits of OXPHOS complexes and other oxygen-utilizing hemoproteins

A coordinated strong reduction in heme biosynthesis and uptake should limit the availability of cellular heme for producing hemoproteins, as well as non-heme proteins, due to the role of heme in coordinating the expression of all proteins involved in oxygen utilization, such as OXPHOS. To test this idea, we detected the levels of several subunits of OXPHOS complexes in treated and control H1299 NSCLC tumor xenografts. Indeed, CycT significantly reduced the levels of cytochrome c (CYCS, acting between OXPHOS Complex III and IV, Fig. 2.7B), UQCRC2 (a subunit of OXPHOS Complex III, Fig. 2.9A), and ATP5F1B (a subunit of OXPHOS Complex V, Fig. 2.9B). Further, CycT strongly decreased the levels of pro-tumorigenic hemoproteins, cyclooxygenase-2 (PTGS2) and cytochrome P450 (CYP1B1) in H1299 tumors, while bevacizumab did not affect or reduced their levels to a lesser extent (Figs. 2.9C and 2.9D). Interestingly, another heme-containing, ROS-producing NADPH oxidase, NOX4, was also reduced by CycT and bevacizumab in H1299 tumors (Fig. 2.7C). NOX4 promotes angiogenesis

and is tumorigenic (C. Chen, Li, Zhou, & Min, 2017). Together, these results show that CycT is highly effective in reducing the levels of key tumorigenic proteins required for oxygen metabolism and ATP generation.

CycT effectively reduces the levels of MYC and MCL1—two regulators promoting OXPHOS—in orthotopic lung tumor xenografts

To further probe the mode by which CycT influences OXPHOS, we examined its effect on the levels of two previously identified regulators promoting OXPHOS, MYC and MCL1. Lee et al. showed that MYC and MCL1 cooperate to promote resistance to chemotherapy in breast cancer stem cells by increasing OXPHOS (K. M. Lee et al., 2017). Likewise, it appeared that the levels of MYC and MCL1 were much higher in NSCLC tumor cells relative to adjacent normal lung cells, and CycT strongly decreased their levels in tumor cells (see Fig. 2.10A & 2.10B). These results coincide with the effects of CycT on OXPHOS and enzyme subunits and suggest that one mode by which CycT inhibits OXPHOS is by acting on MYC and MCL1.

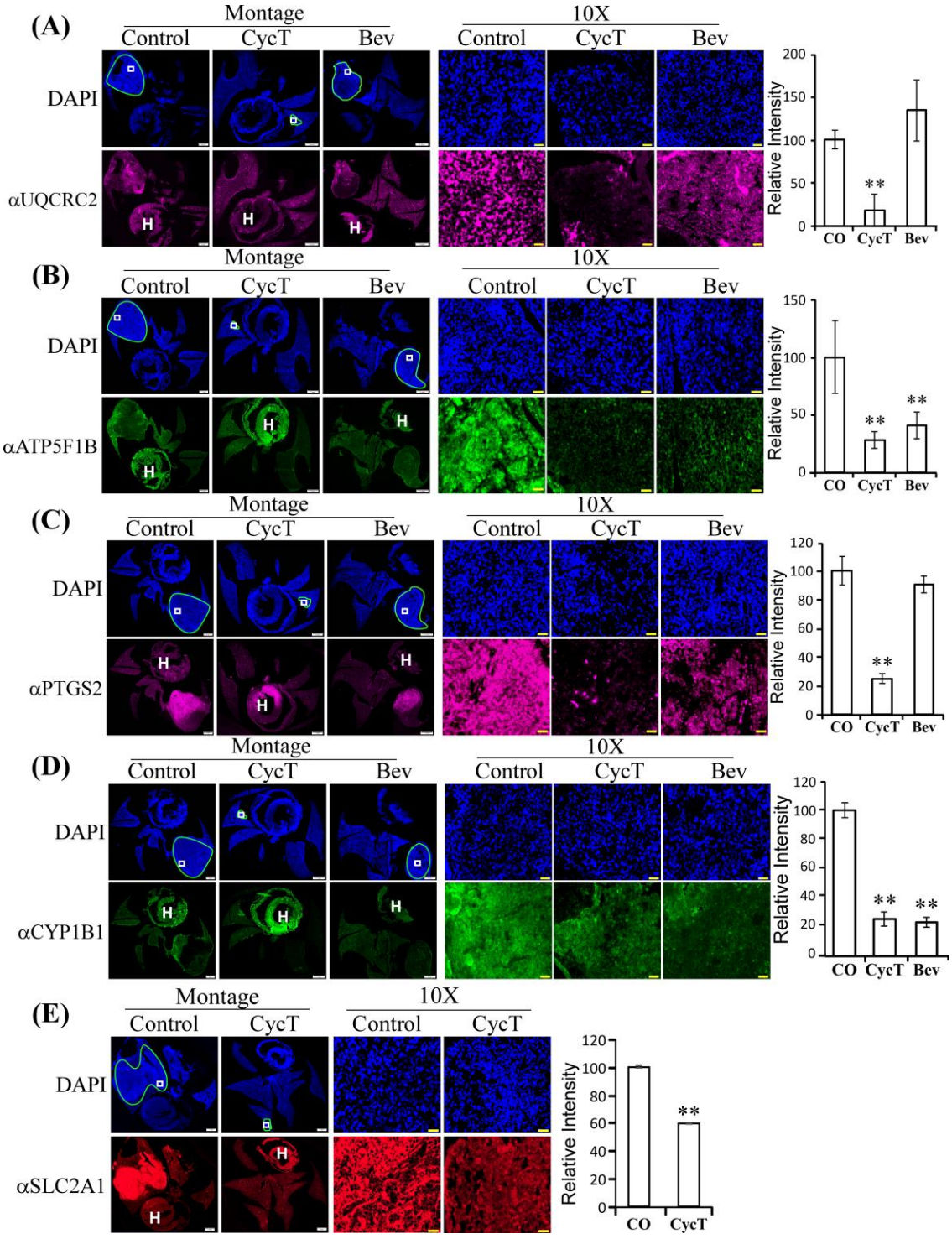

Figure 2.9. CycT diminishes mitochondrial complex proteins and hemoproteins. The effects of CycT and Bev on the levels of UQCRC2 (A), ATP5F1B (B), PTGS2 (C), and CYP1B1 (D) in orthotopic tumor xenografts. (E) The effects of CycT on the levels of SLC2A1 in orthotopic tumor

xenografts. Scale bar: Montage, 1 mm; 10X, 20 μm. Data are plotted as mean ± SEM. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. IHC images are representative of 3 independent experiments.

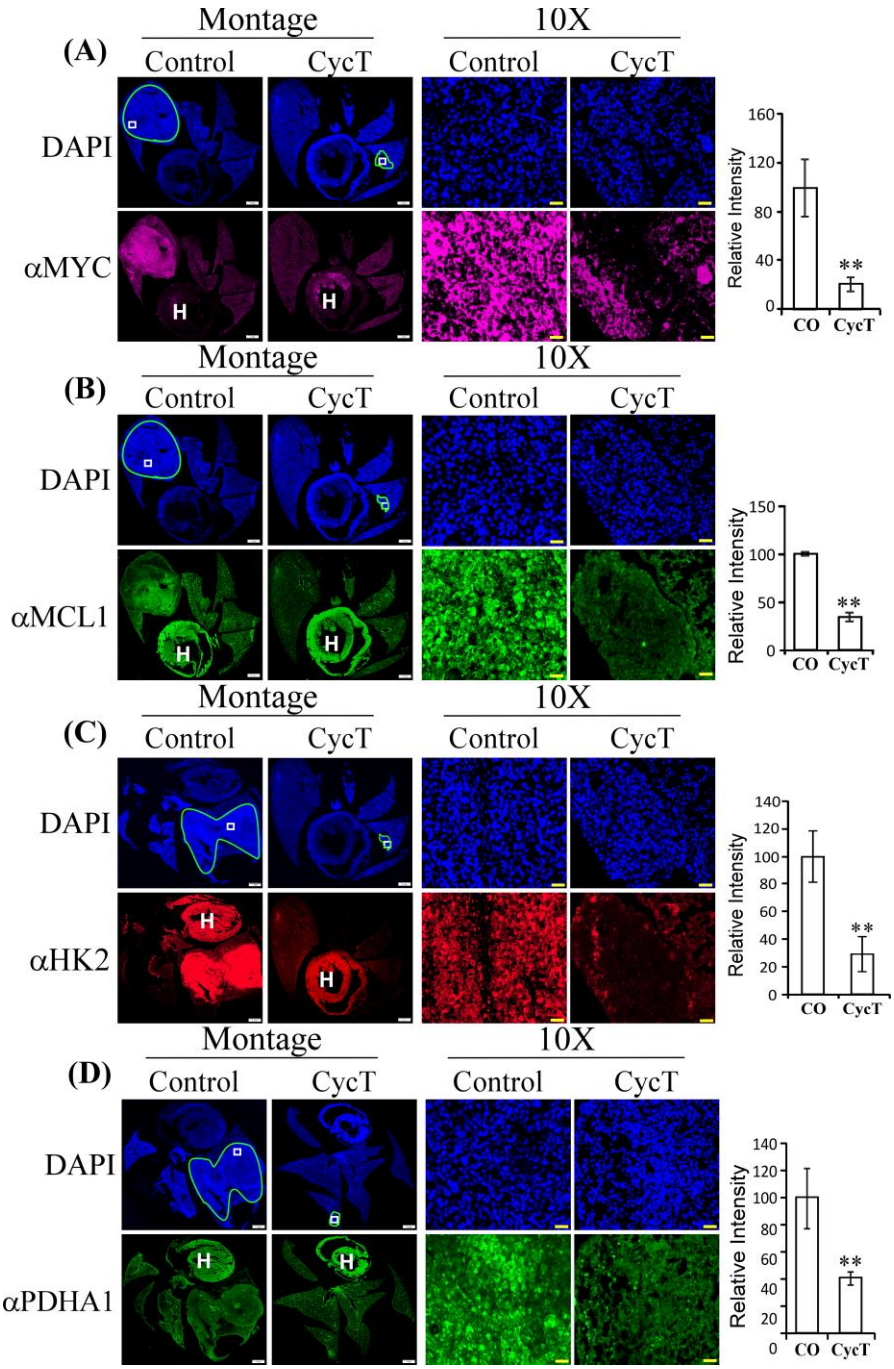

Figure 2.10. CycT diminishes levels of OXPHOS regulators and glycolytic enzymes. The effects of CycT on the levels of OXPHOS-promoting regulators MYC (A) and MCL1 (B) and on the levels of hexokinase II (C) and pyruvate dehydrogenase (D) in NSCLC tumors. Data are plotted as mean ± SEM. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. HK2: hexokinase II; PDHA1: pyruvate dehydrogenase. IHC images are representative of 3 independent experiments.

CycT strongly decreases the levels of proteins and enzymes involved in glucose consumption

While the effect of CycT on OXPHOS is dramatic and pleotropic, it is also worth noting that CycT strongly inhibited ECAR (Fig. 2.1B). To further verify this, we examined the effect of CycT on the levels of proteins and enzymes involved in glucose consumption and glycolysis. Fig. 2.10C shows that CycT strongly reduced the levels of the first enzyme in glycolysis, hexokinase II (HK2), the main hexokinase in lung cancer cells. Likewise, CycT also strongly reduced the levels of pyruvate dehydrogenase (PDHA1) (Fig. 2.10D) and glucose transporter SLC2A1 (GLUT1, Fig. 2.9E) in tumor cells. The effects of CycT on enzymes involved in glucose consumption and glycolysis are in accord with its effect on ECAR (Fig. 2.1B).

CycT alleviates hypoxia in orthotopic xenograft lung tumors

Tumor hypoxia promotes several processes critical for cancer progression, including angiogenesis, epithelial-mesenchymal transition (EMT), migration/invasion, metastasis, immune surveillance, and resistance to chemotherapy and radiotherapy (Gillies, Verduzco, & Gatenby, 2012; C. T. Lee, Boss, & Dewhirst, 2014; McDonald, Chafe, & Dedhar, 2016; Parks, Chiche, & Pouyssegur, 2013). It is an independent marker of poor prognosis in many types of human cancer (Brahimi-Horn, Chiche, & Pouyssegur, 2007; Semenza, 2014). Particularly, substantial tumor hypoxia exists in NSCLC, even in early-stage tumors (Le et al., 2006). Targeting hypoxia is crucial

for improving therapeutic outcome for NSCLC (Salem et al., 2018). Therefore, we examined hypoxia in NSCLC tumors and the effect of CycT on tumor hypoxia. To this end, we used endogenous hypoxia marker Carbonic Anhydrase 9 (CA9) and exogenous hypoxia marker pimonidazole (Rademakers et al., 2008; Sun et al., 2015). Notably, in the control lungs, both pimonidazole labeling and CA9 protein showed higher intensities in tumor regions relative to normal lung regions, indicating the existence of tumor hypoxia (see the control samples in Figs. 2.11A & 2.11B). In contrast, the levels of pimonidazole labeling and CA9 protein are both significantly reduced in CycT-treated cells. These results show that the NSCLC tumors were hypoxic and CycT reduced tumor hypoxia consistently.

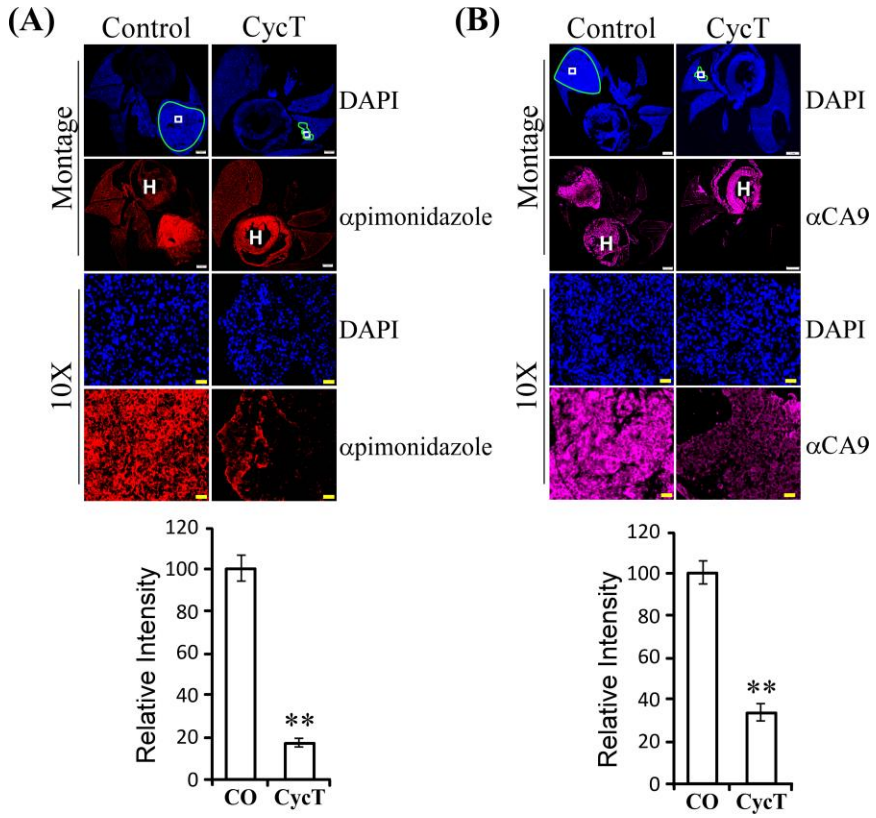

Figure 2.11. CycT alleviates hypoxia. The effects of CycT on the levels of exogenous hypoxia-marker pimonidazole labeling (A) and the levels of hypoxia-inducible CA9 enzyme (B) in orthotopic tumor xenografts. Scale bar: Montage, 1 mm; 10X, 20 μm. Data are plotted as mean ±

SEM. For statistical analysis, the levels in treated tumors were compared to the levels in control tumors with a Welch 2-sample t-test. **, p-value < 0.005. IHC images are representative of 3 independent experiments.

DISCUSSION

The latest experimental evidence from studies of human NSCLC patients has shown that NSCLC tumor cells exhibit high levels of glucose oxidation and lactate consumption (Faubert et al., 2017; Hensley et al., 2016). The evidence supports the approach of targeting OXPHOS for effective treatment of lung cancer. Evidently, elevated glucose consumption and glycolysis in tumor cells do not necessarily lead to diminished oxidative metabolism and OXPHOS. In fact, elevated glucose consumption in human NSCLC tumors are coupled to intensified glucose oxidation, TCA cycle, and lactate utilization (Faubert et al., 2017; Hensley et al., 2016). Numerous previous studies have shown that high glycolytic rates occur concomitantly with high OXPHOS rates in cells of most tumors and that function of mitochondrial OXPHOS is intact in most tumors (for a review, see (Alam, Lal, FitzGerald, & Zhang, 2016)). Several studies have unequivocally demonstrated the importance of mitochondrial OXPHOS in many tumors. Viale et al. showed that a sub-population of dormant tumor cells surviving oncogene ablation, which are responsible for tumor relapse, rely on OXPHOS for survival (Viale et al., 2014). LeBleu et al. showed that migratory and invasive cancer cells favor mitochondrial respiration and increased ATP production (LeBleu et al., 2014). Tan et al. showed that tumor cells without mitochondrial DNA (mtDNA) exhibit delayed tumor growth and that tumor formation is associated with the acquisition of mtDNA from host cells (Tan et al., 2015). Importantly, several studies demonstrated that oxidative metabolism and OXPHOS are crucial for conferring drug resistance in cancer cells and cancer

stem cells. Farge et al. showed that OXPHOS contributes to acute myeloid leukemia resistance to cytarabine and that targeting mitochondrial metabolism induces an energetic shift toward low OXPHOS and strongly enhanced anti-leukemic effects of cytarabine (Farge et al., 2017). Kuntz et al. showed that targeting mitochondrial OXPHOS eradicates drug-resistant chronic myeloid leukemia stem cells (Kuntz et al., 2017). Lee et al. showed that MYC and MCL1 confer chemotherapy resistance by increasing mitochondrial OXPHOS in triple negative breast cancer stem cells (K. M. Lee et al., 2017). Interestingly, we have shown that viable NSCLC tumor cells resistant to the vascular disrupting agent combretastatin A-4 phosphate exhibit further elevated levels of proteins/enzymes relating to heme metabolism and function (Dey et al., 2018). Clearly, NSCLC cells and drug-resistant cells or stem cells of many cancers require mitochondrial OXPHOS.

Heme is a central factor in mitochondrial respiration and oxygen metabolism (Padmanaban, Venkateswar, & Rangarajan, 1989). It is critical for the biogenesis of OXPHOS complexes II-IV (Kim, Khalimonchuk, Smith, & Winge, 2012). Furthermore, heme serves as a signaling molecule that directly regulates diverse processes ranging from gene transcription to potassium channel activation (Mense & Zhang, 2006; Yao, Balamurugan, Arvey, Leslie, & Zhang, 2010). Recent experimental data from other studies also strongly supported the idea that mitochondrial respiration and heme function are crucial for lung tumorigenicity. For example, Sotgia and Lisanti identified >180 mitochondrial gene probes, including components of the OXPHOS complexes, that effectively predicted significantly reduced overall survival in NSCLC patients (Sotgia & Lisanti, 2017). Another genome-wide expression study in 49 tumors and 42 non-involved fresh-

frozen lung tissues of 64 adenocarcinoma patients identified 232 annotated, differentially-expressed genes, 63 of which (p value <0.001) are involved in heme binding, absorption, transport, and Wnt signaling (Lam et al., 2014). Additionally, epidemiological studies indicated a positive association between intake of heme from meat and lung cancer (Tasevska et al., 2009). Clearly, heme is a unique pro-tumorigenic molecule with both metabolic and signaling functions. Likewise, oxygen-utilizing hemoproteins, such as OXPHOS complexes, are also pro-tumorigenic. Thus, shutting down heme synthesis, heme uptake, and the expression of hemoproteins is a viable strategy for effective suppression of lung tumorigenesis and for overcoming drug resistance.

CycT is a more potent, water-soluble form of cyclopamine (Fan et al., 2011). The toxicological profile of CycT has been previously characterized, and it is well tolerated by humans and mice (Fan et al., 2011). Cyclopamine was initially identified as an inhibitor of smoothened (SMO), a G protein-coupled receptor that positively regulates hedgehog (Hh) signaling (J. K. Chen, Taipale, Cooper, & Beachy, 2002). Since then, an array of SMO antagonists have been developed and tested for cancer treatment (Ruat, Hoch, Faure, & Rognan, 2014; Tremblay et al., 2008). Vismodegib (GDC0449, Curis/Roche) was approved in 2012 by the US FDA for treating locally advanced and metastatic basal cell carcinoma (Rudin et al., 2009). However, it is not effective against other cancers (Berlin et al., 2013; Kaye et al., 2012). Importantly, cocrystal structures of SMO with several small molecules showed that the binding site for cyclopamine is distinct from the sites for other antagonists (Sharpe, Wang, Hannoush, & de Sauvage, 2015). Evidently, the mode by which cyclopamine binds to SMO is more similar to that of SMO agonist SAG1.5 than other antagonists. Indeed, cyclopamine is a partial agonist capable of concomitant inhibition of

canonical and activation of non-canonical hedgehog signaling (Teperino et al., 2012). Therefore, it is very likely that CycT has distinct anti-cancer activities different from other SMO antagonists. Here, we show that CycT effectively diminishes the levels of proteins involved in heme biosynthesis, uptake, and transport (Figs. 2.6 and 2.7). CycT also diminished levels of subunits of OXPHOS complexes and other hemoproteins with pro-tumorigenic functions, including PTGS2, CYP1B1, and NOX4 (Fig. 2.9C, 2.9D, and 2.7C). The observed molecular effects of CycT likely underpin the efficacy of CycT at inhibiting NSCLC cell tumorigenic functions (Figs. 2.1 & 2.2) and suppressing the growth and progression of subcutaneous (Fig. 2.3) and orthotopic tumors (Fig. 2.4). Evidently, the capability of CycT to reduce the levels of proteins relating to heme metabolism and function and OXPHOS are not attributable to its function as a SMO antagonist (Fig. 2.8). This is consistent with previous studies showing that the mode of cyclopamine binding to SMO is closer to that of SMO agonist SAG1.5 than other antagonists (Sharpe, Wang, Hannoush, & de Sauvage, 2015) and that it is a partial agonist capable of concomitant inhibition of canonical and activation of non-canonical hedgehog signaling (Teperino et al., 2012). Therefore, other SMO antagonists or inhibitors of Hh signaling are unlikely to be as effective as CycT at limiting the expression of proteins relating to heme metabolism and OXPHOS in lung tumors.

CycT also strongly reduces the levels of MYC and MCL1 in NSCLC tumors. MYC and MCL1 promote OXPHOS in breast cancer stem cells (K. M. Lee et al., 2017). MYC increases the expression of OXPHOS complexes. Perhaps increased MYC levels in NSCLC tumors contribute to increased levels of heme-related proteins and enzymes and OXPHOS subunits in NSCLC tumors. The decreased levels of MYC in CycT-treated tumors also likely result in decreased levels

of heme-related proteins and enzymes and OXPHOS subunits (Figs. 2.6, 2.7, 2.9, and 2.10). In summary, CycT exerts multiple effects on the pathways of ATP generation in NSCLC tumors. Firstly, CycT acts quickly to inhibit OCR in NSCLC cells. Secondly, CycT strongly reduces the levels of OXPHOS-promoting regulators MYC and MCL1. Thirdly, CycT strongly decreases the levels of heme-related proteins/enzymes and OXPHOS complex subunits in NSCLC tumors. This effect of CycT may be mediated by its effect on MYC. Fourthly, CycT strongly reduces the levels of glycolytic enzymes and the major glucose transporter SLC2A1 (GLUT1). These pleiotropic effects of CycT on glycolysis and OXPHOS make it a uniquely effective agent to suppress ATP generation in NSCLC tumors.

Interestingly, bevacizumab is not an inhibitor of Hh signaling; it does not affect Gli1 levels, as expected (Fig. 2.5B). However, it reduces the levels of certain proteins involved in heme metabolism and OXPHOS, such as HRG1, HCP1, PGRMC1, Cytochrome c, UQCRC2, ATP5F1B, CYP1B1, and NOX4, albeit to lesser extents than CycT. These results are consistent with the idea that the efficacy of CycT at suppressing mitochondrial and heme functions are not attributable to its function as a SMO antagonist. The lesser molecular effects of bevacizumab on diminishing the levels of proteins involved in mitochondrial and heme functions correlate with its lower efficacy relative to CycT at suppressing lung tumor growth and progression (Fig. 2.4). The overlapping effects of CycT and bevacizumab on these proteins relating to OXPHOS are consistent with their roles on reducing ATP generation via OXPHOS in tumor cells. The effects of CycT and bevacizumab likely involve the complex interactions among tumor cells and stromal cells. Thus,

such effects are less prominent in cell lines or in subcutaneous tumor xenografts (Fig. 2.3 and data not shown).

CONCLUSIONS

In summary, we show here for the first time that targeting heme function and mitochondrial respiration with CycT can eradicate NSCLC tumors *in vivo.* CycT acts via multiple molecular mechanisms to disrupt heme function and OXPHOS. Targeting OXPHOS is an effective strategy to overcome drug resistance of other cancers, such as leukemia and triple-negative breast cancer (Farge et al., 2017; Kuntz et al., 2017; K. M. Lee et al., 2017). Our data showing the effects of CycT on lung tumors demonstrate a viable therapeutic strategy to combat lung cancer and other drug-resistant cancers.

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CHAPTER 3

DISCUSSION AND CONCLUSIONS

DISCUSSION

CycT exhibited anti-proliferative and anti-invasive properties in NSCLC cell lines. CycT reduced oxygen consumption rate and extracellular acidification rate of NSCLC cell lines. This suggests that cells enter a metabolically inactive state in response to CycT treatment. However, *in vitro* experiments seldom provide insights into drug efficacy or mechanism of action that could be extrapolated to a more realistic *in vivo* setting (Graves et al., 2010). *In vitro* experiments do not factor in vasculature, bioavailability or bioactivity of the drug. Therefore, it is necessary to utilize *in vivo* models to assess the feasibility of a therapeutic strategy or therapeutic agent of interest. Subcutaneous xenograft models are the simplest *in vivo* models to assess the anti-proliferative action of a drug (Graves et al., 2010). This is because introducing cancer cells right under the surface of the skin provides a visual assessment of drug efficacy (Graves et al., 2010). CycT significantly decelerated the growth of subcutaneously implanted xenografts of NSCLC cell lines. Subcutaneous xenograft models facilitate the growth of tumors that are genetically identical to the human lung tumors. However, the subcutaneous space does not offer the tumor microenvironment of the lung in terms of vasculature and oxygen perfusion (Graves et al., 2010). Further, subcutaneous tumors are shown to be more hypoxic than lung orthotopic tumors (Graves et al., 2010). Hypoxic environment facilitates the initiation of several pathways associated with survival and apoptosis thereby confounding the effects of the drug.

Orthotopic tumor models exhibit metastasis and closely mimic therapeutic responses of human lung cancer in the clinic (Bibby, 2004; Hoffman, 1999; Killion, Radinsky, & Fidler, 1998; Kuo et al., 1993). CycT significantly decreased the tumor growth in lung orthotopic xenografts. This suggests that CycT is an effective drug in targeting the growth of NSCLC. Further, there were no visible effects of toxicity in the CycT-treated mice as shown by previous studies (Fan et al., 2011). Although lung orthotopic xenografts mimic the microenvironment of the lung in terms of oxygen perfusion, these models do not account for the immune cells of the lungs as the mice are immunocompromised (Kim, 2018). CycT treatment reduced expression of proteins involved in mitochondrial complexes that carry out OXPHOS. These results concur with results of oxygen consumption rate. Some proteins involved in mitochondrial OXPHOS require heme as prosthetic group. Therefore, we probed for proteins involved in heme metabolism. CycT reduces proteins involved in synthesis, uptake, and degradation of heme along with other pro-oncogenic hemoproteins. CycT also reduced the heme synthesis and degradation *in vitro*. Taken together, these results suggest that lower expression of hemoproteins in response to CycT might be due to lower intracellular heme levels. As hemoproteins require heme as prosthetic group, it is possible that lower intracellular heme might affect expression of hemoproteins. Further studies are required to clarify whether lower heme levels cause lower OXPHOS or vice versa in response to CycT. Additionally, mice bearing orthotopic xenografts were also treated with succinyl acetone, an inhibitor of heme synthesis. Data from bioluminescence imaging show that CycT is significantly more effective than SA in curtailing the growth of the lung orthotopic tumors. Although SA affects heme synthesis, more heme could be generated intracellularly via heme uptake and heme degradation, which might ultimately fuel OXPHOS. Since CycT diminishes both proteins involved

in heme synthesis and uptake, it is possible that the overall diminishing effect on heme renders CycT more efficient than SA. CycT also alleviates hypoxia as indicated by lower expression of the hypoxia markers CA9 and pimonidazole. The hypoxia alleviation could be a result of inhibition of OXPHOS (Ashton, McKenna, Kunz-Schughart, & Higgins, 2018). OXPHOS is shown to occur even in environments with low oxygen tension leaving the surrounding tissues with inadequate oxygen, thereby, resulting in hypoxia (Larman et al., 2012; Weinberg et al., 2010). Inhibition of OXPHOS makes the oxygen utilized in OXPHOS available to the surrounding areas of the tumor thereby alleviating hypoxia in those areas(Ashton et al., 2018).

Although CycT is a known hedgehog antagonist, it has been shown to exhibit some hedgehog pathway agonism (Sharpe, Wang, Hannoush, & de Sauvage, 2015; Teperino et al., 2012). SANT-1, a hedgehog inhibitor, did not affect heme synthesis or degradation in NSCLC cell lines. This suggests that the effect of CycT on heme metabolism is independent of its antagonist effect on hedgehog pathway. Further, the proteins downregulated by CycT are not known targets of hedgehog pathway. Further studies are required to investigate if this effect can be attributed to partial agonist property of CycT.

Many recalcitrant and drug-resistant cancers have been shown to rely on OXPHOS for survival (Bosc, Selak, & Sarry, 2017; Datta et al., 2017; Davidson et al., 2016; Lee et al., 2017; Zhang et al., 2016)}. Additionally, molecular targeted therapies can cause cancer cells to rely on OXPHOS (Ashton et al., 2018). Therefore, targeting OXPHOS is a viable strategy for intervention in NSCLC. CycT lowers OXPHOS regulators: MYC and MCL1. There are several drugs that target

particular complexes of mitochondrial respiration chain (Ashton et al., 2018). For instance, metformin, an inhibitor of mitochondrial respiratory complex I, has been shown to effective against cancer progression initially until the tumors gain resistance (Griss et al., 2015). Since CycT targets OXPHOS regulators and has an overall reducing effect on expression of mitochondrial complexes that carry out OXPHOS, CycT might make a better drug candidate than drugs that target individual complexes. For example, NSCLC cells with mutations in LKB-1 tumor suppressor, cannot rely on glycolysis for loss of ATP production mediated by inhibition of OXPHOS (Ashton et al., 2018). CycT could be used to target NSCLC cells with mutated LKB-1.

CONCLUSIONS

There were no observed effects of toxicity in response to CycT. However, more studies are required to assess the possibility of off-target effects. Results from this study suggest that CycT has great potential to be used as a therapeutic agent. Further studies are required to assess efficacy of CycT as a stand-alone therapy or in combination with other drugs.

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BIOGRAPHICAL SKETCH

Sarada Preeta Kalainayakan was born in Chennai, India. She received her Bachelor in Technology (B.Tech) degree in Biotechnology (First class) from Anna University, Chennai in 2008. She went on to pursue a Master in Technology (M.Tech) in Biopharmaceutical Technology in the same university. She graduated with first class and second rank in 2010. In August 2010, she joined the Molecular and Cell Biology program in the Department of Biological Sciences at The University of Texas at Dallas to conduct her doctoral research. She earned her Master of Science (MS) degree in Molecular and Cell Biology from The University of Texas at Dallas in 2013.

CURRICULUM VITAE

SARADA PREETA KALAINAYAKAN

EDUCATION

PhD	University of Texas at Dallas Richardson, TX	Fall 2010-2018
MS, Molecular and Cell Biology	University of Texas at Dallas Richardson, TX	2013
M.Tech, Biopharmaceutical Technology	Anna University, Chennai, India	2010
B.Tech, Biotechnology	Anna University, Chennai, India	2008

RESEARCH EXPERIENCE

P.I. Dr. Li Zhang University of Texas at Dallas	Graduate Student	2014 – Present
P.I. Dr. Deborah Clegg University of Texas Southwestern Medical Center Adjunct faculty, University of Texas at Dallas	Graduate Student	2011-2014
P.I. Dr. Lawrence Reitzer University of Texas at Dallas	Laboratory Rotation	Spring 2011
P.I. Dr. Zhenyu Xuan University of Texas at Dallas	Laboratory Rotation	Fall 2010
P.I. Dr. Sharmila Anishetty Anna University Chennai-600025, India	Graduate Research Assistant (Masters)	2008-2010
P.I. Dr. Eevera Tamilmani The Periyar Technology Business Incubator Thanjavur- 613403, India	Undergraduate Research Assistant	2007-2008

RESEARCH PROJECTS

Zhang Lab Projects

Assessing drug efficacy in murine models using In-vivo Bioluminescence Imaging

Goal: To assess anti-tumorigenic properties, drug efficiency, and mechanisms of action of mitochondrial targets and heme sequestrants in subcutaneous and lung orthotopically implanted xenografts of various luciferase expressing NSCLC cell lines in NOD/SCID mice.

Assessing efficacy of mitochondrial targeting drugs in Genetically Engineered Mouse Model (GEMM) using In-vivo Bioluminescence Imaging

Goal: To assess anti-tumorigenic properties, and drug efficiency of mitochondrial targeting agents in firefly luciferase expressing KRAS(G12D) mice, that are intra-tracheally infected with replication-resistant adenoviruses expressing Cre recombinase.

Investigating the effect of cyclopamine tartrate on hemoproteins in NSCLC cell lines

Goal: To study the effect of cyclopamine tartrate on heme synthesis, degradation and hemoproteins.

Investigating the role of heme related enzymes in tumorigenicity of NSCLC cell lines

Goal: To generate knockouts of heme synthesis and degradation proteins by CRSIPR/Cas9 system to dissect their role in NSCLC tumorigenesis and progression.

Clegg Lab Projects

Investigating role of High Fat Diet (HFD) on Prostate Cancer

Goal: To study the inflammatory effect of palmitic acid, the most common saturated fatty acid, on prostate cancer cell lines *in vitro*. To study the hypothesis that HFD induced inflammation fosters prostate cancer initiation and progression by placing C57BL6 male mice on 42% HFD for a period of 4weeks, 16 weeks, and 8 months.

Investigating mechanism of action of estrogen via estrogen receptor alpha in prostate cancer

Goals:
To investigate the action of 17β-estradiol on proliferation and inflammation *in vitro* and *in vivo* by utilizing whole body Estrogen receptor alpha (ERα) knockout mice, and whole body GPER30 knockout mice.
To generate prostate specific ERα knockout mice, and prostate specific ERα and PTEN dual knockout mice for the same.

Regulation of miR21 by estrogen and High fat diet on prostate cancer

Goal: To study the regulation of the onco-miRNA, miR21 by 17β-estradiol via ERα in whole body Estrogen receptor alpha (ERα) knockout mice, whole body GPER30 knockout mice and prostate specific ERα knockout mice.

To Study the effect of phyto-estrogens: Enterolactone and Enterodione, on prostate cancer cell lines

Goal: To investigate whether phytoestrogens attenuate expression of pro-inflammatory cytokines in prostate cancer cell lines.

Reitzer Lab Project

Studying the biochemical pathways of the pathogen Moraxella catarrhalis

Goal: To assess the role of various amino acids on multiplication of *Moraxella catarrhalis*.

Anishetty Lab Project

Developing parameters for in-silico design of ligands for α/β-hydrolases

Goal: To study the structural and functional diversity of the α/β-hydrolases. To determine the part of the sequence necessary to maintain the α/β-hydrolase fold and enzymatic activity using computational methods. To utilize the sequence-structure-function data for limiting the pool of possible drug inhibitors for further *in-vitro* and *in-vivo* testing.

Eevera Lab Project

To confer abiotic-stress tolerance in crops using bacteria that synthesize 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Deaminase

Goal: To implement a cost-effective and farmer friendly method to grow crops in areas (especially Thanjavur district) rendered un-arable by tsunami that hit eastern coast of Tamil Nadu, India in 2004. To study the ability of ACC Deaminase synthesizing soil bacteria to confer salinity resistance in sprouting and growth of *Vigna mungo* (green gram) and *Abelmoschus esculentus* (okra) seeds.

TEACHING EXPERIENCE

Served as graduate teaching assistant at University of Texas at Dallas for the following courses:
Taught workshops, graded and supervised exams, and conducted labs as part of TA duties.

Biol 3456 (4cr): Human Anatomy and Physiology with Lab II	Fall 2017
Biol 6345 (3cr): Molecular Basis of HIV/AIDS	Summer 2017
Biol 3361 (3cr): Biochemistry I	Spring 2017
Biol 3456 (4cr): Human Anatomy and Physiology with Lab II	Summer 2014-2015

Biol 3362 (3cr): Biochemistry II	Spring 2014
Biol 3456 (4cr): Human Anatomy and Physiology with Lab II	Summer 2012-2013
Biol 3301 (3cr): Classical and Molecular Genetics	Fall 2011
Biol 3380 (3cr): Biochemistry Lab	Summer 2011
Biol 3380 (3cr): Biochemistry Lab	Spring 2011
Biol 2311 (3cr): Introduction to Modern Biology I	Fall 2010

PEER-REVIEWED PUBLICATIONS

Dey, S., Kumari, S., **Kalainayakan, S.P.,** Campbell, J., Ghosh, P., Zhou, H., FitzGerald, K.E., Mason, R.P., Zhang, L., and Liu, L. The vascular disrupting agent combretastatin A-4 phosphate causes prolonged elevation of proteins involved in heme flux and function. *Oncotarget,* Jan 2018, 9(3). DOI: 10.18632/oncotarget.23734

Alam, M. M., Sohoni, S., **Kalainayakan, S.P**., Garrossian, M., and Zhang, L. (2016). Cyclopamine tartrate, an inhibitor of Hedgehog signaling, strongly interferes with mitochondrial function and suppresses aerobic respiration in lung cancer cells. *BMC Cancer*, Feb 2016, *16*(1), [150]. DOI**:** 10.1186/s12885-016-2200-x

Morselli, E., Fuente-Martin, E., Finan, B., Kim, M., Frank, A., Garcia-Caceres, C., Navas, C.R., Gordillo, R., Neinast, M., **Kalainayakan, S.P.,** Li, D. L., Gao, Y., Yi, C., Hahner, L., Palmer, B. F., Tschöp, M.H., and Clegg, D. J. (2014). Hypothalamic PGC-1α Protects Against High-Fat Diet Exposure by Regulating ERα. *Cell Reports.* October 2014-Volume 9- Issue 2. DOI: 10.1016/j.celrep.2014.09.025

MANUSCRIPTS IN REVISION

Kalainayakan, S.P., Ghosh, P., Dey, S., FitzGerald, K., Konduri, P. C., Sohoni, S., Liu, L., and Zhang, L**.** Developing therapeutic strategies for lung cancer treatment by targeting heme and mitochondrial function.

Sohoni, S., Ghosh, P., Wang, T., **Kalainayakan, S.P.,** Dey, S., Vidal, C., and Zhang, L**.** Elevated intracellular heme levels underpin intensified oxidative metabolism and tumorigenic functions of non-small cell lung cancer cells.

MANUSCRIPT IN PROGRESS

Kalainayakan, S.P., Morselli, E., and Clegg, D. J. High Fat Diet induced Estrogen Receptor Alpha dysregulation promotes pro-carcinogenic environment in the prostates of mice.

BOOK CHAPTERS

FitzGerald, K. E., Lal, S., **Kalainayakan, S.P.,** and Zhang, L. (2016) Molecular Mechanisms Underlying heme action in promoting the pathogenesis of Alzheimer's disease. SM Group Publishers. (Published date: April 2nd, 2016)

ABSTRACTS

Kalainayakan, S.P., Ghosh, P., Dey, S., Liu, L., and Zhang, L**.** Targeting mitochondrial function in lung tumor growth and progression [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018;78(13 Suppl): nr 5490, DOI: 10.1158/1538-7445.AM2018-5490 (Poster)

Ghosh, P., **Kalainayakan, S.P.,** Dey, S., Sohoni, S., and Zhang, L**.** To examine the effect of limiting heme bioavailability on lung tumor growth & progression [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018;78(13 Suppl): Abstract nr 786, DOI: 10.1158/1538-7445.AM2018-786.

Dey, S., Kumari, S., **Kalainayakan, S.P.,** Ghosh, P., Zhang, L., and Liu, L. Elevated expression of heme related proteins in viable tumor cells treated with vascular disrupting agent, combretastatin A-4 phosphate in xenograft models of NSCLC [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018;78(13 Suppl): Abstract nr 3533, DOI: 10.1158/1538-7445.AM2018-3533 (Poster)

Kalainayakan, S.P., Ghosh, P., Dey, S., FitzGerald, K.E., Liu, L., and Zhang, L**.** Developing therapeutic strategies for lung cancer treatment by targeting heme and mitochondrial function. 2017 Innovations in Cancer Prevention and Research Conference (Poster)

Kalainayakan, S.P., Ghosh, P., Dey, S., and Zhang, L**.** Assessing the efficacy of targeting mitochondrial respiration in delaying lung tumor growth by using subcutaneous xenografts in mouse models, Proceedings of the American Association for Cancer Research 2017, DOI:10.1158/1538-7445.AM2017-2129 (Poster)

Dey, S., **Kalainayakan, S.P.,** Ghosh, P., and Zhang, L. Non-invasive monitoring of the effıcacy of anti-cancer therapeutic agent in lung orthotopic xenograft models of NSCLC using Bio-Luminescence Imaging (BLI) Proceedings of the American Association for Cancer Research 2017, DOI: 10.1158/1538-7445.AM2017-2871(Poster)

Ghosh, P., **Kalainayakan, S.P.,** Dey, S., and Zhang, L. Evaluating the efficacy of limiting heme availability on growth and progression of lung tumor. Proceedings of the American Association for Cancer Research 2017, DOI: 10.1158/1538-7445.AM2017-1500(Poster)

Alam, M.M., Sohoni, S., **Kalainayakan, S.P.,** and Zhang, L. Cyclopamine tartrate, an anti-cancer agent targeting Hedgehog signaling, strongly interferes with mitochondrial function and suppresses aerobic

respiration. Proceedings of the American Association for Cancer Research 2016, DOI: 10.1158/1538-7445.AM2016-3086 (Poster)

Kalainayakan, S.P., Hooda J., Brekken R., and Zhang, L. Elevated heme flux and function promote lung tumor development and progression. Keystone Symposia, Vancouver (2015)

Morselli, E., **Kalainayakan, S.P**, Hahner. L., and Clegg, D.J. Anti-inflammatory axis of Estrogen-Estrogen receptor alpha in prostate cancer. Keystone Symposia, Dublin (2013) (Poster)

ORAL PRESENTATIONS

Kalainayakan, S.P., S. Rajasree, G. Shanthi, Ge Wang, W. Cong, E. Namati, G.McLennan, E. Hoffman, K. Damodharan and D. Kumar "**Characterization of Multidrug resistant Strains by Bioluminescence Imaging**" at International conference on Nanomedicine and its Applications, 18th-19th October 2007, Sastra University, India.

HONORS/AWARDS

Betty and Gifford Johnson Scholarship for attending AACR Annual Meeting 2018.
Second Rank in Master of Technology in Biopharmaceutical Technology, degree conferred by Anna University, Chennai, India.
First class with distinction in Bachelor of Technology in Biotechnology, degree conferred by Anna University, Chennai, India.

SCHOLARSHIPS

Graduate Scholarship awarded by UT Dallas (Fall 2010- Summer 2011, Spring 2012-Present)
Merit Scholarship awarded by Neyveli Lignite Corporation (2004-2008)

MEMBERSHIPS IN PROFESSIONAL SOCIETIES

Delta Epsilon Iota Academic Honor Society	2013-Present
Associate member in American Association of Cancer Research	2015-Present
Society for Cancer Immunotherapy	2018
American Society for Cell Biology	2018