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Biomarker and Target Discovery in Cancer

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BIOMARKER AND TARGET DISCOVERY IN CANCER

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BIOMARKER AND TARGET DISCOVERY IN CANCER

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ABSTRACT

Cancer is a complex disease characterized by uncontrolled growth of transformed cells that can arise in many tissue types throughout the body (e.g., breast, lung, prostate, pancreas, lymph nodes) and is a major cause of death worldwide. Cancer progression resulting in aggressive or metastatic disease accounts for one of the leading causes of death worldwide, second only to heart disease in the US. Incidentally, cancer-related mortality has been on a steady decline, dropping 25% over the last 25 years [1]. This could be attributed to improved and earlier diagnoses and better treatment options being developed over the past few decades. Molecular profiling, transcriptome sequencing and data integration from many high-throughput platforms unraveled dysregulation in many metabolic pathways in cancer. Since cancer cells are fast-growing, their metabolic needs are enhanced, hence the requirement for *de novo* synthesis of essential metabolites. One critical requirement of fast-growing cells and a historically important pathway in cancer is the nucleotide biosynthetic pathway and its enzymes are valuable targets for small molecule inhibition. Hanahan and Weinberg have proposed other characteristics, collectively called hallmarks of cancer [2], based on extensive research. Hallmarks that we have sought to target with these studies are cell proliferation, invasion, and deregulated cellular energetics. We embarked on data analysis and identification of targets that are involved in de novo purine biosynthesis as well as those involved in cancer metastasis. The work presented in this thesis discusses research in the areas of *de novo* purine biosynthesis, describing the role of methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 1-like **(**MTHFD1L), an enzyme involved in nucleotide metabolism, overexpressed in breast cancer, as well as, describing the expression and role of a collagen modifying enzyme prolyl 4-hydroxylase subunit alpha 1 (P4HA1) in lung adenocarcinoma. We found P4HA1 to play an oncogenic role in lung adenocarcinoma. Knockdown of expression resulting in significantly reduced cell proliferation, colony formation, and invasion. Likewise, with MTHFD1L, in the context of breast cancer, we found reduced cell

proliferation and invasion upon knockdown of expression. Overall, this thesis describes two potential therapeutic targets that are amenable to small molecule inhibition.

Keywords: Purine biosynthesis; cancer; MTHFD1L; P4HA1; cellular energetics

DEDICATION

This is dedicated to my mother, who has always supported me; my family and friends, who have loved me and helped me through difficult times; and myself, who endured.

ACKNOWLEDGMENTS

This final year of my doctoral journey has been one to remember. The year twenty-thousand and twenty has swung wildly from the happiness to heartache. From the bonding of good friends in marriage, the birth of my nephew, Black Lives Matter protests, SARS-CoV2 decimating my hometown, infecting my grandmother and aunt, my grandfather's heart attack, and the list goes on. I've made it to the end of this year, despite everything, and I'm able to present this work to all of you. I would like to thank everyone who I interacted with during my doctoral journey. Each of you have touched my life in big and small ways, though significantly, nonetheless. Thank you to my committee for being patient and understanding and working with me to make this possible. Thank you to the Cancer Biology Theme and Graduate Biomedical Sciences for allowing me this opportunity. Lastly, thank you to UAB for serving as my home all these years.

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LIST OF ABBREVIATIONS

5FU	Fluorouracil
ADSL	Adenylosuccinate lyase
AGO2	Argonaute 2
AHCY	Adenosylhomocysteinase
AICARFT	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
AMP	Adenosine monophosphate
APRT	Adenine phosphoribosyltransferase
ATCase	Aspartate carbamoyl transferase
ATIC	IMP cyclohydrolase
BRAF	B-RAF proto-oncogene, serine / threonine kinase
CAD	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
COMMD4	COMM domain-containing protein 4
CPS	Carbamoyl phosphate synthetase II
DHFR	Dihydrofolate Reductase
DHODH	Dihydroorotate dehydrogenase
EGFR	Epidermal Growth Factor Receptor
GART	Phosphoribosylglycinamide formyltransferase
GMP	Guanosine monophosphate

HER2	Human epidermal growth factor receptor 2
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HYP	4-hydroxyproline
IMP	Inosine monophosphate
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAT	Methionine adenosyltransferase
MTHFD1	Methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 1
MTHFD1L	Methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 1-like
MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 2
MTHFR	Methylenetetrahydrofolate reductase
MTX	Methotrexate
MYC	MYC proto-oncogene, BHLH transcription factor
NSCLC	Non-small cell lung carcinoma
NTRK1	Neurotrophic receptor tyrosine kinase 1
ODCase	Orotidine monophosphate (OMP) decarboxylase
OMP	Orotidine monophosphate
OPRTase	Orotate phosphoribosyltransferase
P4HA1	Prolyl-4-hydroxylase, alpha subunit 1
P4HA2	Prolyl-4-hydroxylase, alpha subunit 2
P4HA3	Prolyl-4-hydroxylase, alpha subunit 3
P4HB	Prolyl-4-hydroxylase, beta subunit
PAICS	Phosphoribosylaminoimidazole succinocarboxamide synthase
PD-L1	Programmed death-ligand 1
PFAS	Phosphoribosylformylglycinamidine synthase

PFS	Progression-free survival
PPAT	PRPP amidotransferase
PRA	5-phosphoribosyl-1-amine
PRPP	5-phosphoribosyl-1-pyrophosphate
ROS1	ROS proto-oncogene 1, tyrosine kinase receptor
SAM	S-adenosylmethionine
SCLC	Small cell lung carcinoma
SHMT1	Serine hydroxymethyltransferase-1
SHMT2	Serine hydroxymethyltransferase-2
THF	Tetrahydrofolate
TNBC	Triple negative breast cancer
UMP	Uridine monophosphate
UMPS	Uridine monophosphate synthetase

GENERAL INTRODUCTION

Cancer is a disease initiated in and affecting various organ systems driven by an amalgam of different genetic drivers. For many decades, research has been geared towards strengthening our understanding of the mechanisms for initiation, earlier detection, and more effective treatments with less detrimental side effects. For 2020, there are a projected 1.8 million new cases and 600,000 cancer related deaths for the United States [1]. Recent advances, including high-throughput technology-based analysis, suggest that diverse genetic, epigenetic, and environmental factors influence this initiation and uncontrolled growth, which can lead to incurable metastatic disease. Many avenues of research have focused on efforts to characterize the expression and regulation of potential oncogenes in aggressive cancers. Lung cancer, breast cancer, and prostate cancer are the three leading malignancies in new occurrences and mortality in the United States. Patients often respond initially to treatment; however, the heterogeneity of the tumor and the multitude of genetic mutations contribute to resistance and tumor recurrence.

Lung Adenocarcinoma

Lung cancer is the leading cause of cancer-related deaths globally [3] with non-small cell lung cancer (NSCLC) accounting for 80% of all lung cancers [4]. In the United States, the overall five-year survival rate of patients with lung cancer has increased compared to 2011, when it ranged from 13% to 15% [5]. The five-year survival rate of

patients diagnosed with non-small cell lung cancer is 24%, and with small cell lung cancer is 6%. This estimate was calculated based on the number of patients diagnosed with lung cancer between 2009 and 2015 [1]. A decisive factor in the high mortality of lung cancer is the late diagnosis of the disease. More than 50% of the cases are diagnosed at advanced stages when the disease is incurable. In order to reduce the high mortality rate, early diagnosis of the disease is imperative.

In each individual, the occurrence and development of cancer is influenced by a unique combination of endogenous and exogenous factors. High throughput characterizations have led to the identification of driver mutations including epidermal growth factor receptor (*EGFR*), observed in 10–30% of patients, Kirsten rat sarcoma viral oncogene homolog (*KRAS*), observed in 15–30% of patients, and fibroblast growth factor receptor 1 (*FGFR1*), observed in 20% of patients, among others [3, 6]. Epidermal growth factor (EGFR) receptor mutations in adenocarcinoma have been well studied. Studies have shown that in patients with lung adenocarcinoma, the probability of an EGFR mutation increases with age, from 3.7% in people aged 18 to 30, to 18.5% in people between 81 and 100 years of age. Research has also shown that women are more likely to have EGFR mutations if they smoke compared to men, but also that men which are not smokers are more likely to have EGFR mutations than men that smoke (Dogan et al., 2012). EGFR tyrosine kinase inhibitors, erlotinib and gefitinib are used to treat lung cancer with EGFR activating mutations [3, 7-10]. Similarly, lung cancer patients harboring *ALK* (anaplastic lymphoma kinase) gene fusions are treated with crizotinib [4, 11-14]. Immunotherapy targeting PD1 and PD-L1 is showing varied response in lung adenocarcinoma [6, 15]. For accurate diagnosis, treatment, and drug development,

biomarkers specific to all histological types of lung cancer are important [16, 17]. Technological advances in the field along with bioinformatics is making personalized medicine a reality in the treatment of lung cancer. However, in order to treat advanced lung cancers, there is a need to develop additional biomarkers as well as therapeutic targets that are amenable to small molecule inhibition.

Breast Cancer

Breast cancer is a leading cause of cancer-related death in women, accounting for around 40,000 deaths per year in the United States [1]. Despite advances in the early detection and treatment of breast cancer, the mortality rate is still high. Currently, the most important prognosticators for patients with breast cancer in the clinical setting are components of the staging system, such as primary tumor size and the presence of lymph node metastases [18-20]. However, there is a need for novel molecular predictors of tumor behavior, as well as, therapeutic targets that can be used in treatment. There are few biomarkers that can be used in breast cancer diagnosis and prognosis. Estrogen receptor (ER) and progesterone receptor (PR) are commonly used and highly predictive of breast cancer patients that will benefit from endocrine therapy [18, 21-23]. ERBB2 amplification is used in prognostication of breast cancer. Triple negative breast cancer (TNBC) is an aggressive form of breast cancer and, while well-studied, there are no approved therapeutic targets [21, 23-25]. Thus, there is a critical need for new prognostic factors and therapeutic targets that are more efficacious.

Cancer Diagnostics and Treatment

Lung cancer is typically only detected in the late stages of disease when symptoms such as cough, chest pain, shortness of breath, coughing up blood appear.

Mostly, the early stages of lung cancer are discovered by chance. Computed tomography and chest radiography are commonly used to diagnose lung cancer. But these methods can only identify visible changes in the lungs that are mostly irreversible; therefore, there is a great need to develop additional methods for early diagnosis of lung cancer. In order to overcome the problem of late diagnosis, it is necessary to discover new and highly sensitive, specific biomarkers [16]. The diagnostic significance of protein biomarkers is defined by specificity and sensitivity. The specificity of biomarkers was determined by the percentage of instinctively negative test results in the group of patients with benign diseases and in the group of healthy people, while the specificity of biomarkers was determined by the percentage of true positive test results in the group of cancer patients. However, 100% sensitive specific and sensitive biomarkers were not found [26].

Certain biomarkers have also been found in healthy individuals in plasma. Exhausted condensate, blood, tumor tissue, urine, and sputum are mainly used for noninvasive biomarker detection. Exhaled breath condensate consists of DNA, cytokines, and proteins [26]. The best source for detecting biomarkers is blood. This is because the cellular remains from the tumor penetrate into the blood. Therefore, blood can be used as a minimally invasive liquid biopsy. Blood is a complex matrix consisting of RNAs, DNAs, endothelial cells, immune cells, cancer cells, stromal cells, miRNAs, tumor-associated circulating lipids, and proteins [27]. Biomarkers can serve as indicators of pathogenic processes or pharmacodynamic or pharmacological response to treatment [28]. Different oncogenic markers can be used to distinguish between normal and pathogenic processes [29].

Cancer therapies vary between patients and can take the form of surgery, radiation, and chemotherapeutics. Surgery is the standard of care for most solid cancers and proves more effective in those patients with early stage disease. However, there are some drawbacks to surgery. Aside from the risk of undergoing surgery itself, research shows a possibility for the generation of metastases [30-32]. There is also a lack of efficacy for surgery in late stage tumors which have more chances to spread into surrounding tissues and metastasize. Adjuvant therapies have been paired with surgical resection to address these and other concerns.

Typically, the adjuvant therapies of choice, which are given after surgery, are radiotherapy, which can be used locally, and chemotherapy, a systemic treatment. These methods have their own positive and detrimental effects. Chemotherapeutics, for example, target rapidly dividing cells, which includes many of normal cell types. Thus, it is imperative to understand any molecular events and signaling pathways that contribute to cancer growth, metastasis, and therapy resistance in order to develop effective therapeutic options.

Therapeutic Targets in Lung Cancer

EGFR belongs to the ErbB family of receptor tyrosine kinases (RTKs). EGFR peptides are generally overexpressed in human cancers, and research has shown that they are able to induce cellular transformation [33]. Receptor heterodimerization or homodimerization is generated by an extracellular ligand when bound to EGFR. This further leads to phosphorylation of sites in the cytoplasmic tyrosine kinase and activation of various intracellular pathways, including phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and RAS/RAF/mitogen-activated

protein kinase pathway. And activation of this pathway leads to cell proliferation, metastasis, and prevention of apoptosis [34]. In 62% of NSCLC cases, EGFR is overexpressed, and its severity is associated with poor prognosis. Lung tumors associated with EGFR mutations are present in approximately 30% of patients with lung adenocarcinoma in East Asia, and about 10% in the United States [35]. These mutations occur in exons 18-21 which encode a part of EGFR kinase [34].

Missense mutations in exon 21 and in-frame deletions in exon 19 make up about 90% of EGFR mutations [36]. Hyperactivation of downstream antiapoptotic signaling pathways occurs when a mutation in the kinase domain of EGFR is activated that triggers ligand-independent activation of tyrosine kinase. EGFR mutations are mainly found in women who do not smoke with adenocarcinoma with lepidic characteristics [34]. Treatment with tyrosine kinase inhibitors (TKIs) has high treatment rates, ranging from 55% to 78%. Such high rates are for TKIs such as afatinib, erlotinib, and gefitinib in patients with mutant tumors. These are becoming standard treatments for EGFR TKI because survival in these patients is significantly higher and without disease progression [37, 38]. However, most patients develop resistance and relapse in a very short time, because a new mutation (T790M) occurs in exon 20 of EGFR, a PI3KCA mutation, or an amplification of the MET oncogene [36].

In addition to EGFR, there is rearrangement of a member of the tyrosine kinase receptor in the insulin receptor family is ALK [39]. In aplastic lymphoma, the rearrangement of the ALK gene was first found [40]. Subsequently, rearrangement of the ALK gene was found in a subset of NSCLC tumors containing fusion of genes similar to protein 4 (EML4) associated with echinoderm and ALK [36]. The chimeric protein encodes this rearrangement. This protein has constitutive kinase activity and promotes

proliferation and malignant growth. EML4-ALK fusion was detected in 3.7% to 7% NSCLC. It is mainly detected in adenocarcinomas with cribriform histology features and sealed ring cells and is more common in young patients who have never smoked [40]. There are several variants of ALK fusion with other rarer partners (TRK-fused gene (TFG), huntingtin-interacting protein 1 (HIP1) genes, kinesin light chain 1 (KLC1), and kinesin family member 5B (KIF5B)) and several variants of EML4 rearrangement -ALK. ALK molecular alterations, KRAS, and EGFR have been described with the same molecular changes in 2.7% of lung adenocarcinoma cases, although they have been shown to be mutually exclusive. The special population of patients with lung adenocarcinoma is defined by ALK fusion. A high response to ALK inhibitors such as crizotinib has been shown. Compared with patients receiving chemotherapy, patients receiving crizotinib showed a much higher response rate and a better median PFS. However, in a number of patients, resistance develops, and secondary mutations occur within the kinase domain of EML4-ALK (C1156Y, L1196M, F1174L). Newly developed ALK inhibitors targeting ALK positive NSCLC are currently under investigation. These new ALK inhibitors are AP26133, ceritinib, and alectinib [41]. While EGFR and ALK are valuable therapeutic targets, they are useful for only the responsive patients with specific mutations or gene rearrangement. Additional target discovery is essential as well as understanding the biology of lung cancer progression and metastasis. In this thesis, we will describe the identification of an extracellular matrix modifying enzyme Prolyl collagen hydroxylase P4HA1 and its role in lung cancer progression.

Collagen Modifying Enzyme P4HA1 in Cancer

The extracellular matrix (ECM) plays a key role in tumor growth and progression. ECM remodeling contributes to tumor pathogenesis including metastasis. The tumor microenvironment is characterized by imbalances in collagen modification, ECM homeostasis by matrix metalloproteinases, leading to cancer progression and metastasis [42]. Changes in the tumor microenvironment along with ECM disruption leads to cancer aggressiveness and metastasis [42, 43]. Collagen-modifying prolyl-4 hydroxylase (P4H) enzymes have been studied for their role in various cancers [42-47]. The most abundant subunit, P4HA1, catalyzes 4-hydroxyproline formation necessary for proper collagen polypeptide folding. P4HA1 expression has been shown to play a significant role in colorectal, prostate, and breast cancers [42-44, 48]. Targeting the enzymes that are overexpressed and involved in tumor-based collagen modification can be a viable therapeutic strategy to target aggressive cancer. We describe one such target P4HA1 overexpression in lung cancer in this thesis and also evaluate a strategy to target this enzyme using specific inhibitor of P4HA1.

Nucleotide Biosynthesis in Cancer

Cancer cells grow rapidly and continuously divide. This necessitates an increased nucleotide demand for DNA, RNA and other critical metabolites for cell division. While nucleotide salvage is common in normal cells, cancer cells show enhanced utilization of de novo biosynthetic pathway [49]. A detailed description of this pathway and potential targeting strategy have been discussed later in the thesis, both as a review and initial work on one of the enzymes involved in nucleotide biosynthesis (MTHFD1L). The significance of these pathways has been shown and supported by many studies.

Thus, further investigation into the role of their regulators has proven necessary for understanding their mechanisms and importance in cancer. The studies presented in this work aim to contribute to the field by bolstering these findings.

DYSREGULATION OF DE NOVO NUCLEOTIDE BIOSYNTHETIC PATHWAY ENZYMES IN CANCER

by

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INTRODUCTION

Cancer is a multifaceted disease characterized by uncontrolled growth of transformed cells that can arise in many tissue types throughout the body (e.g., breast, lung, prostate, pancreas, lymph nodes). Disease progression resulting in aggressive or metastatic burden accounts for one of the leading causes of death worldwide, second only to heart disease in the US. Incidentally, cancer-related mortality has been on a steady decline, dropping 25% over the last 25 years. This could be attributed to improved and earlier diagnoses and better treatment options being developed over the past few decades. However, there are still an estimated 1.8 million new cases and 600,000 deaths predicted for 2019 in the US alone [1]. Efforts have been made to elucidate the mechanism by which cancer cells function and become aggressive.

Research has shown that most cancers are genetic in nature and arise from mutations in classes of genes known as oncogenes and tumor suppressors that lead to an oncogenic phenotype. These mutations can be spontaneous or induced, driving a single cell to undergo rapid proliferation to form a tumor. These errors are often corrected by DNA repair pathways, but these pathways can also be mutated, allowing problems that arise to go unchecked. Hanahan and Weinberg have proposed other characteristics, collectively called hallmarks of cancer [2], based on extensive research. One of the most important metabolic hallmarks is deregulated cellular energetics. Due to the need for cancer cells to synthesize large amounts of energy and nucleotides for DNA and RNA, upregulated *de novo* nucleotide biosynthesis enables cells to proliferate rapidly [2]. There have been numerous efforts to target many enzymes of these pathways. In this review, we

give an overview of folate metabolism and *de novo* purine and pyrimidine biosynthetic pathways.

One-carbon (1C) Metabolic Pathway in Cells

One-carbon (1C) metabolism is a biosynthetic process integral to cellular function across cell types due to the role it plays in both amino acid and nucleotide metabolism. Its name is derived from the fact that it involves the transfer of 1C-containing groups, facilitated by the cofactors tetrahydrofolate (THF), vitamin B12 (Cobalamin), and s-adenosylmethionine (SAM), which are produced via folate metabolism [50]. Folate is used to describe any of the various forms of folic acid, the synthetic form of vitamin B9; an essential nutrient that plays an important role in cell proliferation, red blood cell production, and methylation. Folate deficiency is a serious condition that can cause megaloblastic anemia and accumulation of toxic metabolites and is typically seen in pregnant women and those with gastrointestinal diseases [51]. However, research has shown that inhibition of folate metabolism or folate restriction in cancer blocks cell proliferation and these inhibitors, such as methotrexate, are widely used for chemotherapeutics [52, 53].

Tetrahydrofolate

Tetrahydrofolate is the activated form of folate and has the potential to transfer various 1C-containing groups, ranging from methyl groups to formyl, formimino, and methenyl groups. Methylene-THF is used for thymidine synthesis, which is oxidized and used for purine synthesis or reduced to methyl-THF that is then used to form methionine [54]. Alternatively, serine hydroxymethyltransferase (SHMT) utilizes methylene-THF in

the reversible conversion of glycine to serine. 1C units produced from serine can then be used reintroduced into the folate cycle for adenosine, guanosine, and thymidylate synthesis along with the regeneration of the coenzymes NADPH, NADH, and ATP (**Figure 1**) [55]. However, methyl-THF is typically utilized as the 1C donor for the conversion of S-adenosylmethionine [56].

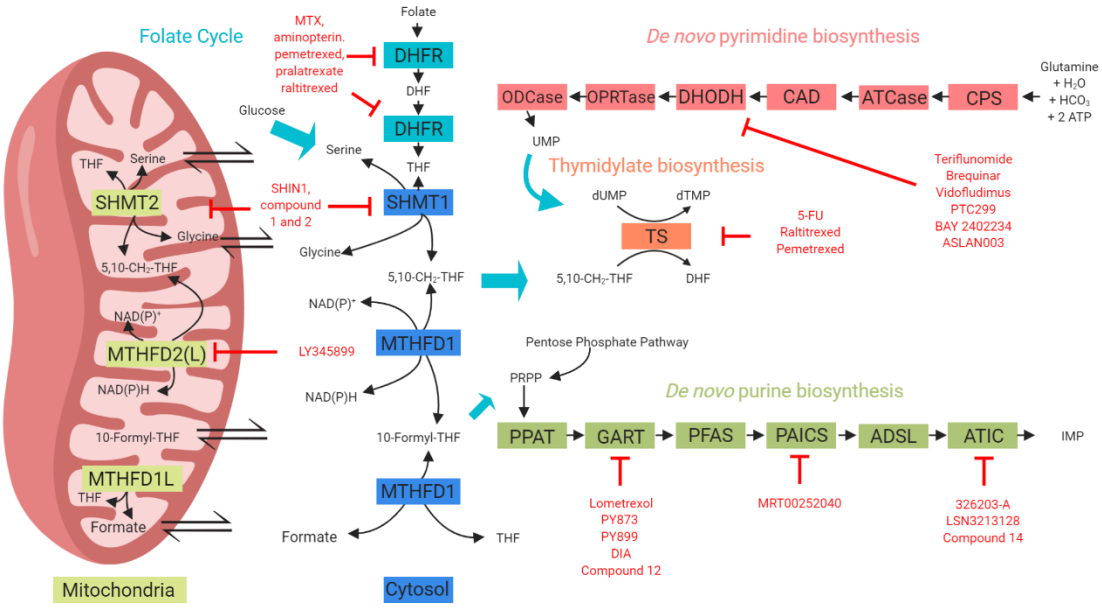

Figure 1. Folate, Purine, and Pyrimidine Metabolic Pathways and Inhibitors. One carbon metabolism or the folate cycle utilizes four enzymes, dihydrofolate reductase (DHFR), serine hydroxymethyltransferase-1 (SHMT1), and methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 1 (MTHFD1) resulting in various tetrahydrofolate (THF) species, such as, 5, 10-CH_2-THF and 10-formyl-THF. 5, 10-CH_2-THF is used in thymidylate biosynthesis and is produces dihydrofolate (Vitamin B9) via thymidylate succinate. 10-formyltetrahydrofolate goes on to play a role in *de novo* purine biosynthesis. *De novo* nucleotide biosynthesis consists of two pathways. *De novo* purine biosynthesis is comprised of ten steps (green). The process converts 5-phosphoribosyl-1-pyrophosphate (PRPP) from the Pentose Phosphate Pathway into inosine monophosphate (IMP). The pathway consists of six different enzymes and various cofactors, including PRPP amidotransferase (PPAT), phosphoribosylglycinamide formyltransferase (GART), phosphoribosylformylglycinamidine synthase (PFAS), phosphoribosylaminoimidazole succinocarboxamide synthase (PAICS), adenylosuccinate lyase (ADSL), and IMP cyclohydrolase (ATIC). A product of one carbon metabolism, 10-formyltetrahydrofolate, also serves as a cofactor for this cycle. *De novo* pyrimidine biosynthesis is comprised of six steps (pink) catalyzed by three genes (*CAD, DHODH, and UMPS*) coding for six enzymes, including, carbamoyl phosphate synthetase II (CPS), aspartate carbamoyl transferase (ATCase), dihydroorotase (CAD), dihydroorotate dehydrogenase (DHODH),

orotate phosphoribosyltransferase (OPRTase), and orotidine monophosphate (OMP) decarboxylase (ODCase). CPS, ATCase, and CAD are all coded by the gene *CAD*, while OPRTase and ODCase are a part of the bifunctional enzyme, uridine monophosphate synthetase (UMPS). Inhibitors have been developed to target key points within these pathways.

S-adenosylmethionine (SAM)

A universal donor of methyl groups, S-adenosylmethionine transfers methyls to nucleic acids, proteins, and lipids. SAM is produced and consumed via the SAM cycle (**Figure 2**). In the first step of the SAM cycle, which is the rate-limiting step, 5,10-methylenetetrahydrofolate is irreversibly reduced to 5-methyltetrahydrofolate via methylenetetrahydrofolate reductase (MTHFR). Methionine adenosyltransferase (MAT) drives the formation of SAM from methionine and adenosine triphosphate (ATP). Methylases utilize SAM to produce S-adenosyl-homocysteine, which is hydrolyzed by adenosylhomocysteinase (AHCY) to homocysteine that can either be recycled back into methionine or degraded irreversibly into cysteine [55, 57]. Within this cycle, methyl-THF is created and acts as a methyl donor for the methylation of homocysteine, resulting in THF formation. This THF can then again be converted into methylene-THF via SHMT or methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 1 (MTHFD1), feeding back into the folate metabolic cycle. MTHFD1 has three functional units: methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, and formyltetrahydrofolate synthase [55, 58]. These functions yield THF derivatives that give rise to methionine, thymidylate, and *de novo* purine synthesis.

One-Carbon (1C) Metabolism and Cancer

As previously stated, 1C metabolism, facilitated by THF, B12, and SAM, is essential for nucleotide synthesis, methylation, and amino acid synthesis in normal cells. Due to an increased proliferative index, these processes are especially important for cancer cells [25, 59, 60]. Drugs targeting 1C metabolism have long since been used as a cancer therapy, as cancer cells are highly susceptible to the deprivation of 1C groups. This has been shown by the inhibition of *de novo* purine synthesis [61, 62].

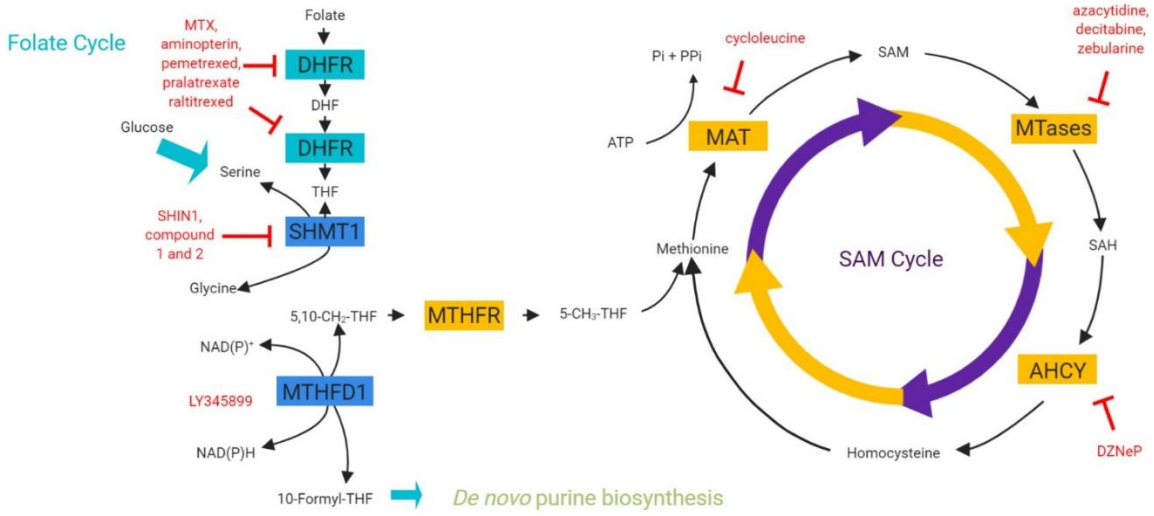

Figure 2. S-adenosylmethionine (SAM) Pathway and Inhibitors. 5, 10-CH_2-THF from the folate cycle is irreversibly reduced into 5-CH_3-THF. Methionine is converted into S-adenosylmethionine, which is a common one-carbon donor, via methionine adenosyltransferase (MAT). Upon donating a methyl group through methyltransferase reactions (MTases), SAM becomes s-adenosylhomocysteine (SAH). SAH is then converted into homocysteine via adenosylhomocysteinase (AHCY). Homocysteine can then be converted back into methionine or used to form other amino acids. Inhibitors have been developed to target key points within these pathways.

De novo Purine and Pyrimidine Biosynthesis and Cancer

Nitrogenous bases are the building blocks of DNA and RNA. The bases cytosine, thymine, and uracil are classified as pyrimidines and are composed of a ring containing nitrogen atoms in the first and third positions, while adenine and guanine are classified as

purines and are composed of a pyrimidine ring attached to an imidazole ring [63]. These nucleotides play roles throughout the cell from providing energy to coenzymes necessary for numerous biochemical pathways. ATP is the most regularly used form of energy, with guanosine triphosphate driving processes like protein synthesis. Metabolism relies heavily on the presence of these bases as they are found in the coenzymes FAD, NAD, and NADP and regulators AMP, and ADP, along with ATP. These molecules are vital to cellular function and there are two unique pathways by which they are synthesized.

The nucleotide salvage pathway uses free bases that are derived endogenously from nucleotide turnover during the degradation of DNA and RNA or exogenously from dietary intake. This recycling is facilitated by adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) attaching 5-phosphoribosyl-1-pyrophosphate (PRPP), and uridine phosphorylase and thymidine phosphorylase attaching ribose-1-phosphate and 2-deoxy-alpha-D-ribose-1-phosphate to their associated bases [49]. Cytosine is salvaged via the uracil pathway by cytidine deaminase or uridine-cytidine kinase. This salvage pathway is beneficial for cells due to its reduced energy expense.

Alternatively, purines and pyrimidines can be produced via *de novo* pathways which do not utilize free bases in the manner of salvage pathways. Instead, purines are synthesized directly onto ribose, and pyrimidines are converted into their final states after being synthesized and attached to ribose phosphate. The purine pathway begins with the formation of inosine monophosphate onto ribose-5-phosphate, leading from PRPP to inosine monophosphate (IMP) through a series of 11 biochemical reactions that adds the atoms of the ring successively. The atoms of the purine ring are derived from glycine, glutamine, and aspartate. The rate-limiting step in this pathway is the very first step

where PRPP is catalyzed by PRPP amidotransferase (PPAT) to form into 5-phosphoribosyl-1-amine (PRA). The resulting IMP is then used for the synthesis of both guanosine monophosphate (GMP) and adenosine monophosphate (AMP). These nucleotides can then participate in a feedback inhibitory loop, of PRPP synthase and PRPP amidotransferase, the rate-limiting step of this pathway. Steps 4 and 10 of the purine pathway utilize formyl-THF, from which the last atom necessary for completing the purine ring is taken (**Figure 1**). This allows for purine biosynthesis to be greatly affected by chemotherapeutics aimed at altering folate metabolism, indirectly inhibiting cell growth, DNA and RNA synthesis, and cell proliferation.

The *de novo* pyrimidine biosynthesis pathway begins in a reverse fashion of sorts. In contrast with purine *de novo* biosynthesis, pyrimidines are constructed as free rings that are then attached to ribose-5-phosphate. The six-step process is initiated by three gene products of gene *CAD*, which encodes for CAD, a trifunctional protein that functions as **C**arbamoyl-phosphate synthetase (CPS II), **A**spartate transcarbamoylase, and **D**ihydroorotase, associated with the enzymatic activities of the first three reactions [64]. Pyrimidine synthesis occurs in the cytoplasm, where bicarbonate and ammonia from glutamine are catalyzed by CPS II to form carbamoyl phosphate. This is considered the rate-limiting step of this pathway. The following step, the combination of carbamoyl phosphate with aspartate, commits the molecule to proceed through the remaining reactions. The resulting open ring is dehydrated and dehydrogenated by dihydroorotase and dihydroorotase dehydrogenase, respectively. The ring is then attached to ribose-5-phosphate via orotase phosphoribosyltransferase and decarboxylated via orotidylic acid decarboxylase (**Figure 1**). The resulting molecule is uridine monophosphate (UMP). UMP acts in an inhibitory feedback loop, inhibiting CPS II, the enzyme that catalyzes the

rate-limiting step of pyrimidine biosynthesis and is also used in turn for *de novo* thymidylate synthesis.

Cancer cell utilization of de novo nucleotide biosynthesis

Such as nucleotides play various important roles in the cellular function, cancer cells, with their high energy expenditure, tend to acquire means to exploit these pathways. Cancer cell metabolism is commonly altered in such a manner characterized by increased glucose uptake and fermentation to lactose. This occurs even though the cells have mitochondria and the ability to undergo normal oxidative phosphorylation. This phenomenon was observed by Otto Heinrich Warburg and is coined as the "Warburg Effect" [65, 66]. Briefly, typical cell metabolism and ATP production takes place via oxidative phosphorylation within the mitochondria, resulting in pyruvate production that enters into the Krebs cycle, which provides the largest portion of ATP resulting from the phosphorylation of ADP. Most cancer cells produce their energy through rapid glycolysis followed by lactose fermentation, even in the presence of oxygen. This means of energy production is much less efficient at generating ATP compared to oxidative phosphorylation, though it provides metabolites that benefit the increased proliferation of cancer cells. This shift in metabolism has been attributed to causing cancer, but modern research postulates that it is the result of mutations in oncogenic and tumor-suppressive genes rather than a cause of it. Pathways involved in cell proliferation are regularly dysregulated. Oftentimes, these same pathways are metabolic pathways that cancer cells can exploit to acquire nutrients at levels to facilitate their rapid cell proliferation.

Cancer cells can be highly proliferative, and research has shown that they are in constant need of nucleotides to support DNA replication and RNA production. For this reason, *de novo* purine and pyrimidine biosynthesis are upregulated to obtain the substantially larger quantities of nucleotides needed to meet the demand for nucleic acid synthesis [67]. Thymidylate synthase and inosine synthetase are upregulated via MYC expression, along with metabolism and glutamine uptake, which is used as a nitrogen source for these *de novo* processes and the replenishment of tricarboxylic acid components that were spent in the process [60].

Researchers have shown that the upregulation of metabolic enzymes, such as phosphoribosyl aminoimidazole succinocarboxamide synthase (PAICS), correlate with upregulated cancer phenotypes, such as proliferation, invasion, and metastasis [59]. This enzyme and other key players within these *de novo* biosynthesis pathways serve as potential druggable targets for therapy. That being said, there are a plethora of drugs being investigated that are aimed at inhibiting metabolic pathways.

Targeting Strategy *de novo* Nucleotide Biosynthetic Pathways in Cancer

The importance of folate in cell growth was recognized in 1947 by Sidney Farber who hypothesized that as cancer cells divide rapidly, they must be sensitive to folate inhibition [68]. He first utilized this concept to treat children with acute lymphoblastic leukemia (ALL) with the antifolate aminopterin. However, single-agent therapy only achieved short term ALL remissions until combination chemotherapy found its way into clinical practice. Due to its toxicity, aminopterin was soon replaced by methotrexate (MTX). MTX acts by inhibition of the enzyme dihydrofolate reductase (DHFR), which catalyzed the reactions from folic acid to THF. In 1956, Hertz and Li first applied

methotrexate treatment in solid tumors. Their study was the first report of radiographic regression of tumor metastasis by utilizing chemotherapy [69].

A rapid expansion of anti-metabolite development followed within the next decades with the production of DHFR inhibitors pemetrexed, pralatrexate, and raltitrexed which are used e.g. in non-small cell lung cancer, pleural mesothelioma, peripheral T-cell lymphoma and metastatic colorectal cancer treatment [56]. However, not only antifolates are popular targets. One of the most widely used antimetabolites in cancer therapy is the pyrimidine analogue 5- Fluorouracil (5FU). By inhibiting the thymidylate synthase, 5FU blocks the conversion of deoxyuridylic acid to thymidylic acid and thus interferes with DNA synthesis. It is most commonly used to treat colorectal, breast, gastric, pancreatic, ovarian, and liver cancer [70]. The deoxycytidine analogue gemcitabine is another widely applied chemotherapeutic agent. Its mechanisms of actions include inhibition of DNA synthesis by chain termination, inhibition of deoxycytidylate deaminase and ribonucleotide reductase [71]. It is clinically used in the treatment of pancreatic, bladder, breast and non-small cell lung cancer [72]. The SAM cycle also has several inhibitors targeting its key enzymes in colorectal, lymphoma, liver, and prostate cancers [73-75].

Often encountered challenges in all these reagents are toxicity and chemoresistance. Within the last two decades, the potential of small-molecule inhibitors, such as erlotinib, has been recognized for their use in cancer therapy [76]. Their advantages include their ability to specifically target explicit receptor-pathway sequences, the rapid inactivation of their target, and the reversibility of this inactivation. Furthermore, enzymes inhibited by these molecules may still interact with their protein binding partners, so that enzymatic and structural roles can be investigated separately. Besides, they are often better tolerated by patients, while being more effective than conventional chemotherapeutic agents geared

toward cytotoxicity as is seen with paclitaxel and temozolomide, for example [76, 77]. As enzymes involved in the folate cycle and *de novo* purine and pyrimidine biosynthesis are among the most overexpressed proteins across cancers, they are promising targets for these inhibitors. Two of these enzymes are SHMT1 and 2, with SHMT1 being the cytoplasmic and SHMT2 the mitochondrial protein. They both catalyze the conversion of serine and THF into glycine and 5, 10-methylene–THF. The mitochondrial part of the folate pathway is required in nutrient-poor conditions. However, both the mitochondrial and the cytosolic C1 metabolism can enhance tumorigenesis. When both pathways are disabled, tumor formation was precluded in colon cancer xenografts [78]. Ducker et al. have recently developed small molecular inhibitors against SHMT1/2. *In vitro* studies showed cell growth inhibition when treated with these inhibitors, although rapid clearance rates make them currently unusable *in vivo* [79]. Other groups have also focused on developing and validating SHMT1/2 inhibitors for cancer therapy in preclinical studies [80, 81]. Further, drugs inhibiting other components of the folate cycle are currently under development [82, 83].

Enzymes involved in the *de novo* purine biosynthesis are PPAT, GART, PAICS, ADSL, and ATIC. GART inhibitors have been available for over two decades [84]. Lomotrexol is one such specific inhibitor, which does not affect enzymes like DHFR or TS [84]. However, as it showed marked side effects in clinical trials, lometrexol was never approved for clinical application [85]. Another GART inhibitor, AG2034, showed a better tumor growth inhibition than lomotrexol while causing similarly high toxicity [86, 87]. New inhibitors are PY873, PY899, DIA and compound 12 [62, 88-90]. However, it remains to be seen whether GART-inhibition is feasible without causing severe side effects. Other studies have focused on developing drugs targeting additional components

of the *de novo* purine biosynthesis. Through virtual ligand screening of the National Cancer Institute Diversity Set, Xu et al. identified a novel non-folate inhibitor (326203-A) in complex with 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT), which is a subunit of ATIC, proving a screening approach for discovery of novel inhibitors [91]. Spurr et al. identified a small molecule inhibiting AICARFT homodimerization. They showed a dose-dependent reduction in cell viability when treating breast cancer cells with their compound [92]. Recently, a new AICARFT inhibitor (LSN3213128) [93] was characterized showing significant tumor growth inhibition in breast and lung xenograft models [94]. As the *de novo* purine synthesis enzyme PAICS is consistently overexpressed in many cancer types [17, 20, 59, 95] it is a valid target for inhibition. The recently developed small-molecule inhibitor (MRT00252040) [96] targeting PAICS is currently being evaluated.

In the *de novo* pyrimidine biosynthesis, the most common targeted enzyme is the dihydroorotate dehydrogenase (DHODH). Several preclinical studies have shown that inhibition of DHODH resulted in decreased growth in different cancer types [97-100]. In pancreatic xenograft models, DHODH inhibitors selectively inhibited KRAS mutant cell lines [98]. Moreover, inhibition of the *de novo* pyrimidine pathways was shown to sensitize triple-negative breast cancer cells to chemotherapy [25]. Several DHODH inhibitors such as teriflunomide, brequinar, and vidofludimus have been studied in cancer [98-101]. However, some display off-target effects and a narrow therapeutic window [102, 103]. A novel DHODH inhibitor PTC299 had a broad potency in hematologic malignancies in preclinical studies. This novel inhibitor displayed some advantages, including good oral bioavailability and lack of off-target kinase inhibition and myelosuppression [104]. An ongoing phase 1b clinical trial investigates PTC299 in relapsed/refractory acute leukemia

(NCT03761069). Another novel DHODH inhibitor (BAY 2402234) applied as monotherapy showed efficacy and differentiation induction across multiple acute myeloid leukemia (AML) subtypes [105]. The small-molecule inhibitor ASLAN003 has shown good tolerance and pharmacokinetic profile in a phase 1 clinical trial (NCT01992367). The drug is now being evaluated in a phase 2 trial in acute myeloid leukemia in patients not eligible for standard treatment (NCT03451084). Preliminary results showed encouraging signs of clinical activity in AML patients, while the optimal dose has yet to be determined [106]. Several other ongoing trials investigate DHODH inhibition in hematologic malignancies (NCT03404726, NCT03760666, NCT03834584, and NCT02509052). DHODH inhibition is further evaluated in solid tumors. One example is a clinical trial investigating leflunomide in previously treated metastatic triple-negative cancers (NCT03709446). To date, no specific inhibitor for the other *de novo* pyrimidine synthesis enzymes was evaluated in preclinical or clinical studies.

Further understanding of the nucleotide biosynthetic pathways and finding novel targeting are still needed to improve therapy in cancer patients. Other strategies than direct enzyme inhibition mentioned in this section, including dietary restriction of serine or glycine, are currently investigated in cancer treatment [107, 108].

Future Perspective

In the era of high throughput data generation and integration through sequencing, proteomics, metabolomics, and electronic medical records, precision medicine and targeting are becoming a reality in cancer diagnosis and treatment. One of the limitations to achieve greater success in precision targeting is the relatively low number of targets and drugs available to treat cancer patients. Many efforts are being made in this direction

including the development of easy cancer data analysis from large cancer datasets for target identification such as cBioportal, Oncomine and UALCAN [109-113] and others. One of the emerging themes in these data analyses has been the observation of consistent upregulation of purine nucleotide biosynthetic pathway. Historically, nucleotide synthesis has been the pathway of choice to target cancer as the nucleotides form the building blocks for DNA synthesis in cells, and cancer cells have a heightened need for them due to the fast growth. Our studies and others have shown and validated the role of many enzymes that are more specific to cancer cell nucleotide synthesis, making them valuable emerging targets for small molecule inhibition.

There is potential to target the nucleotide biosynthetic pathway enzymes in various cancers either in isolation or in combination with other therapies, including immunotherapy. Therefore, there is a need for the development of small molecules that are highly specific and that show low toxicity to target these enzymes to effectively target cancers.

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COLLAGEN MODIFYING ENZEME P4HA1 IS OVEREXPRESSED AND PLAYS A ROLE IN LUNG ADENOCARCINOMA

by

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ABSTRACT

Lung cancer is the leading cause of cancer-related deaths globally and is histologically defined as either small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC), with the latter accounting for 80% of all lung cancers. The 5-year overall survival rate for lung cancer patients is low as it is often discovered at advanced stages when potential cure by surgical resection is no longer an option. To identify a biomarker and target for lung cancer, we performed analysis of multiple datasets of lung cancer gene expression data. Our analyses indicated that the collagen-modifying enzyme Prolyl 4-Hydroxylase Subunit Alpha 1 (P4HA1) is overexpressed in NSCLC. Furthermore, our investigation found that overexpression of enzymes involved in this pathway predicts poor outcome for patients with lung adenocarcinoma. Our functional studies using knockdown strategies in lung cancer cell lines *in vitro* indicated that P4HA1 is critical for lung cancer growth, migration, and invasion. Additionally, diethyl pythiDC (PythiDC), a small molecule inhibitor, decreased the malignant phenotypes of lung cancer cells. Moreover, we found that miR-124 regulates and targets P4HA1 in lung cancer cells. Thus, our study suggests that collagen-modifying enzymes play an important role in lung cancer aggressiveness. Furthermore, our studies showed that P4HA1 is required for lung cancer cell growth and invasion, suggesting its potential as a valid therapeutic target in lung adenocarcinoma.

INTRODUCTION

The most common subtype of non-small cell lung cancer (NSCLC) is lung adenocarcinoma. The identification of specific molecular alterations present in a patient's lung cancer has changed the way clinicians treat this disease [1-4]. While multiple genomic and epigenomic events converge to trigger unregulated growth, invasion, and metastasis in lung cancer, the exact mechanisms of lung cancer initiation and progression are not fully understood. Thus, there is an urgent need for the identification of biomarkers that may aid in the early diagnosis or stratification of lung cancer as well as new therapeutic targets.

It is first critical to understand the molecular circuitry that plays a role in tumor initiation and progression to target cancer. Smoking has been positively correlated with lung tumor size during diagnosis suggesting an association between smoking and lung cancer [1, 3, 5-8]. *EGFR* (Epidermal Growth Factor Receptor) mutation status is now examined even if the patient is a former smoker because of the proven benefit these therapies have and the substantial improvement seen in patients over standard chemotherapy [5, 6, 9-11]. Those patients who present with localized disease represent the subset that undergo surgical resection and for which extensive molecular analyses have been performed. Tumors from stages 1B to 3B receive surgery followed by chemotherapy that often includes a platinum-based regiment [3, 12, 13]. Studies show EGFR mutations, such as L858R, are differentially responsive to small molecule inhibitors like gefitinib, suggesting differential sensitivity and clinical use of these inhibitors [14-17]. Importantly, high-risk early stage, as well as higher stage patients would benefit greatly from therapies that directly target the driver events present in their cancers.

Recent advances in sequencing technology and high throughput data from lung adenocarcinoma have allowed for the identification of multiple overexpressed enzymes in lung adenocarcinoma, which serve as good therapeutic targets. The current study focuses on one such enzyme P4HA1. Collagen-modifying prolyl-4 hydroxylase (P4H) enzymes have been studied for their role in various cancers [18-23]. The most abundant subunit, P4HA1, catalyzes 4-hydroxyproline formation necessary for proper collagen polypeptide folding. P4HA1 expression has been shown to play a significant role in colorectal, prostate, and breast cancers [18, 19, 24]. Based on these findings, we have conducted studies to assess the importance of P4HA1 in lung cancer growth and progression. The current study shows overexpression of P4HA1 in lung adenocarcinoma. Furthermore, we show the mechanism of regulation and functional role of P4HA1 in lung adenocarcinoma. Our study suggests that P4HA1 is an excellent therapeutic target.

MATERIALS AND METHODS

Cell Lines

Lung cancer cell lines A549, H1437, and H23 were obtained from ATCC (Manassas, VA, USA) and cultured in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) supplemented with penicillin-streptomycin (100U/ml) and 10% FBS (Life Technologies). BEAS-2B cells were obtained from ATCC and cultured in BEBM (Lonza, Basel, Switzerland) supplemented with BEGM (Lonza, Basel, Switzerland). All cells were grown at 37°C in 5% CO_2.

Gene expression analysis using TCGA data

P4HA1 gene expression levels of lung adenocarcinoma compared to normal samples were obtained via the UALCAN cancer analysis tool (http://ualcan.path.uab.edu) [25] which utilizes TCGA transcriptome sequencing datasets.

Gene Expression Analysis

We used 26 frozen primary tumors and associated non-malignant lung samples from lung adenocarcinoma patients who underwent resection at the University of Michigan Health System from 2000-2012. Informed consent was obtained for each subject and was approved by the Institutional Review Board. Tumor specimens were immediately frozen following resection and stored at -80°C. Regions containing a minimum of 70% tumor cellularity were used for RNA isolation and RT-PCR. None of the patients included in this study received preoperative radiation or chemotherapy.

Affymetrix microarray U133Plus2 data set from Okayama [26] representing 226 lung adenocarcinomas was used in the survival analysis for genes P4HA1, P4HA2, and P4HA3. The CEL files of microarray data were normalized using Robust Multi-array Average (RMA) method. Overall survival is the outcome and censored at 5 years.

For *in vitro* assays, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was generated via reverse transcription of the resulting RNA. Quantitative real-time polymerase chain reaction (q-RTPCR) was performed using Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR (Polymerase Chain Reaction) (Thermofisher Scientific, Waltham, MA) System. SYBR green probes were used to determine mRNA expression levels. Primers used in the study were acquired from

Integrated DNA Technologies (Coralville, IA). β-actin served as a normalized control. All q-RT-PCR reactions were performed in triplicate.

MicroRNA-124-3p analysis

Frozen lung tissue was obtained from the UAB tissue collection and banking facility and Vanderbilt Thoracic Biorepository with IRB approval. RNA was isolated from tissue using a Qiagen miRNeasy kit (Cat# 217004). 10ng RNA input was used for cDNA synthesis using Applied Biosystems TaqMan™ MicroRNA Reverse Transcription Kit (Cat#4366596). MiR-124-3p expression was quantified from cDNA using TaqMan qRT-PCR and normalized to endogenous control RNU6B. Mean miR-124-3p expression (Error bars ± SEM: $*P = 0.048$, one-sided student's t-test with unequal variance). Precursor microRNAs were obtained from Ambion (Thermofisher, Austin, TX).

RNA Interference and miRNA transfection

P4HA1 small interfering RNA (siRNA) and non-targeting control duplexes were obtained from Dharmacon (GE Healthcare, Lafayette, CO). Reverse transfection was accomplished using Lipofectamine RNAiMAX (Life Technologies) per manufacturer instructions. P4HA1 shRNAs were produced by System Biosciences (Mountain View, CA). Lentiviruses were generated by the University of Alabama at Birmingham Neuroscience NINDS Protein Core (P30 NS047466). P4HA1 shRNA expressing cells, along with scramble controls, were selected for by treatment with 1 μg/ml puromycin (Life Technologies).

Small Molecule Inhibition

The P4HA1-specific inhibitor, Pythi-DC, was obtained from MedChemExpress (Monmouth Junction, NJ). Serial dilutions (10-100μM) were tested over 6 days to observe the effect of Pythi-DC on P4HA1 expression, cell viability, colony formation, and invasion.

Immunoblot Analyses

Protein lysates were collected using NP-40 lysis buffer (Boston BioProducts, Ashland, MA) with 1X Halt™ Phosphatase and Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA). Samples were separated by SDS-PAGE and transferred onto Immobilon®-P PVDF membrane (MilliporeSigma, Billerica, MA). Membranes were blocked in a blocking buffer of 5% non-fat dry milk and TBS-T (Tris-buffered saline and 0.1% Tween) and then incubated in primary antibody (P4HA1, Cat#12658-1-AP, 1:1000; P4HA2, Cat#13759-1-AP, 1:1000; P4HA3, Cat#23185-1-AP, 1:1000;P4HB, Cat#11245-1-AP, 1:1000) overnight at 4°C. Blots were then washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody and developed with Luminata Crescendo Western HRP chemiluminescence substrate per manufacturer's protocol (MilliporeSigma, Billerica, MA). Images were taken using the Amersham™ Imager 600RGB (GE Healthcare Life Sciences, PA, USA). Antibody dilutions were optimized in house.

Cell Proliferation Assay

Cells were seeded at 100-200 cells/well and proliferation was measured by cell counting using a Z2 Coulter particle counter (Beckman Coulter, Brea, CA). Proliferation was measured every 48 hours. Media was changed every three days.

Colony Formation Assay

Cells were trypsinized and seeded into 6-well plates at a density of 1000-10000 cells/well, depending on the cell line. Media was changed every three days and colony formation was measured after 9 days. Cells were washed with 1X PBS, fixed with 5% glutaraldehyde, stained with crystal violet, and quantified.

Matrigel Invasion Assay

Cells were trypsinized and seeded at 15K-30K cells/well into Matrigel® invasion chambers (Corning, Corning, NY) in serum-free media in the upper chamber. Media containing 10% FBS was added to the lower chamber as a chemoattractant. After 18-24 hours, non-invading cells were removed along with the Matrigel using a cotton swab. Invaded cells were fixed with 5% glutaraldehyde and stained with crystal violet. Wells were then imaged using an inverted scope (4X) and the number of invaded cells was quantified.

Statistical Analysis

To determine significant differences between two groups, Student's two-tail t-test was used for all experiments. *P*-values <0.05 were considered significant. Kaplan-Meier survival curve with log-rank test was used for overall survival analysis based on gene expression value.

RESULTS

P4H genes are overexpressed in lung adenocarcinoma and are markers of survival

We have previously demonstrated a role for P4HA1 in prostate cancer growth and progression. For this reason, we decided to pursue deciphering the role P4HA1 in lung adenocarcinoma. Upregulation of prolyl-4-hydroxylase (P4H) genes (P4HA1, P4HA2, and P4HA3) in lung adenocarcinoma were observed via transcriptome analysis of TCGA datasets and gene expression microarray profiling (**Figure 1A**; **Supplementary Figure 1A**).

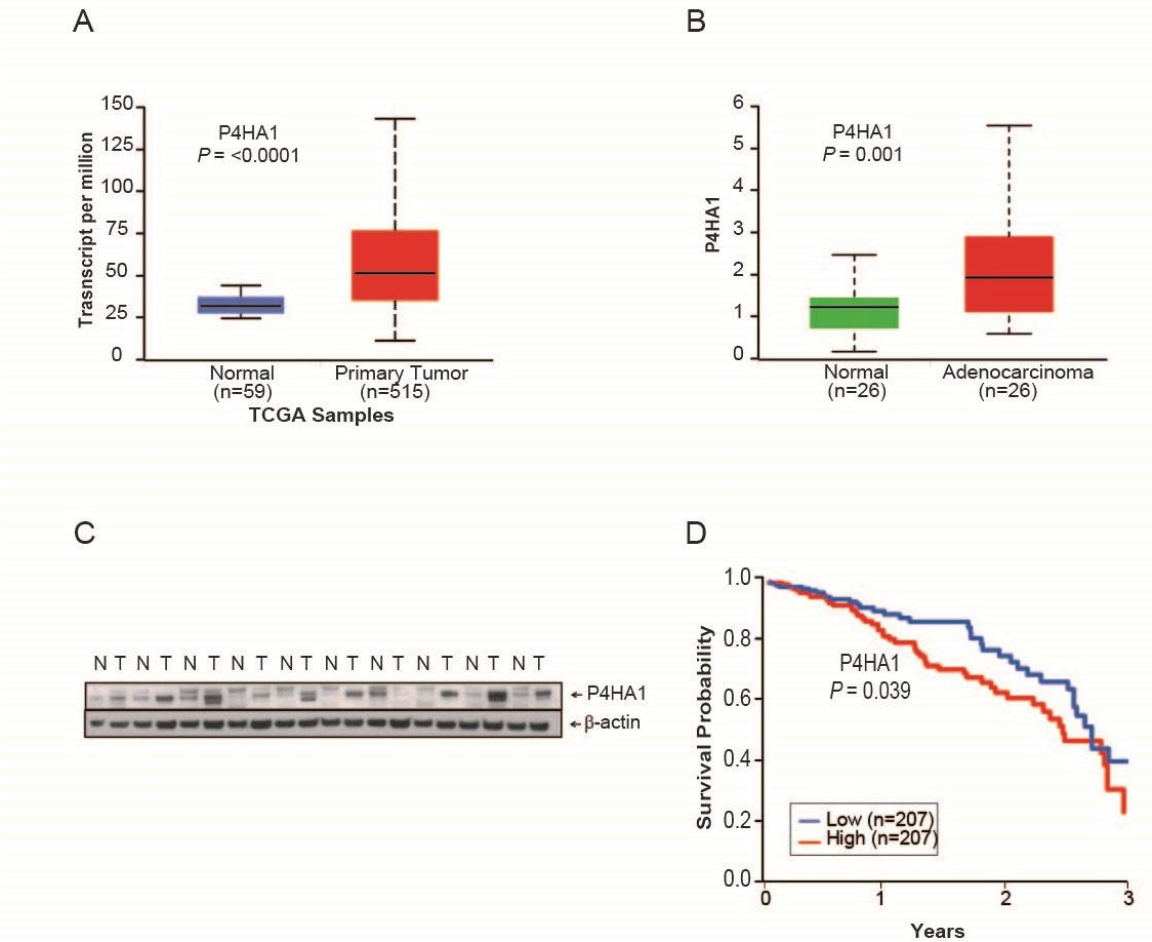


Figure 1. P4HA1 is overexpressed in lung adenocarcinoma and significantly affects survival. (A) P4HA1 gene expression from TCGA data (*P*=<0.0001), (B) quantitative real-time PCR of matched lung adenocarcinoma patient tissues (n=26; *P*=0.001), (C) P4HA1 protein expression in lung adenocarcinoma patient and matched normal tissues by immunoblot analysis using P4HA1 antibody, and (D) Kaplan-Meier plot showing overall survival probability based on P4HA1

expression (*P*=0.02). Affymetrix microarray U133Plus2 data set from Okayama [26] representing 226 lung adenocarcinomas was used in the survival analysis. The CEL files of microarray data were normalized using Robust Multi-array Average (RMA) method. Overall survival is the outcome and censored at 5 years.

To validate these findings, qRT-PCR was performed using RNA isolated from both lung adenocarcinoma and associated normal lung tissues. This analysis confirmed significant P4HA1 overexpression in lung adenocarcinoma (**Figure 1B**). Immunoblot analysis also showed overexpression of P4HA1 in lung adenocarcinoma compared to matched normal tissues (**Figure 1C**). Transcriptome analysis also conveyed the association of P4HA1 with poor overall patient survival (**Figure 1D).** Interestingly, other enzymes belonging to the same family, P4HA2 and P4HA3 expression also showed poor patient survival (**Supplemental Figure 1B**).

P4HA1 plays a role in cell proliferation and invasion in lung adenocarcinoma

The importance of P4HA1's role in lung adenocarcinoma was determined by altering its expression levels in the lung adenocarcinoma cell lines, A549 and H1437. Knockdown was confirmed via immunoblot analysis. Transient and stable knockdown of P4HA1 resulted in a significant decrease in cell proliferation when compared to non-targeting control cells (**Figure 2A; Supplemental Figure 2A**). To test the effect of P4HA1 on invasion, the Boyden Matrigel invasion was used. Transient and stable P4HA1 knockdown effectively reduced the invasive potential of lung adenocarcinoma cell lines (**Figure 2B; Supplemental Figure 2B**). To support the importance of P4HA1 in lung adenocarcinoma, P4HA1 was stably expressed in BEAS-2B cells via adenovirus transduction. Overexpression of P4HA1 in BEAS-2B cells resulted in a significant increase

in invasion (**Figure 2C**). These findings suggest a role for P4HA1 in lung adenocarcinoma proliferation and invasion *in vitro*.

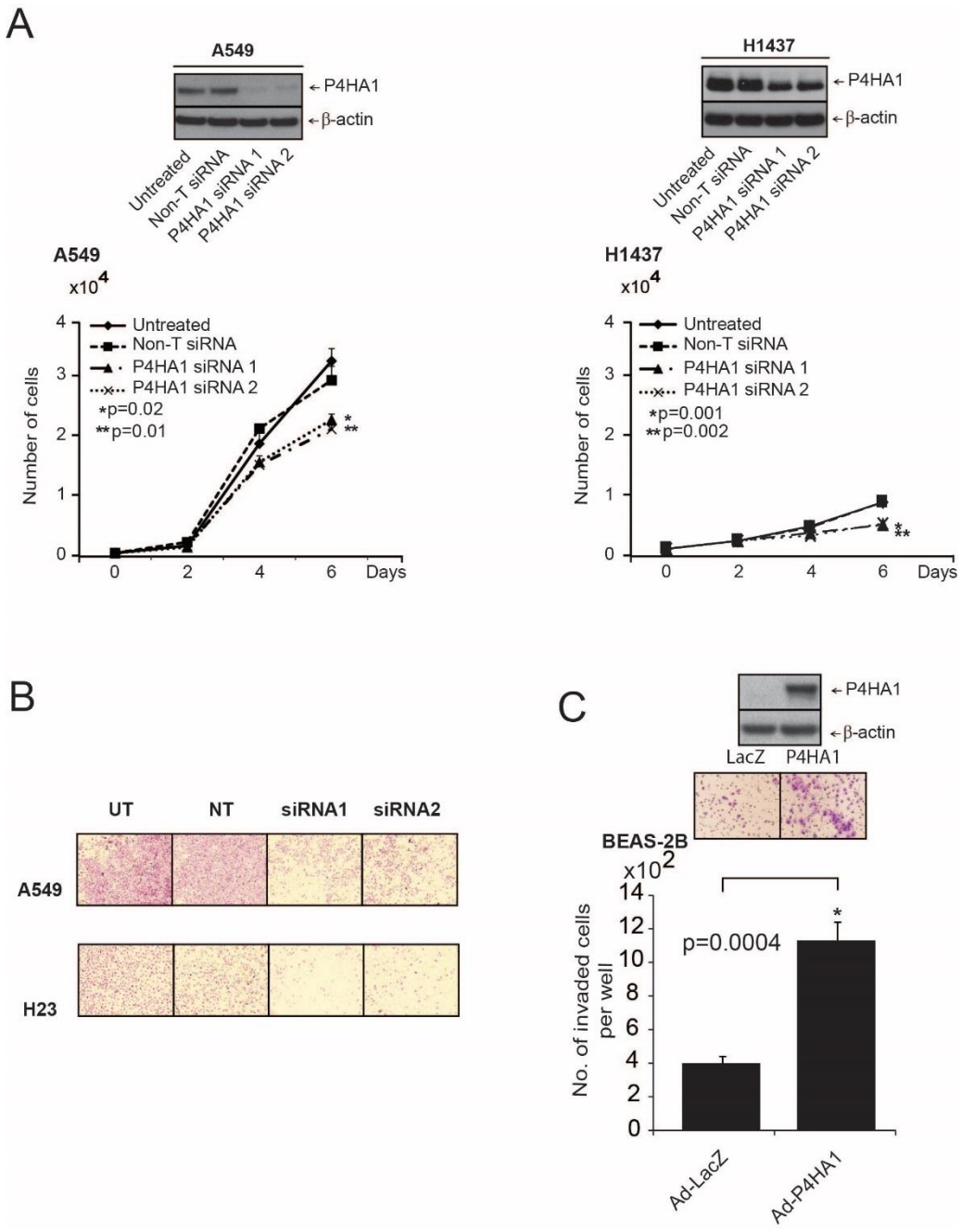


Figure 2. P4HA1 plays a significant role in cell proliferation and invasion for lung adenocarcinoma. (A) Immunoblot analysis of protein lysates from A549 and H1437 cells treated with independent P4HA1 siRNA duplexes. β-actin serves as a loading control. Transient knockdown of P4HA1 in lung adenocarcinoma cell lines (A549 and

H1437) shows reduction in cell proliferation. Cell proliferation assay of P4HA1 siRNA treated A549 and H1437 compared to non-targeting siRNA (Non-T siRNA) controls. (B) Boyden chamber Matrigel invasion assay of P4HA1 siRNA treated A549 and H23 cell lines compared to Non-T siRNA controls. Invaded cells were stained and imaged. (C) Overexpression of P4HA1 via adenovirus transduction in normal human lung cells (BEAS-2B). Invaded cells were quantified.

PythiDC: Target-Specific Small Molecule Inhibition

To validate these findings, assays were repeated utilizing the P4HA1-specific inhibitor, Pythi-DC. Serial dilutions of Pythi-DC were added to lung cancer cell lines and evaluated. Cell viability saw a significant reduction at 75 and 100μM (**Figure 3A**). This trend was also observed with Boyden Matrigel invasion and colony formation assays (**Figure 3B and 3C**).

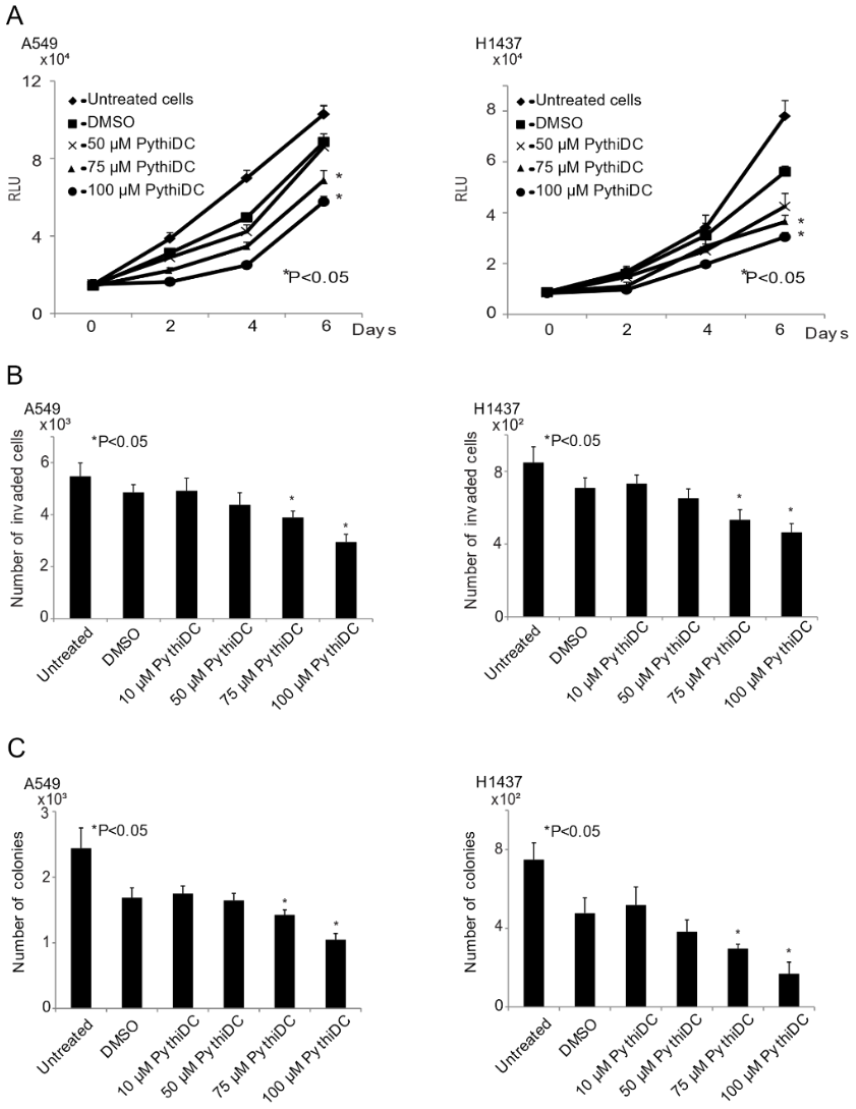
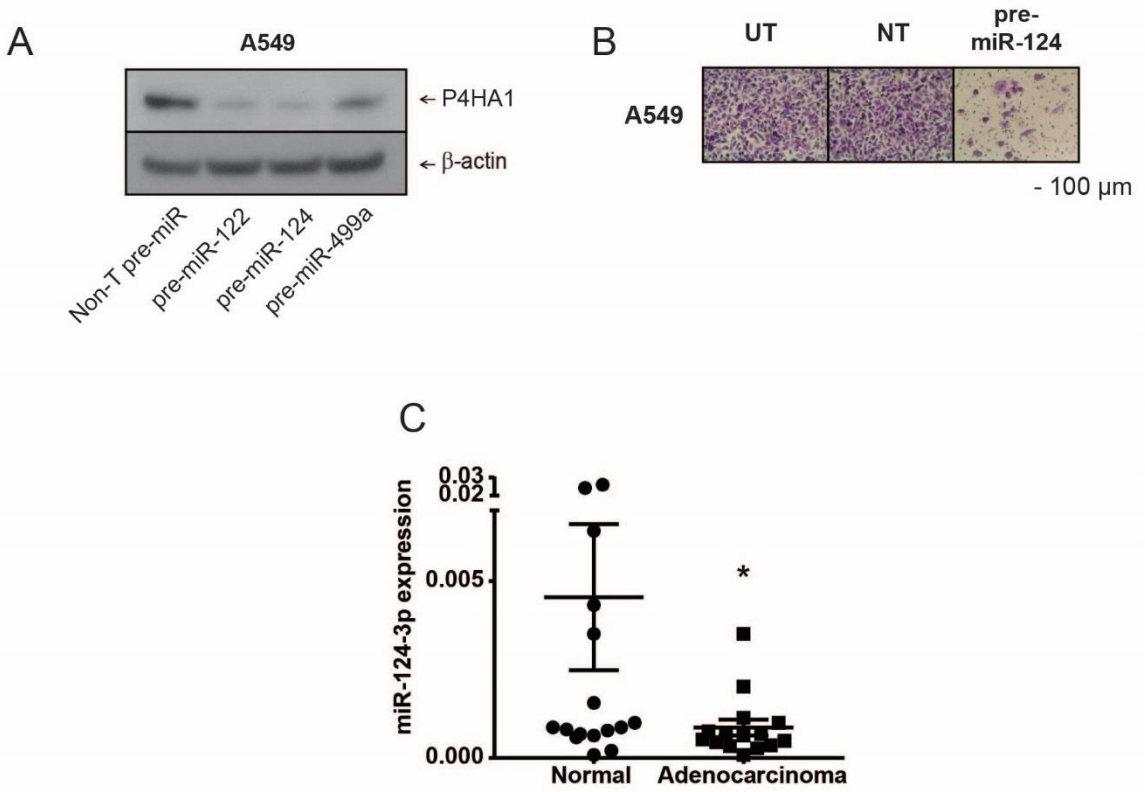


Figure 3. Small molecule inhibition of P4HA1 by Pythi-DC inhibits invasion and colony formation. A549 and H1437 lung adenocarcinoma cell lines show reduced cell proliferation (A), invasion (B), and colony formation (C) with Pythi-DC treatment.

miR-124 Regulates P4HA1 Expression in Lung Cancer

MicroRNAs have been shown to act as tumor suppressors targeting oncogenes, causing their repression, and vice versa. We have previously demonstrated miR-124's tumor-suppressive role in regulating P4HA1 in aggressive prostate cancer, and so sought to investigate the consistency of these findings within lung adenocarcinoma [19]. To determine miR-124's regulation of P4HA1 expression in lung adenocarcinoma, lung

cancer cells were treated with precursor miRs for miR-122, 124, and 499a and P4HA1 protein expression levels were measured via immunoblot analysis. Both precursor miR-122 and 124 resulted in a significant reduction in P4HA1 protein expression, compared to control non-targeting precursor microRNA (**Figure 4A**). This data supports the role of miR-124 as a regulator of P4HA1 in lung adenocarcinoma. Lung cancer cell line A549 was treated with precursor miR-124 and showed a significant decrease in invasion as determined by Matrigel invasion assays (**Figure 4B**). The significance of miR-124 in lung adenocarcinoma patients was determined by ascertaining the microRNA levels in lung adenocarcinomas. RNA was isolated from frozen lung and tumor tissues and qPCR performed using RNU6B as an endogenous control. Results showed a significant decrease in miR-124 levels in lung adenocarcinoma when compared to normal lung tissues (**Figure 4C**).



	Normal (n=16)	Adenocarcinoma (n=15)
Age	60.25 ± 3.16	70.20 ± 2.96
Female	9	6
Male	7	9
Stage I	N/A	12
Stage II	N/A	1
Stage IV	N/A	2

Figure 4. MiR-124-3p targets and downregulates P4HA1 and shows lower levels in lung adenocarcinoma patients. (A) Immunoblot analysis of protein lysates from A549 cells treated with independent pre-miR duplexes. β-actin serves as a loading control. Pre-miR-122 and 124 show reduced P4HA1 expression. (B) Boyden Matrigel invasion assay of A549 treated with pre-Mir-124. Non-targeting microRNA serves as a control. (C) RNA isolated from frozen lung tissue shows decreased levels of miR-124-3p in lung adenocarcinoma patients compared to normal lung. Expression normalized to endogenous RNU6B control. Mean miR-124-3p expression (Error bars ± SEM: **P*= 0.048, one-sided student's t-test with unequal variance). Clinico-pathologic characteristics of lung cancer patients are mentioned in table.

DISCUSSION

Lung cancer is one of the most common cancers affecting both men and women. Lung adenocarcinoma is the common histological subtype of aggressive lung cancers [27]. Many molecular alterations have been identified that initiates and drives lung cancer progression. Effective therapeutic modalities are available targeting specific subtypes of lung adenocarcinoma [13]. Recently, immunotherapy has emerged as an effective method to treat some lung cancers [4, 28], however, there is still an urgent need for additional effective and novel means of targeting lung adenocarcinoma. We have previously shown that P4HA1 can serve as a biomarker and potential therapeutic target in prostate cancer [19]. The current study suggest that P4HA1 is overexpressed and plays a critical role in lung adenocarcinoma.

Prolyl-4-hydroxylase catalyzes the formation of 4-hydroxyproline (Hyp) from proline, which plays a number of roles, one being the stabilization of collagen's triple helix [29]. Post-translational modifications such as this type can have a wide range of effects on various proteins, altering both interactions with other proteins and protein conformation. P4HA1 is also known to effect argonaute 2 (Ago2) [30]. With such widespread possibilities, targeting P4HA1 is a promising therapeutic strategy. Our studies showed microRNA-124 can target P4HA1. Different miRs have been shown to target P4HA1. MiR-122 inhibition has been shown to result in P4HA1 upregulation [31, 32]. MiR-124-3p targets P4HA1 and inhibits collagen synthesis in smooth muscle cells [20]. As shown in the present studies, hsa-miR-124-3p is significantly downregulated in primary lung adenocarcinoma and that the exogenous addition of pre-miR-124-3p can affect the ability

of lung cancer cell lines to invade. Thus, reintroduction of miR-124 can be an effective strategy to target P4HA1.

We have demonstrated significant upregulation of P4HA1 in lung adenocarcinoma and shown its correlation with patient survival. Prolyl-4-hydroxylases are tetrameric proteins made up of alpha subunits (P4HA1, P4HA2, and P4HA3) and a beta subunit (P4HB) [33]; all of which have been shown to be upregulated and predict poor patient survival in lung adenocarcinoma. P4HA2 upregulation can promote cancer progression in breast cancer [34], while low expression of P4HA2 is detrimental for pancreatic cancer patients [21]. P4HB being the only beta subunit is also upregulated in gastric cancer and hepatocellular carcinoma [22, 23, 35]. P4HA3 is upregulated and associated with metastasis and poor survival in gastric cancer and silenced in a subset of melanomas [22]. Therefore, we examined P4HA1 as it is the most abundant of the alpha subunits. The study by Gilkes et al. showed that P4HA1 enhanced invasion and metastasis via alignment of cancer cells along the collagen fibers it helps to stabilize [36]. We have also shown that modulation of P4HA1 levels via small molecule inhibitors, such as Pythi-DC, reduces colony formation. In summary, our study highlights the essential role of P4HA1 in lung cancer progression and suggests P4HA1 as a viable therapeutic target in lung adenocarcinoma patients. We have also shown that modulation of P4HA1 levels via PythiDC, reduces cell viability, invasion, and colony formation. In summary, our study highlights the essential role of P4HA1 in lung cancer progression and suggests P4HA1 as a viable therapeutic target in lung adenocarcinoma patients.

ACKNOWLEDGMENTS

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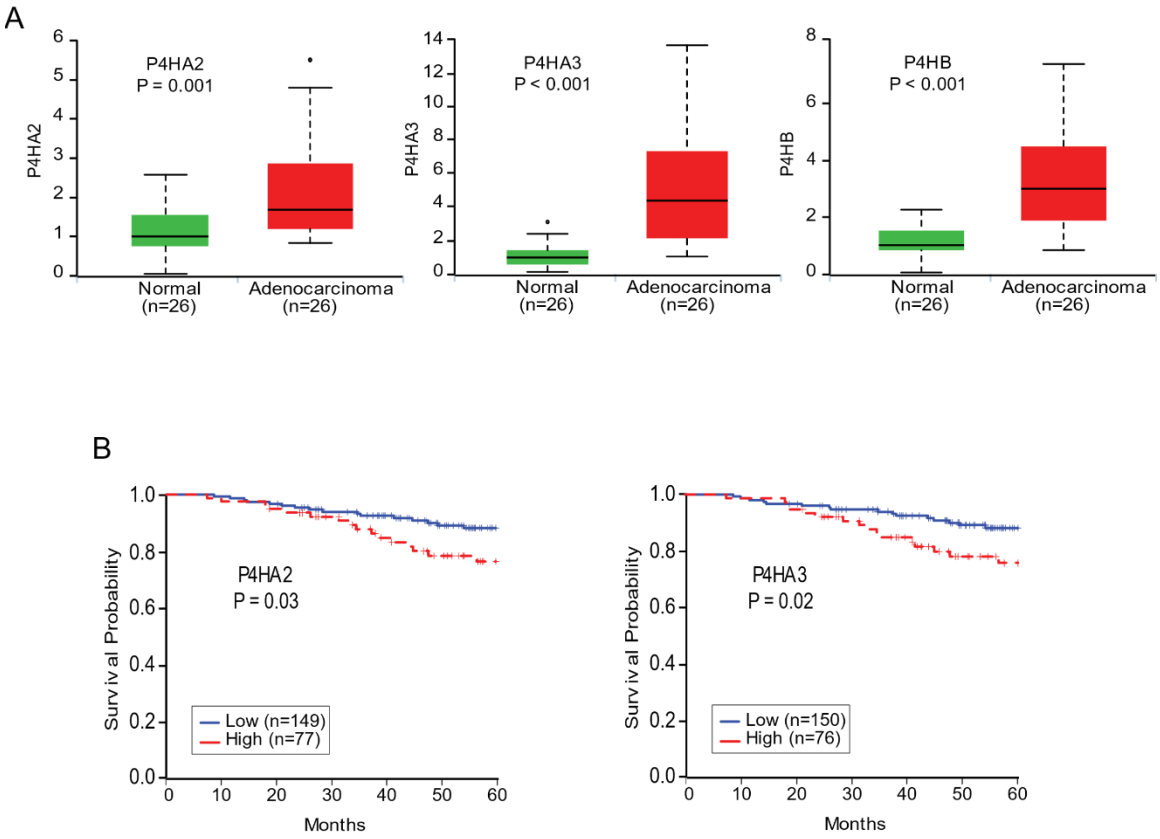
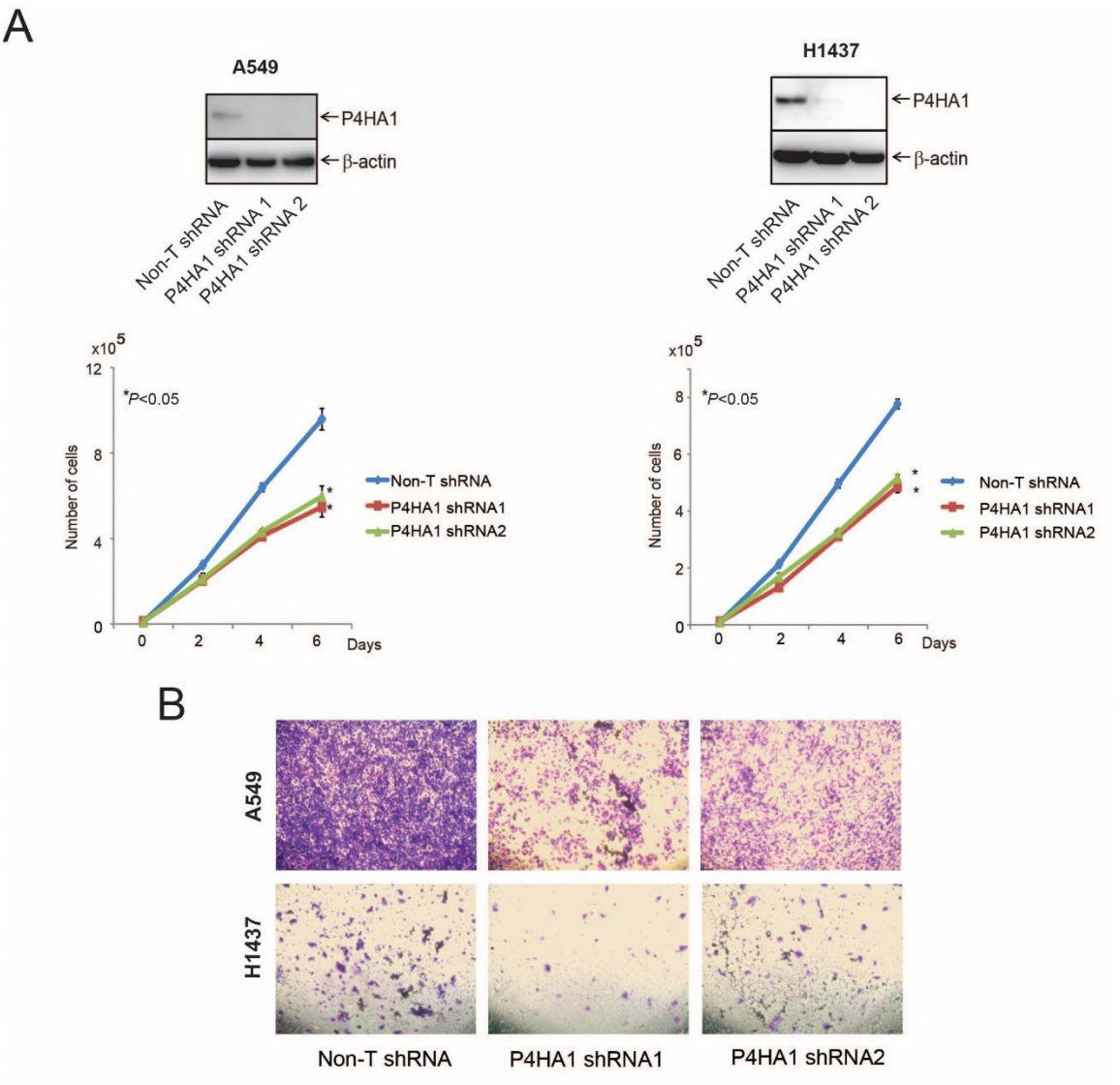


Supplemental Figure 1. Prolyl-4-hydroxylases play a significant role in cell proliferation and invasion for lung adenocarcinoma. (A) P4HA2, P4HA3, and P4HB mRNAs were significantly higher in lung adenocarcinomas (AD) as compared to normal lung tissues measured by RT-PCR (t test, $p<0.01$). Lung tissue samples were obtained from University of Michigan. (B) Kaplan-Meier survival curve analysis indicated that increased P4HA2 and P4HA3 mRNA levels have a poor patient survival in 226 early lung adenocarcinomas (Okayama Affymetrix microarray U133Plus2 dataset). Patients were classified into low (2/3) patients and high (1/3 patients) expressing groups according to gene expression value. Five-year survival time was used.



Supplemental Figure 2. Stable P4HA1 knockdown via shRNA reduces cell proliferation and invasion. (A) Immunoblot analysis of protein lysates from A549 and H1437 cells treated with independent P4HA1 shRNA duplexes. β-actin serves as a loading control. Stable knockdown of P4HA1 in lung adenocarcinoma cell lines (A549 and H1437) shows reduction in cell proliferation. Cell proliferation assay of P4HA1 shRNA treated A549 and H1437 compared to non-targeting shRNA (Non-T shRNA) controls. (B) Boyden chamber Matrigel invasion assay of P4HA1 shRNA treated A549 and H1437 cell lines compared to Non-T shRNA controls. Invaded cells were stained and imaged.

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METHYLENETETRAHYDROFOLATE DEHYDROGENASE (NADP+ DEPENDENT) 1-LIKE (MTHFD1L) IN BREAST CANCER

by

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INTRODUCTION

Breast cancer has consistently been ranked number one in new cases in US women for the past 40 years and is predicted to make up 30% of new cancer cases in 2019 [1]. As diagnostics have improved, this number has increased, with a five-year survival rate of approximately 27% in metastatic disease [2, 3]. Mutations in proto-oncogenes and tumor suppressors have readily been identified as key drivers for tumor progression, increased cell proliferation, and metastasis [4-7]. Research is ongoing to better understand the mechanisms behind these cancer characteristics to find targetable and druggable proteins to alleviate their effects. One characteristic that has been identified as an "emerging hallmark" of cancer is dysregulated metabolism [8]. This ranges from a shift to aerobic glycolysis, also known as the Warburg effect, to increased lipogenesis [9-13]. Another important metabolic pathway is the folate metabolism [14-17]. The name folate encompasses vitamin B and its derivatives. Folate is compartmentalized into cytoplasmic and mitochondrial portions. The gene methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like (MTHFD1L) codes for the protein monofunctional C1-tetrahydrofolate synthase, mitochondrial [18-20]. This enzyme is involved in the one-carbon metabolism process of producing tetrahydrofolate (THF), particularly 10-formyl-THF, in the mitochondria that is utilized for *de novo* purine and thymidine biosynthesis [21-23]. It has been shown that folate metabolism inhibition, as seen with the use of the chemotherapeutic methotrexate, significantly blocks cell proliferation [24-26]. This is linked to its role in amino acid and nucleotide biosynthesis. Nucleotide biosynthesis is a very important process for cancer cells as

unchecked cell proliferation creates a high demand for nitrogenous bases, the building blocks of DNA and RNA [27, 28]. Typically, these bases can be derived endogenously through nucleotide turnover from DNA and RNA degradation through the salvage pathway. The issue can also be resolved via *de novo* biosynthesis pathways [17, 23, 29, 30]. Through these pathways, nucleotides are formed onto ribose-5-phosphate. The purine biosynthesis pathway encompasses the construction of inosine monophosphate (IMP) onto the ribose-5-phosphate ring. There are a series of 11 biochemical reactions, successively adding atoms to the ring. These atoms are donated from molecules like glycine, glutamine, and aspartic acid. The pathway begins with the production of 5-phospho-α-D-ribosyl 1-pyrophosphate (PRPP) by the phosphorylation of ribose-5-phosphate by ribose phosphate pyrophosphokinase. This is the first step committed to *de novo* purine biosynthesis as PRPP can be used in pyrimidine biosynthesis as well. Formyl-THF, produced by MTHFD1L, is utilized in steps 4 and 10 and donates the final atom to complete the purine ring. MTHFD1L has been shown to be upregulated in various cancers [14, 18-20, 31]. In this paper, we aimed to show the importance of MTHFD1L in breast cancer. We hypothesized that the reduction of MTHFD1L levels would decrease cell proliferation, migration, and invasion.

MATERIALS AND METHODS

Cell Lines

Breast cancer cell line MDA-MB-231 (ATCC, Manassas, VA, USA) was cultured in DMEM and cell lines MCF-7 and SKBR-3 (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Life Technologies, Carlsbad, CA, USA). MDA-MB-468 and SUM-159 were graciously donated by the Samant Lab and were cultured in DMEM-F/12

(Corning, Corning, NY). SUM-159 media was supplemented with penicillin-streptomycin (100U/ml), 5% FBS (Atlanta Biological, Flowery Branch, GA), 5ug/ml insulin, and 1ug/ml hydrocortisone. The remaining media was supplemented with penicillin-streptomycin (100U/ml) and 10% FBS. All cells were grown at 37°C in 5% CO_2.

Gene Expression Analysis using TCGA Data

MTHFD1L gene expression in breast cancer tissue compared to normal tissues was obtained using the UALCAN web database [32] which utilizes TCGA transcriptome sequencing for *in silico* analysis.

siRNA Transfection

MTHFD1L siRNA and non-targeting control duplexes (GE Healthcare Life Sciences, Marlborough, MA, USA) were reverse transfected using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA) per manufacturer's protocol. After 72 h transfected cells were seeded for proliferation assays and for RNA and protein isolation.

Immunoblot Analysis

Protein lysates were isolated from NTsi and MTHFD1L siRNA duplex treated cells using NP-40 lysis buffer (Boston BioProducts, Ashland, MA) with 1X Halt™ Phosphatase and Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA). SDS-PAGE was used to separate samples and then transferred to Immobilon®-P PVDF membrane (MilliporeSigma, Billerica, MA). Membranes were blocked in 5% nonfat dry milk and TBS-T (Tris-buffered saline and 0.1% Tween) and then incubated in primary

antibody overnight at 4°C. Blots were incubated with horseradish peroxidase-conjugated secondary antibody and developed with Luminata Crescendo Western HRP chemiluminescence substrate per manufacturer's protocol (MilliporeSigma, Billerica, MA). Images were taken using the Amersham™ Imager 600RGB (GE Healthcare Life Sciences, Marlborough, MA, USA). Anti-MTHFD1L at 1:1000 (#16113-1-AP, Proteintech Group Laboratories, Rosemont, IL), anti-β-actin at 1:20,000 (#HRP-60008, Proteintech Group Laboratories, Rosemont, IL), and anti-rabbit IgG (#SA00001–2, Proteintech Group Laboratories, Rosemont, IL) were used.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from breast cancer cells according to manufacturer protocol using the Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA). cDNA was generated via reverse transcription of the resulting RNA. Quantitative real-time polymerase chain reaction (q-RTPCR) was performed using Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System. SYBR green probes were used to determine mRNA expression levels. Primers used in the study were acquired from Integrated DNA Technologies (Coralville, IA). β-actin served as a normalized control. All q-RTPCR reactions were performed in triplicate.

Cell Proliferation Assay

A Z2 Coulter particle counter (Beckman Coulter, Brea, CA) was used to measure cell proliferation via cell counting. Cell counts were taken every 48 hours and media was changed every three days.

Colony Formation Assay

Cells were seeded at a density of 500-2000 cells/well, depending on the cell line. Media was changed every three days and the assay was harvested after 9 days. Cells were fixed with 5% glutaraldehyde, stained with 5% crystal violet, and then quantified. Images were taken using the Amersham™ Imager 600RGB (GE Healthcare Life Sciences, Marlborough, MA, USA).

Matrigel Invasion Assay

Cells were seeded into Matrigel® invasion chambers (Corning, Corning, NY) in serum-free media in the upper chamber. Media containing 10% FBS was used as a chemoattractant in the lower chamber. Cells invaded for 18-36 hours. Non-invading cells and Matrigel matrix were removed using a cotton swab. Invaded cells were fixed with 5% glutaraldehyde and stained with crystal violet.

Statistical Analysis

To determine significant differences between two groups, Student's two-tailed t-test was used for all experiments. P-values <0.05 were considered significant.

RESULTS

MTHFD1L is Overexpressed in TNBC and Significantly Affects Survival

TCGA analysis demonstrated the overexpression of MTHFD1L in breast cancer (**Figure 1A**). Expression of MTHFD1L was significantly higher still in TNBC (**Figure 1B**). Kaplan-Meier survival analysis showed significantly reduced overall patient survival in those patients with higher MTHFD1L expressing tumors (**Figure 1C**). *In silico* analysis

was validated in breast cancer cell lines via qRT-PCR and immunoblot analysis. Comparing breast cancer cell lines, MTHFD1L expression was significantly higher in TNBC cell lines MDA-MB-231 and SUM-159 (**Figure 1D**). MCF-7 showing increased expression and the TNBC cell line MDA-MB-468 showing decreased expression, highlights tumor heterogeneity. Similarly, these trends were seen with immunoblot analysis as well (**Figure 1E**).

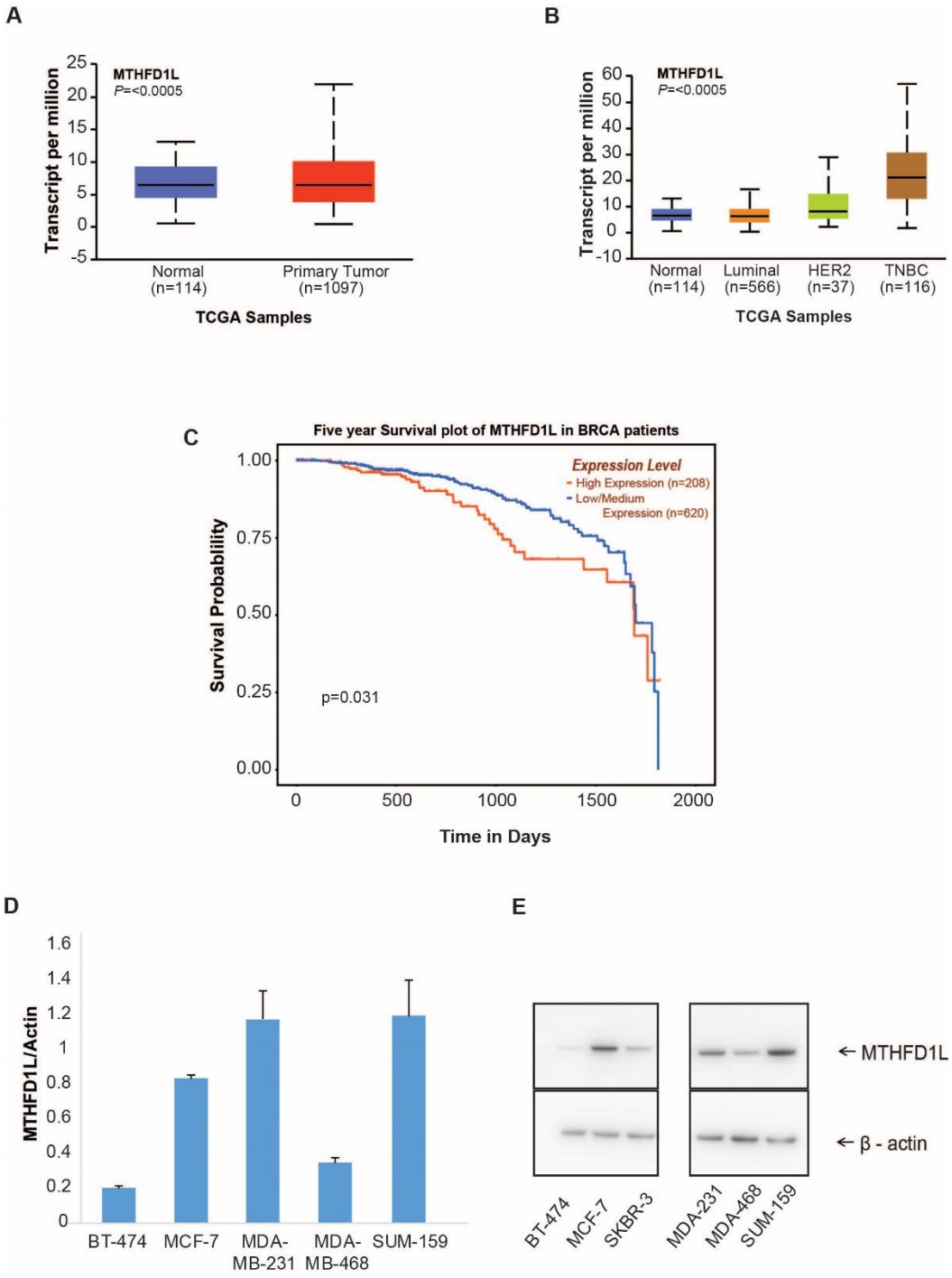

Figure 1. MTHFD1L is overexpressed in TNBC and significantly affects survival. (A) MTHFD1L gene expression from TCGA data of primary breast cancer compared to normal tissues (*P*=<0.0005), (B) MTHFD1L gene expression of breast cancer molecular subtypes (luminal, HER2, and TNBC) in comparison to normal tissues from TCGA data quantitative (*P*=<0.0005), (C) Kaplan-Meier plot showing overall survival probability based on MTHFD1L expression over five years (1825 days) (p=0.031), (D) Breast cancer cell line panel showing MTHFD1L RNA expression, (E) Immunoblot analysis of breast cancer cell line panel showing MTHFD1L expression.

MTHFD1L Significantly Affects Cell Proliferation and Invasion of TNBC

The role of MTHFD1L was evaluated using multiple breast cancer cell lines. Modulation of MTHFD1L was performed using transient transfection of gene-specific unique siRNA duplexes in breast cancer cell lines MDA-MB-231 and SUM-159. Knockdown efficiency was confirmed by immunoblot analysis (**Figure 2A**) and qRT-PCR (**Figure 2B**). Cell proliferation assay spanned 6 days and showed a significant reduction in cell growth with siRNA treatment (**Figure 2C**). This supports the importance of MTHFD1L in breast cancer proliferation. Colony formation and invasion was investigated to assess the role of MTHFD1L. Invasion showed marked reduction upon MTHFD1L knockdown (**Figure 2D**). Knockdown of MTHFD1L resulted in reduced colony formation compared to NTsi-treated cells (**Figure 2E**).

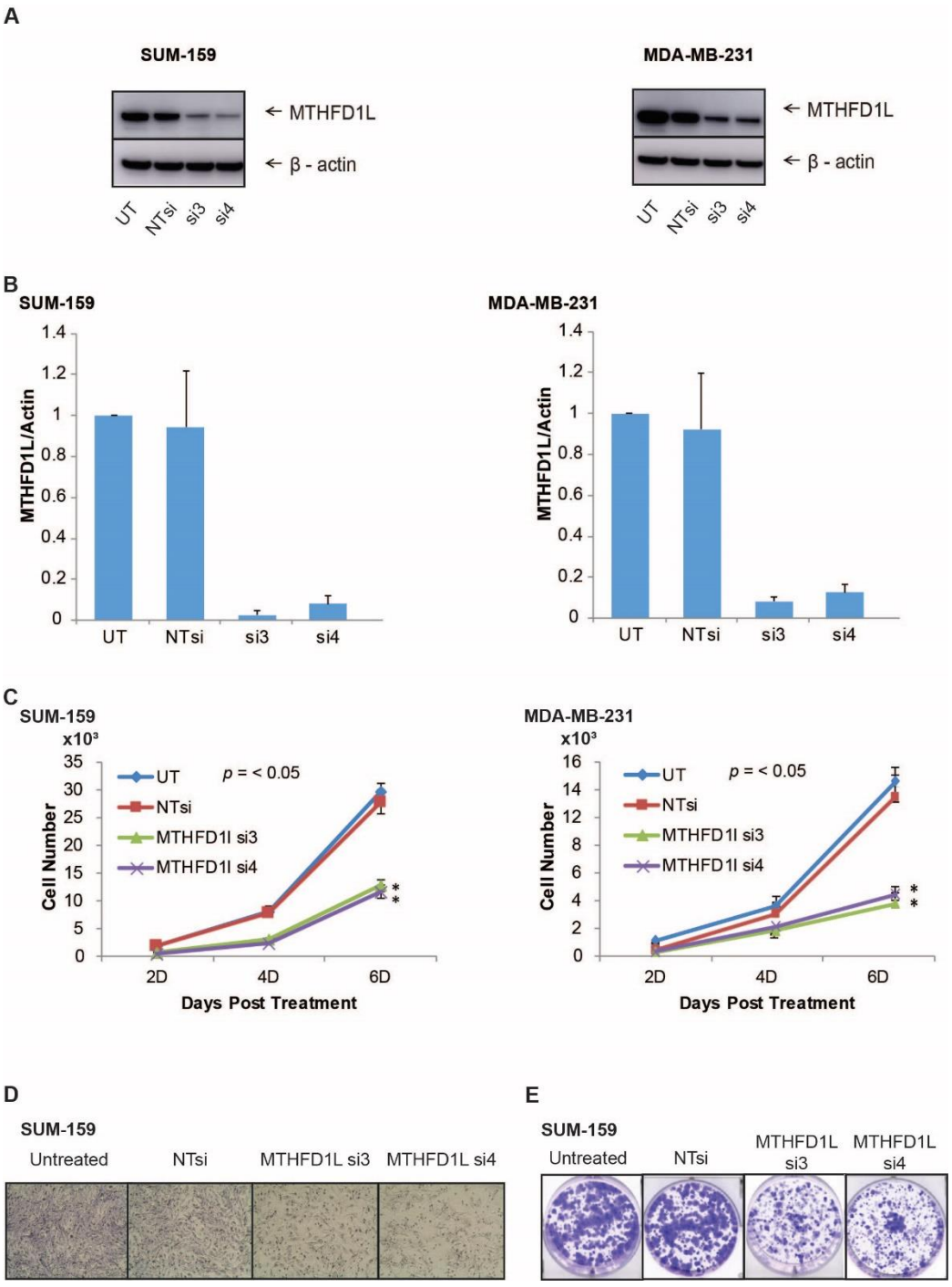

Figure 2. MTHFD1L significantly affects cell proliferation and invasion of TNBC. (A) Immunoblot analysis of protein lysates from SUM-159 and MDA-MB-231 cells treated with independent MTHFD1L siRNA duplexes. β-actin serves as a loading control, (B) Verification of transient MTHFD1L transient knockdown compared to non-targeting siRNA (NTsi), (C) Cell proliferation assay of MTHFD1L siRNA-treated SUM-159 and MDA-MB-231 compared to NTsi controls, (D) Boyden chamber Matrigel invasion assay of MTHFD1L siRNA treated SUM-159 compared to NTsi. Invaded cells were stained and imaged, (E) Colony formation assay MTHFD1L siRNA treated SUM-159 compared to NTsi.

DISCUSSION

Triple-negative breast cancer has proven to be difficult to treat and has a higher probability of recurrence resulting in mortality [21, 33]. Research is constantly striving to find means of improved diagnosis and more targeted therapies. As triple-negative breast cancer lacks the more druggable proteins, progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2) receptor, the discovery of alternative druggable targets is highly necessary [2, 3, 34-37]. It has been shown that cancer cells are characterized by dysregulated cellular energetics and targeting these pathways opens up new possibilities for novel treatments [27, 38-40]. Increased proliferation creates an increased demand for molecules, such as nucleotides and amino acids. This increased need for these molecules is met in a number of different ways. *De novo* purine and pyrimidine biosynthesis is closely linked to folate metabolism[29, 30, 38, 41, 42]. Drugs like the early chemotherapeutic, methotrexate, have been developed to inhibit this pathway in cancers through dihydrofolate reductase (DHFR) [43, 44]. This enzyme is responsible for the creation of tetrahydrofolate which plays a role in both the mitochondrial and cytoplasmic portions of folate metabolism. MTHFD1L, among other mitochondrial enzymes, make up ideal targets to aim for reduced detrimental effects via treatment.

In this study, we highlight the potential role of MTHFD1L in TNBC proliferation, invasion, and metastasis. We show significant upregulation of MTHFD1L through *in silico* analysis of publicly available TCGA RNA sequencing data in breast cancer tissues. These findings were validated via immunoblot analysis and qRT-PCR. When evaluating

the importance of MTHFD1L in oncogenic phenotypes, knockdown of MTHFD1L resulted in a significant reduction of cell proliferation, colony formation, and invasion, suggesting a potential role in metastasis. The upregulation of MTHFD1L has been observed in additional cancer types, making it an appealing target for pan-cancer application.

In summary, our studies show that MTHFD1L plays a significant role in breast cancer proliferation and invasion, making it an ideal target for drug therapies. Small molecule inhibition specific to MTHFD1L will be imperative to assess the feasibility of these claims.

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GENERAL DISCUSSION

Cancer is a devastating disease with complex molecular alterations. Both genomic and environmental factors play a role in cancer initiation, progression and metastasis. The recent advances in next generation sequencing and other high throughput technologies have enhanced the pace of cancer biomarker and target discovery. There is a need for concomitant effort to develop new therapies. This involves identifying valid therapeutic targets. The identification of valid therapeutic target increases the potential of precision medicine and targeting. The cancer biomarkers show differing expression between tumor and normal cells. These biomarkers cancer be use as tools for early detection, diagnosis, and prognosis. Tumor biomarkers are divided into several types, namely epigenetic (changes in the DNA methylation profile), genomic (changes in the number of copies, mutations, RNA expression), proteomic (change in protein levels) and metabolic (changes in the spectrum and levels of low molecular weight metabolites). To detect many of these molecular correlates in cancer, one can measure RNA and DNA circulating in blood plasma, proteomic (changes in protein expression profiles and levels), miRNA synthesis profile and levels (microRNAs), circulating tumor cells, protein biomarkers, endothelial cells, stromal cells, immune cells, and exosomal miRNAs [26]. Due to their participation in cellular processes and relative stability, proteins represent the best biomarkers for the diagnosis of lung cancer [16]. Because cancer is a chronically active inflammatory condition, its microenvironment often contains infiltrated proinflammatory cytokines and inflammatory cells. Acute phase reactants (APRP) are produced in response to inflammation [50].

Due to proximity to tumor cells, proteins associated with lung cancer may be present in pleural effusions and may therefore be a useful source of biomarkers [51]. When proteomic pleural and serum effusions were compared, more biomarkers of cancer were found in pleural effusions than in serum [52]. Among the potential biomarkers, pigment epithelium (PEDF) and gelsolin were the most promising. PEDF is involved in the inhibition of angiogenesis and gelsolin is a protein that inhibits metalloproteinase 2. Gelsolin may also be involved in cancer invasion and is involved in the disorganization of the lung parenchyma. Niemann-Pick disease type C2 protein (NPC2) is another biomarker found in pleural effusions. NPC2 is a protein most likely involved in the regulation of cholesterol transport [53].

Prolyl 4-hydroxylase subunit alpha-1 (P4HA1) is a potential prognostic and diagnostic biomarker for lung cancer [54]. P4HA1 is also known as procollagen-proline 2-oxoglutarate 4-dioxygenase alpha 1. P4HA1 is a member of the family of tetrameric α-ketoglutarate enzymes dependent on dioxygenase. P4HA1 is a key enzyme in collagen synthesis and consists of two identical beta subunits and two alpha subunits [55]. P4HA1 catalyzes the incorporation of oxygen into organic substrates. In various protein substrates, P4HA1 catalyzes the 4-hydroxylation of proline in -X-Pro-Gly-motifs [55]. Collagen is a more well-known protein substrate. Of essential importance is the modification of P4HA1 proline to 4-hydroxyproline, for proper three-dimensional bending of newly synthesized procollagen chains. Other potential substrates for P4HA1 are elastin, Argonaute 2, C1q, and prion protein, which means P4HA1 can play many important roles in various biological functions. Increased regulation of P4HA1 has been reported in many cancers [56]. In lung cancer, high P4HA1 mRNA expression has a poor prognosis [57]. The study that conducted Li et al. showed that high levels of P4HA1 were

important for the clinical characteristics of the patients and were regulated in the tumor tissue. Adverse clinical outcomes were seen in patients who had elevated P4HA1 levels. High P4HA1 expression is an independent prognostic factor that is poor for relapse-free survival and overall survival in lung cancer patients. This study showed that high levels of P4HA1 could be used as a prognostic and early diagnostic biomarker for patients with lung cancer [58].

In the work presented in this dissertation, P4HA1 and MTHFD1L have been highlighted to show their feasibility as targets for cancer therapeutics. Novel therapeutics will always have advantages and shortcomings, with the desire to effectively treat cancer patients while reducing off-target effects. More and more methods are being geared towards not only downstream proteins directly, but also regulators of DNA and RNA that can play more of an initiating role. With improvements in sequencing and analysis, more personalized treatments are being developed. As highlighted here, there is likely to be overlap in ideal targets for treatment between cancer types, but with the inherent heterogeneity of the disease, there will continue to be a need for more efficient and more precise targeting of therapeutic molecules.

Here we've made an argument for P4HA1 as a promising therapeutic target. Prolyl-4-hydroxylase, being as versatile as it is, can potentially offer different routes to convey therapeutic effects. We instigated therapeutic intervention via small molecule inhibition through Pythi-DC treatment, as well as, miR modulation. This resulted in demonstrated significant upregulation of P4HA1 in lung adenocarcinoma and, as well as, correlation with patient survival. We've highlighted P4HA1, but there is reason to study the other subunits as well, in the context of additional cancers. P4HA3, though it didn't correlate significantly with lung adenocarcinoma patients, could play a much bigger role in other cancer types.

MTHFD1L being a part of the nucleotide biosynthetic pathway is also a promising target. There are decades of research identifying new antimetabolites and they've been able to have accurate drug targeting with better tolerance in patients, compared to traditional chemotherapy. Being highly dependent on energy production, disruption of cellular energetics can render cancer cells highly sensitive to dell death. We've shown that a significant upregulation of MTHFD1L is seen in breast cancer patient with a correlation with decreased patient survival. Discovery of a small molecule inhibitor specific to MTHFD1L will be ideal to narrow down its feasibility for drug therapy.

Personalized cancer therapy or precision medicine involves adapting antitumor treatments to individual clinical characteristics, associated patient microcirculation, and molecular tumor profiles, in order to more effectively treat cancer with as little toxicity as possible [59]. Although in the last decade, immunotherapeutic and molecularly targeted agents have been revolutionary, only a small number of patients respond to these therapies. However, even those patients who respond to these therapies, after some time acquire resistance to drugs used in therapy and develop progressive disease. Therefore, there is a great need to detect biomarkers that can predict resistance and sensitivity to therapies [60]. Serum peptide profiling in patients treated with cisplatin-gemcitabine in combination with the proteasome inhibitor bortezomib revealed a 13-peptide signature that distinguishes patients with short versus long-term progression-free survival (PFS). This 13-peptide signature makes a distinction with great specificity, accuracy, and sensitivity [61]. Patients with a partial response versus those who do not respond may be separated by a 5-peptide signature. The long duration of PFS was closely related to tumor response to treatment. This suggests that relative to the outcome of therapy, the signature

of survival has a greater power of prediction. It is assumed that expressed peptides, whose various activities contribute to the production of serum peptides specific for lung cancer, are generated from common serum proteins that accompany cleavage by certain exopeptidases. These blood proteins are a source of surrogate biomarkers, because these proteins are only the basis for real biomarkers or proteases [62].

Advances in elucidating the molecular biology of lung cancer are of great importance for the clinical treatment of the patient NSCLC, because this progress has led to the identification of a large number of potential markers [41]. Potential markers that may be helpful in the treatment of lung cancer are the COMMD4 (COMM domain-containing protein 4) gene [63], EGFR [64], ROS1 (ROS proto-oncogene 1, tyrosine kinase receptor) [65], HER2 [66], NTRK1 (neurotrophic receptor tyrosine kinase 1), FGFR [67], ALK), KRAS, MET proto-oncogene, RET proto-oncogene, BRAF (B-RAF proto-oncogene, serine / threonine kinase) [41], PD-L1 (programmed death-ligand 1) [38], etc. Several key advantages over the all-comers approach, it offers a biomarker-guided treatment approach. An approach guided by biomarkers is now considered essential for the production of drugs to treat cancer. To increase the likelihood of a successful reading, biomarkers can be used to select patients to participate in clinical trials. The three times higher rate of approval for participation in clinical trials was in the application for drugs with biomarkers compared to drugs without biomarkers in the period from 2006 to 2015. Regulators can provide accelerated approval of drugs that use biomarker testing as part of their clinical development programs, depending on stage data and medical needs [38].

In summary, to reduce mortality in this disease, it is necessary to understand the mechanisms of tumor development, but also to investigate specific biomarkers that would

lead to improved treatment and earlier diagnosis. In order to achieve the lowest possible toxicity, and the best possible response to treatment, it is necessary to adapt individual anticancer treatments. Because the response to therapy occurs only in a small number of patients, and even when the response does occur, resistance to therapy usually develops after some time, it is necessary to detect biomarkers that will show sensitivity and resistance to therapy. Molecular abnormalities that have been identified in recent years have led to the development of individual therapies and provided new options to patients. Predictive biomarkers have led to a better diagnosis of lung cancer. The concerted efforts of the cancer biology field continue to highlight new avenues to explore. The push for precision medicine creates a demand for more accessible data analysis for target identification and being able to tease apart how these different pathways interact with one another.

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