Type Ⅱ Interleukin-4 Receptor Activation in Basal Breast Cancer Cells Promotes Tumor Progression

By

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List of Abbreviations

2-deoxy-D-glucose	2DG
6-phosphogluconate dehydrogenase	PGD
Adenosine triphosphate	ATP
ATP citrate-lyase	ACLY
Epithelial to mesenchymal transition	EMT
Estrogen receptor	ER
Gamma-aminobutyric acid	GABA
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Glycogen synthase kinase	GSK-3β
Guanosine 5’-monophosphate synthase	GMPS
Histone acetyltransferase	HAT
Histone deacetylase 3	HDAC3
Human epidermal growth factor receptor 2	HER2
Insulin receptor substrate	IRS
Interleukin 4	IL4
Mitogen-activated protein kinase	MAPK
Phosphohexose isomerase	PHI
Progesterone receptor	PR
Prolyl-hydroxylase	PDH
Pyruvate kinase M2	PKM2
Succinate dehydrogenase 5	SDH5
Succinate dehydrogenase subunit b	SDHB
Triple-negative breast cancer	TNBC

Publications

1. Demond Williams and Barbara Fingleton, “Non-Canonical Roles for Metabolic Enzymes and Intermediates in Malignant Progression and Metastasis,” *Clinical & Experimental Metastasis* 36, no. 3 (June 2019): 211–24, https://doi.org/10.1007/s10585-019-09967-0.

2. Demond Williams and Barbara Fingleton, “Measurement of Metabolites from Migrating Cells,” *Methods in Molecular Biology (Clifton, N.J.)* 2294 (2021): 143–50, https://doi.org/10.1007/978-1-0716-1350-4_10.

3. Demond Williams et al., “Type II Interleukin-4 Receptor Activation in Basal Breast Cancer Cells Promotes Tumor Progression via Metabolic and Epigenetic Modulation,” *International Journal of Molecular Sciences* 25, no. 9 (April 24, 2024): 4647, https://doi.org/10.3390/ijms25094647.

4. Bindeman W*, Williams D*, Kassel S, & Fingleton B. Dominant-negative IL13 attenuates metastatic colonization in triple negative breast cancer *Manuscript In Preparation* *indicates equal contribution

Chapter Ⅰ: Introduction

This dissertation is focused on identifying mechanisms by which expression of the type Ⅱ interleukin 4 (IL4) receptor can contribute to breast cancer progression, and whether it can be targeted to improve outcomes in breast cancer. In chapter 2 I will discuss my work linking the type Ⅱ receptor to metabolic changes in breast cancer cells that lead to altered gene expression and more aggressive behavior. In chapter 3, I will further explore links between IL4 receptor activation and metabolic changes. In chapter 4, I will present some initial work on characterizing and testing the efficacy of an inhibitor of the type Ⅱ IL4 receptor. Finally in chapter 6, I will present an overall discussion of my work and some ideas for future directions. To provide context for the studies to be presented in chapters 2, 3, and 4, this introductory chapter will focus on 3 main topics: (Ⅰ) a general overview of how altered metabolic pathways can impact cancer metastasis (reproduced from a published review article); (Ⅱ) a brief introduction to breast cancer specifically; and (Ⅲ) an introduction to the IL4 receptor and its activity, expression and potential as a therapeutic target.

(Ⅰ) Expanded Roles for Metabolic Pathways in Metastasis –

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Introduction

Despite recent exciting developments in cancer treatments, it is evident that metastatic disease is still a fundamental barrier to improved outcomes for the majority of patients. Indeed, metastatic disease is the main cause of cancer deaths. Metastatic progression is a multi-step process, that recent work suggest may actually begin early in cancer development, although this is still being investigated(1). In order to establish metastatic colonies, cancer cells must alter themselves in a number of ways, including acquisition of a motile phenotype; transition from an epithelial to mesenchymal phenotype; acquisition of ability to enter, survive in, and exit the vasculature; and

development of mechanisms to enable survival and ultimately outgrowth at distant sites. Cellular metabolism underlies many of those alterations and dysregulation of energy metabolism has been well established as a hallmark of cancer biology(2). For example, the Warburg-effect, or the observation that highly proliferative cells such as tumor cells are largely dependent on glycolysis to meet their energetic needs even in the presence of oxygen, is frequently associated with cancer development. Outside of the bioenergetic consequences of altered metabolism however, recent research has established non-canonical functions of metabolic enzymes and metabolites that contribute to cancer progression. Understanding these novel pathways could reveal innovative ways to specifically target the metastatic process. Here we describe evidence for novel mechanisms that link metabolic alterations in cancer and metastatic progression and highlight some potential therapeutic strategies that can arise as a result.

Atypical Intracellular Roles of Metabolic Enzymes in Metastasis

Metabolic alterations observed in cancer are often accompanied by dysregulation of the expression of metabolic enzymes. Secondary functions of these metabolic enzymes have been shown to contribute to metastatic progression through altering the signaling and genetic landscape of cancer cells. Glycolysis is a basic process that links glucose uptake with the initial steps of energy production as well as biosynthesis. However, several glycolytic enzymes also have a number of effects outside of their classical enzymatic activity. For example, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can form a complex with the transcription factor Sp1 that binds to the SNAIL minimal promoter to drive its expression(3). The expression of SNAIL contributes to metastasis through induction of epithelial to mesenchymal transition (EMT) and a more stem-like phenotype. EMT is an important process in metastasis that can cause epithelial cells to lose E-cadherin mediated cell-cell adhesion and gain an invasive phenotype allowing them to move away from the primary tumor into surrounding stroma and potentially into the vasculature. Suppression of GAPDH resulted in loss of stem cell markers that was linked to decreased tumor-forming ability in a colorectal cancer

model(3). Another example is Pyruvate kinase M2 (PKM2), which is an isoform of the enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate. This isoform is frequently upregulated in cancers and has many pro-tumorigenic roles(4). One way that PKM2 can contribute to metastatic progression is through EMT. PKM2 is able to translocate to the nucleus and form complexes with TGIF2, a repressor of Transforming Growth Factor (TGF)-β signaling, and histone deacetylase 3 (HDAC3)(5). The PKM2 nuclear complex was shown to bind to the CDH1 promoter and deacetylate it leading to repression of E-cadherin expression.

Although not strictly acting non-canonically, an atypical role of glycolysis important for invasive phenotypes relates to the finding of glycolytic enzymes in abundance in invadopodia(6). Glycolysis appears to be the primary energetic pathway for cytoskeleton remodeling in several breast and prostate cancer models(7). When glycolysis was inhibited through treatment with 2-deoxy-D-glucose, there was a decrease in focal adhesions and motility in PC3 prostate cancer cells. In contrast, inhibition of oxidative phosphorylation with oligomycin had no effect on the motility of prostate cancer cell lines. In addition, the association of glycolytic enzymes with the cytoskeleton is important for the viability of cancer cells(8–10). Treatment of Lewis lung carcinoma, colon carcinoma, or breast cancer cells with clotrimazole, a calmodulin antagonist, resulted in decreased cell viability(9). The decrease in cell viability was preceded by dissociation of glycolytic enzymes from the cytoskeleton, resulting in a reduction of local adenosine triphosphate (ATP) supply to the cytoskeleton and subsequent altered morphology. Citric acid cycle enzymes are also associated with increased metastasis. Enhanced expression of ATP citrate lyase, which catalyzes the conversion of citrate to acetyl-CoA and oxaloacetate, is linked to increased lipogenesis. Normally, the majority of lipids used for cellular functions including lipid membranes are obtained from the diet or produced in the liver. The biosynthesis requirements of rapidly proliferating cancer cells can result in alternative mechanisms including generation of acetyl-CoA through the activity of ATP citrate lyase. The acetyl-CoA is then a substrate for fatty acid synthase, ultimately leading to membrane lipid production. Inhibition of fatty acid synthase and ATP citrate lyase slowed tumor growth and inhibited metastasis in non-small cell lung cancer, cervical cancer, and prostate cancer(11–13). Concordantly,

expression of microRNA 22 (miR-22), which inhibits ATP citrate lyase expression, is downregulated in a number of cancers(14,15). The ectopic expression of miR-22 was shown to decrease *de novo* lipogenesis and metastatic ability in breast, lung, osteosarcoma, cervical, and prostate cancer(14). ATP citrate lyase is a prospective therapeutic target and there are several novel inhibitors under investigation(12,16).

Dysregulation of succinate dehydrogenase activity, which normally catalyzes the conversion of succinate to fumarate, is associated with a number of cancers including pheochromocytoma, renal cell carcinoma, and paragangliomas(17–19). Decreased succinate dehydrogenase activity leads to accumulation of succinate which inhibits prolyl-hydroxylase (PDH)(20). This inhibition of PDH stabilizes hypoxia-inducible factor 1-alpha (HIF-1α), thus activating pro-angiogenic HIF-1α signaling. Additionally, succinate dehydrogenase 5 (SDH5) has been shown to regulate glycogen synthase kinase (GSK)-3β signaling in lung cancer(21). SDH5 forms complexes with GSK-3β, and PP2A, a phosphatase that regulates activity of GSK- 3β. Loss of SDH5 results in increased β-catenin signaling and subsequent EMT in lung cancer. Evidence also suggests that genetic ablation of succinate dehydrogenase subunit b (SDHB), increases TGF-β signaling and activates a complex of the transcription factors SNAIL and SMAD3/4 leading to a metastatic phenotype in colorectal cancer cell lines(22). Indeed, lack of SDHB expression is associated with invasive and metastatic disease in colorectal patient samples.

Glutamine addiction is another emerging metabolic hallmark of cancer cells(23). The glutamine hydrolyzing enzyme, glutaminase has multiple isoforms that have differing effects on disease progression in cancer (Fig 1.1). Increased expression of glutaminase 1 in triple- negative breast cancer is associated with poor disease-free survival, and decreased tumor infiltrating leukocytes(24). The enhanced uptake and

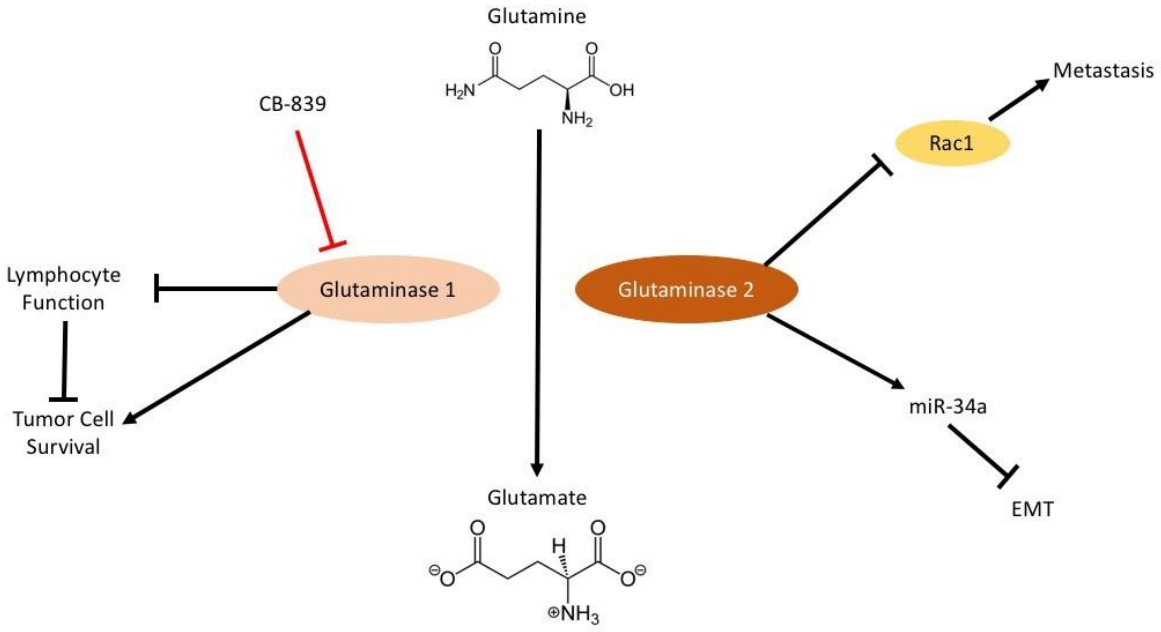


Figure 1.1: Isoforms of glutaminase have opposing roles in cancer metastasis. Glutaminase 1 increases tumor cell survival via its canonical catalytic activity. Opposingly glutaminase 2 inhibits tumor metastasis and EMT via its secondary functions as a binding protein (Details in text). EMT (Epithelial-Mesenchymal Transition); Rac1 (Ras-related C3 botulinum toxin substrate 1)

utilization of glutamine by the tumor cells results in decreased availability of this carbon source in the tumor microenvironment. The lack of environmental glutamine, which is important for lymphocyte function(25,26), may explain the decrease of tumor infiltrating lymphocytes and poor prognosis associated with glutaminase expression in triple negative breast cancer. Glutaminase 2, the liver isoform of glutaminase, appears to have an opposing role to glutaminase 1, as it is able to inhibit metastasis through protein binding instead of its classical catalytic functions. Glutaminase 2 was shown to bind the small GTPase Rac1, a pleiotropic regulator of multiple cellular processes(27). The binding of Rac1 by glutaminase 2 blocks interactions with guanine exchange factors resulting in Rac1 inhibition. Glutaminase 2 is also known to stabilize Dicer, which results in the maturation of miR- 34a(28). MiR-34a can repress the EMT transcription factor SNAIL and inhibit metastasis in hepatocellular carcinoma.

Enzymes associated with nucleotide metabolism are also able to affect metastatic progression. Guanosine 5'-monophosphate synthase (GMPS) was shown to regulate p53 function through altering deubiquitylation complex(29). GMPS is an enzyme normally involved in de novo purine biosynthesis and is usually sequestered in the cytosol by TRIM21. A complex between USP7, MDM2, and p53 is formed in the nucleus that results in the ubiquitylation and degradation of p53. However, upon genotoxic stress GMPS is imported into the nucleus. When in the nucleus GMPS replaces MDM2 in the complex, and induces USP7 mediated deubiquitylation and stabilization of p53, resulting in increased transcription of p53 target genes. Loss of normal p53 function has been associated with metastasis(30). Understanding how to target enzymes such as TRIM21 to promote this secondary function of GMPS and thus induce p53 activity has potential as a therapy for metastasis.

Extracellular Roles of Metabolic Enzymes in Metastasis

A number of metabolic enzymes can actually be secreted and drive cancer progression through alternative roles as signaling molecules. The most studied example of a secreted metabolic enzyme acting as a signaling molecule is phosphohexose isomerase (PHI), also known as autocrine motility factor (AMF), neuroleukin, or

maturation factor. The canonical role of PHI is early in glycolysis where it catalyzes the conversion of glucose-5-phosphate to fructose-6-phosphate. The expression of PHI is under the control of HIF-1α, and phosphoinositide 3-kinase (PI3K)(31,32). When secreted from cells, PHI exhibits functions outside of its normal enzymatic role by binding and signaling through its cognate receptor gp78(33) (Fig 1.2). PHI expression enhances metastasis in pancreatic and colorectal cancer(34,35). One of the ways that PHI contributes to metastasis is through induction of EMT. Ectopic expression of PHI is sufficient to drive EMT in a number of cancers including breast cancer(36) and endometrial cancer via mitogen-activated protein kinase (MAPK) signaling(37). PHI can also signal through NF-κB resulting in increased expression of the mesenchymal transcription factors ZEB1, and ZEB2 in addition to decreasing expression of miR-200, a microRNA that represses expression of ZEB1 and ZEB2(38).

Expression of PHI was shown to increase motility of cancer cells by regulating expression of microtubule associated proteins such as kinesin-like protein KIF3A(39). PHI also plays a role in cytoskeletal dynamics by modulating expression of Rho GTPases and Rac1(40). Expression of PHI in melanoma cells leads to the formation of stress fibers that are importance for cell migration. Additionally, PHI can increase the invasiveness of hepatoma cells by increasing expression of integrin β1, which is important for cellular adhesion to extracellular matrix, and secretion of MMP2, which plays a role in degrading the surrounding extracellular matrix and allowing tumor cells to invade(41). In all these cases, PHI is thought to act in an autocrine manner, binding to the same cells from which it is secreted. Outside of intrinsic cancer cell signaling PHI has also demonstrated paracrine activity. Expression of the PHI receptor, gp78, was reported in normal endothelial cells(42). PHI secreted by tumor cells signaled in an autocrine manner to increase expression of vascular endothelial growth factor (VEGF) in cancer cells. Simultaneously, PHI acted in a paracrine manner on endothelial cells in order to increase expression of VEGF receptor FLT-1 and endothelial cell motility. Together these events increased the permeability of endothelial vessels and contributed to formation of ascites in a mouse model(43).

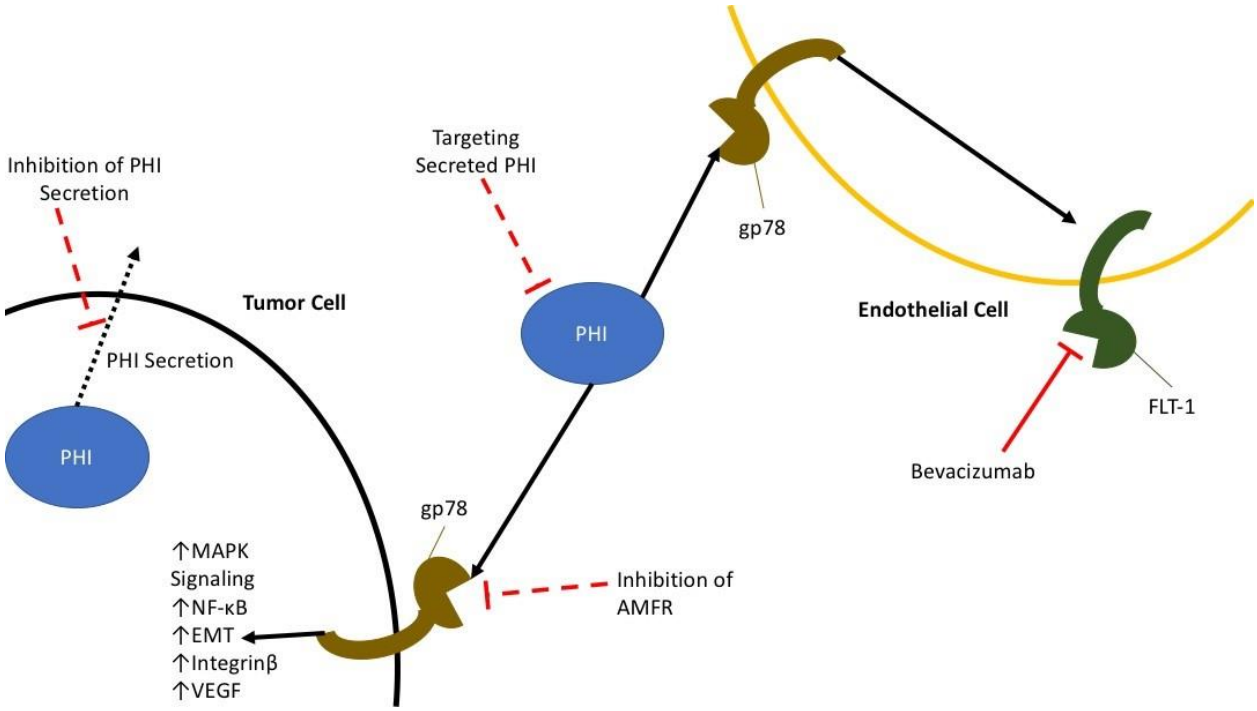


Figure 1.2: Secreted PHI has autocrine and paracrine signaling roles. PHI drives pro-tumorigenic MAPK signaling, EMT, and secretion of angiogenic factors. PHI sensitizes endothelial cells to angiogenic signaling by increasing expression of VEGF receptors. PHI (Phosphohexose Isomerase); MAPK (Mitogen-activated protein kinase); EMT (Epithelial-Mesenchymal Transition); AMFR (Autocrine motility factor receptor); VEGF (Vascular Endothelial Growth Factor)

A number of other metabolic enzymes may contribute to cancer progression upon their secretion. Secreted phospholipase A2 (sPLA2) has been shown to have differing roles dependent on its localization. When expressed intracellularly, sPLA2 can inhibit Wnt signaling through activation of Yap in intestinal tissue(44). Upon inflammation however sPLA2 is secreted into the lumen where it increases Wnt signaling, and prostaglandin E2 synthesis via the sPLA2 receptor Plar2r1 which is associated with increased susceptibility to colon cancer(44). Increased Wnt signaling is also associated with metastasis, and EMT in cancer(45). In addition, sPLA2 has been shown to confer protection against lipotoxic stress, and nutrient deprivation in breast cancer cell lines(46). Peroxredoxin 4 (PRDX4), the only secreted member of a family of peroxidase enzymes, was shown to induce osteoclastogenesis in a RANKL independent manner(47). The secretion of PRDX4 led to increased ERK, and calcium/NFATc1 signaling which is mediated by the IgG like receptors OSCAR and TREM-2. Genetic ablation of PRDX4 expression led to decreased oseteoclastogenesis *in vitro*, and decreased osteolytic lesions in mice in the setting of breast-prostate-to-bone metastasis. Changes in metabolism commonly observed in cancer often result in the accumulation of metabolites. These metabolites can act as intracellular or extracellular signaling molecules that have multiple effects, which are sometimes contrary to each other. For example, incubation of metastatic prostate cancer cell lines with citrate has been shown to enhance motility and invasion, as well as inhibit cell adhesion(11). Moreover, in lung adenocarcinoma and squamous cell lung cancer, expression of SLC25A1, a transporter responsible for transporting citrate out of the mitochondria into the cytosol, could drive cancer cells to a stem-cell like phenotype, and increase colony formation(48). In contrast, there is some evidence that treatment with citrate can slow tumor growth in a number of tumor models including breast, lung and pancreatic cancer(49). The treatment with citrate was shown to inhibit glycolysis, and insulin-like growth factor 1 receptor phosphorylation. This corresponds with evidence of decreased citrate levels being a biomarkers in prostate cancer(50).

Glutamine is imported into cells through various transporters, including ASCT2 (also known as SLC1A5) (51) (Fig 1.3). Blocking glutamine uptake by ablation of ASCT2 expression causes decreased proliferation, and activation of mTORC1 signaling in

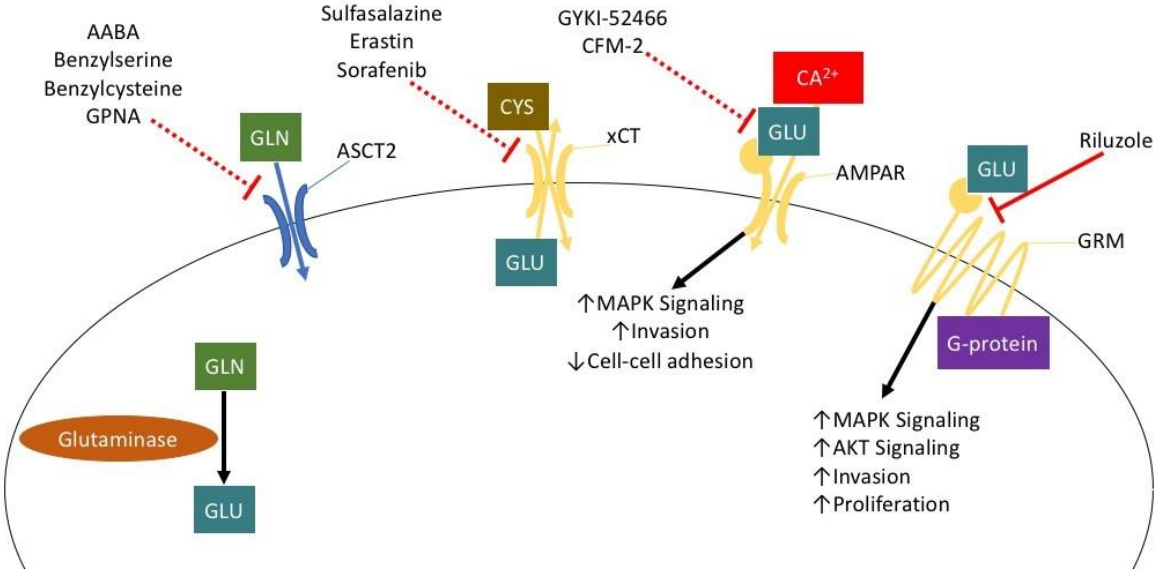


Figure 1.3: Glutamate is a pro-metastatic signaling molecule. Glutamate produced by the hydrolyzation of glutamine can be exported from tumor cells via transporters like xCT. This secreted glutamate may drive pro-tumorigenic signaling by binding to ionotropic and metabotropic glutamate receptors AMPAR, and GRM. GRM (Metabotropic Glutamate Receptor); AMPAR (α-amino-3-hydroxy-5methyl-4isoxazolepropionic acid receptor); GLN (Glutamine); GLU (Glutamate); MAPK (Mitogenactivated protein kinase); AKT (Protein kinase B)

prostate cancer cells(52), as well as decreased migration in osteosarcoma, and triplenegative breast cancer(53). Pharmacological blockade of ASCT2 mediated glutamine uptake by GPNA and benzylserine was shown to have anti-tumor effects in endometrial carcinoma(54). A novel class of 2-amino-4-bis(aryloxybenzyl)amino butanoic acid (AABA) derived drugs designed to target ASCT2, such as V-9302, have been demonstrated to decrease proliferation, and increase cell death, and oxidative stress(55). Recent evidence however, suggests that V-9302 may instead block glutamine uptake mediated by redundant glutamine transporters that show increased expression in some cancers such as SNAT2(56). In melanoma cells, glutamine can inhibit platelet-activating factor-induced MAPK signaling(57), resulting in decreased metastasis and angiogenesis downstream of platelet-activating factor signaling.

The first step in the catabolism of glutamine is the conversion from glutamine to glutamate. Glutamate can also play an important role in regulating the metastasis of cancer by acting as a signaling molecule (Fig 1.3). Disruption of the glutamate-cysteine antiporter xCT (also known as SLC7A11) leading to the retention of cellular glutamate and reduction of cysteine consumption, results in decreased proliferation, and decreased invasion in non-small cell lung cancer(58). In addition, inhibition of xCT leads to decreased viability in glucose deprived states(59). When xCT is functional and glutamate is exported, glutamate can signal through multiple types of receptors. The first class of glutamate receptors are metabotropic glutamate receptors(60), which are G protein-coupled receptors that are able to activate multiple pro-tumorigenic signaling pathways such as MAPK and AKT signaling. Genetic manipulation of metabotropic glutamate receptor 1 (GRM1) to reduce its expression led to decreased proliferation of ER positive breast cancer cells(61). Treatment of the oral cancer cell line B88-SDF-1 with an antagonist of metabotropic glutamate receptor 5 resulted in decreased metastasis and invasion *in vivo* and *in vitro*, respectively. Another class of glutamate receptor is the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are ionotropic receptors that act as ion channels. Activation of AMPA receptor was shown to drive invasion in pancreatic cancer cells via MAPK signaling(62). Knockdown of the glutamate receptor AMPA4 reduced expression of genes associated with adhesion, and invasion(63).

Another important metabolic fate of glutamate is its conversion to gammaaminobutyric acid (GABA) by glutamate decarboxylase. Expression of glutamate decarboxylase 1 increased the ability of breast cancer metastases to utilize glutamine(64). GABAergic signaling in breast cancer was also found to increase migration and invasion in breast cancer(65). The increase in migration, and invasion was mediated through ERK1/2 signaling. GABA receptor activation also affected the survival of the chondrosarcoma cell line OUMS-27(66). When the cells were exposed to the GABA antagonist CGP, the activities of apoptotic proteins caspase 3 and caspase 9 were elevated.

Along with alterations in cancer cell metabolism, perturbations in the metabolism of tumor stroma are emerging as a key driver of metastatic progression. The interactions between cancer-associated fibroblasts, and tumor cells is described as a 'reverse Warburg effect' where cancer cells induce metabolic reprogramming of fibroblasts leading to increased aerobic glycolysis(67), as well as increased expression of monocarboxylate transporter-4 (MCT4) (68)resulting in release of lactate into the tumor microenvironment. This is correlated with upregulated expression of monocarboxylate transporter-1 (MCT1) mediated lactate uptake in cancer cells which has been shown to contribute to survival and growth(68), as well as tumor migration(69). The role of secreted lactate in disrupting innate and adaptive immune responses has been comprehensively reviewed elsewhere(70). Novel roles of lactate in modulating immune response are also constantly emerging such as its ability to activate NF-κB in $CD4^+$ T-cells and drive their polarization to the immunosuppressive T_{reg} subtype driving prostate carcinoma progression(71). Metabolic symbiosis has been shown to work both ways with fibroblasts reprogramming cancer cell metabolism to increase glycolytic metabolism and secretion of lactate to support metastasis(72) as well drive secretion of hepatocyte growth factor (HGF) from fibroblasts inducing resistance to tyrosine kinase inhibitor therapy(73).

Metabolism and Epigenetic Regulation in Metastasis

An emerging area of research in cancer is alterations in epigenetic activity that are controlled by metabolic changes(74,75). A striking example is the association of

epigenomic reprogramming and metabolism with distance metastases in pancreatic cancer(76). The development of distant metastases was associated with global epigenetic changes including increases in histone acetylation, and decreased histone methylation. Metastatic lesions with these epigenetic changes frequently exhibited increased oxidative pentose phosphate pathway activity driven by overexpression of 6phosphogluconate dehydrogenase (PGD). Inhibition of PGD in distant metastases reversed the epigenetic changes, indicating that increased oxidative pentose phosphate pathway activity is essential for disease progression in pancreatic cancer. Further work from the same authors showed that the metastatic capable cells evolved a pentose conversion pathway to provide substrate for PGD thus maintaining its hyperactivity(77). This conversion pathway is distinct from the rate-limiting pentose phosphate pathway and is evidence of a novel metabolic program that appears to especially promote the metastatic phenotype via regulating the epigenome.

Altered metabolism can contribute to changes in the epigenomic state of cancer cells by providing cells with substrates for epigenetic enzymes (Fig 1.4). ATP citratelyase (ACLY), which catalyzes the conversion of citrate to acetyl-CoA has been identified as an important enzyme for producing the nuclear pools of acetyl-CoA used by enzymes that control histone acetylation such as histone acetyltransferases(78). Such a pathway has been suggested as necessary for polarization of macrophages to an ‘M2’ or alternatively activated tumor-promoting phenotype(79), although there is some question as to whether this is relevant in human macrophages(80). Alternatively, activated macrophages are clearly associated with tumor progression and metastasis(81,82), however it is not yet clear if those dependent on ACLY activity are a true metastasis-promoting subtype(83). In hepatocellular carcinoma, acetyl-CoA increases and associated histone acetylation were demonstrated to be downstream of Acyl-CoA thioesterase 12 (ACOT12) activity(84). This increased histone acetylation was shown to drive expression of the transcription factor Twist2 which induced EMT.

Metabolites may also act as competitive inhibitors of epigenetic enzyme activity. Dysregulation of the citric acid cycle in cancer has been associated with accumulation of a number of metabolites that can affect methylation of the epigenome, such as the

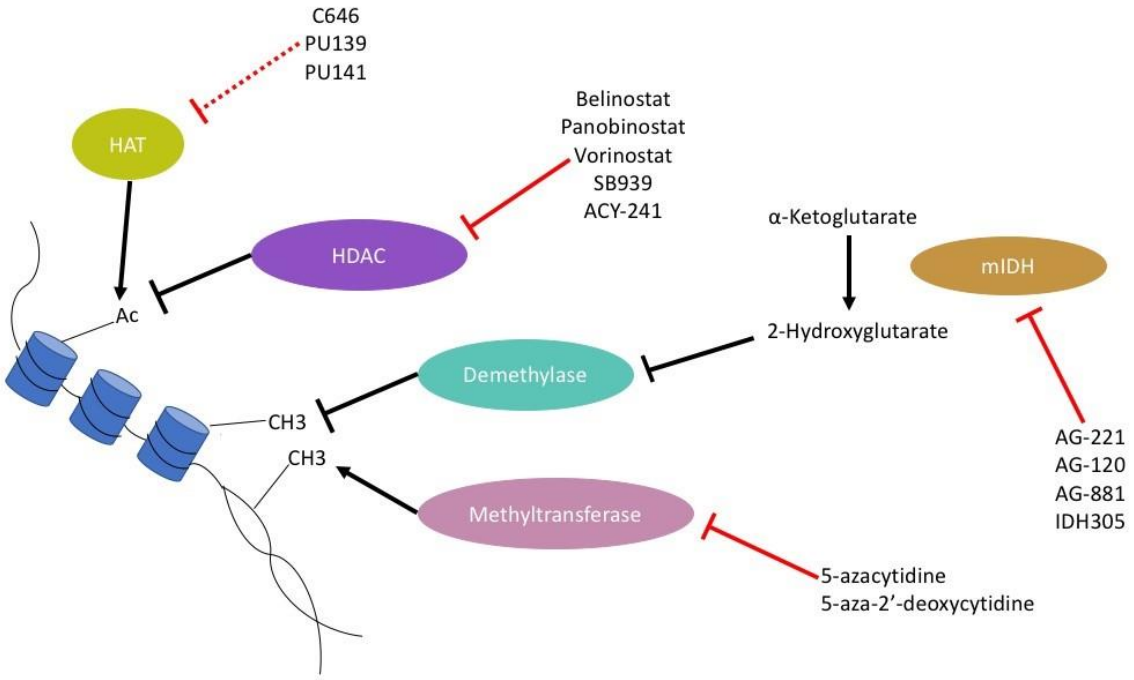


Figure 1.4: Altered metabolism impacts regulation of cancer epigenome via production of substrates and allosteric regulators of epigenetic enzymes. IDH mutations can produce 2-hydroxyglutarate which alter the function of demethylase enzymes. In addition, altered metabolism has been linked to changes in production of Acetyl-CoA the substrate of histone acetyltransferases. Also shown in this figure are agents or drugs that can block the various activities, including the HAT inhibitor C646 that will be used in later experiments. HAT (Histone Acetyltransferase); HDAC (Histone Deacetylase); mIDH (mutant IDH); Ac (Acetylation)

oncometabolite 2-hydroxyglutarate. During normal metabolism, isocitrate is converted to α-ketoglutarate by the enzyme isocitrate dehydrogenase (IDH). Mutations in IDH are common in acute myeloid leukemia where they may play a role in pathogenesis, and in gliomas(85,86). IDH R132, IDH2 r140, and IDH2 R172 mutations result in increased production of 2-hydroxyglutarate, via the action of the mutated enzymes on αketoglutarate(87). 2-hydroxyglutarate can inhibit the function of α-ketoglutarate dependent dioxygenases, such as the demethylase KDM4C(88), and dysregulate the methylation status of cancer cells(89). Mutant IDH was also shown to lower expression of ATM and interfere with DNA repair in acute myeloid leukemia(90). IDH mutations that produce 2-hydroxyglutarate can cause EMT in colorectal cancer cells through driving the expression of the transcription factor ZEB1(89,91). Conversely, IDH mutant gliomas can have better prognosis due to promotion of methylation and thereby suppression of invasion-promoting genes such as G0S2(92).

Deficiency and inhibition of succinate dehydrogenase causes hypermethylation in ovarian cancer, pheochromocytomas, and paragangliomas(93,94). Fumarate has also been associated with progression in a number of cancers including renal cell carcinomas, paragangliomas, and nasopharyngeal cancers(95,96). Loss of fumarate hydratase expression stabilized HIF-1α and HIF-2α, leading to EMT and upregulation of an anti-oxidant response in renal cancer(97,98). In addition, fumarate can inhibit TET demethylases and cause a hypermethylation phenotype in renal cancer(99). Finally, fumarate may cause senescence through oxidative stress(100).

Implications and Future Directions

Altered metabolism is well established as a hallmark of cancer biology(2) and associated with multiple aspects of cancer progression including metastasis(101,102). The clear tumor-promoting roles of dysregulated metabolic pathways have led to a development of a number of therapies(103). However, these therapies present particular challenges in their utilization in the clinic. Most critically, many of the metabolic pathways that may be dysregulated in cancer cells are still used by other cell types, thus identifying a reasonable therapeutic index has proven difficult. For example, inhibition of

the key cancer associated metabolic phenotype aerobic glycolysis, using 2deoxy-D-glucose (2DG) results in toxic effects similar to hypoglycemia(104). However, understanding and targeting non-canonical functions of enzymes related to aerobic glycolysis, such as PHI, may present promising therapeutic strategies that lack the unintended toxicity of targeting ubiquitous metabolic pathways (Table 1.1). On the other hand, there are a number of therapies in development for mutant IDH isoforms, which have the advantage of being distinct from the normal enzyme(105).

The opposing roles of GLS1 and GLS2 in metastasis(24,27,28) highlight the importance of understanding and targeting the isoform specific effects of metabolic enzymes. While pharmacologic inhibition, and genetic ablation of GLS1 slows cancer growth(106) the expression of GLS2 attenuates metastasis. These opposing roles might suggest that specificity of glutaminase inhibitors is critically important. However, the fact that the metastasis-promoting effects of glutaminase 1 are dependent on its catalytic activity while the metastasis-suppressing effects of glutaminase 2 are catalysisindependent enables possible methods of differential modulation.

The significance of changes in metabolism is not just limited to cancer cells, but also extends to the tumor stroma. As illustrated by interactions between cancerassociated fibroblasts and tumor cells, altered metabolism in the stroma can directly support metastatic progression by supplying tumors with high energy metabolites such as lactate(68,69). In addition, lactate has been shown to affect the signaling of immune cells and produce an immunosuppressive tumor microenvironment(71). Changes in cancer cell metabolism can also contribute to changes in signaling in the stroma that enhance metastasis, such as PHI paracrine signaling promoting angiogenesis(42). These changes emphasize the importance of understanding changes in metabolism in the context of the tumor microenvironment and not just considering the cancer cells.

Finally changes in metabolism can sensitize cancer cells to other forms of therapy. Mutant IDH has been shown to sensitize glioma cells to inhibition of glutaminase(107). Inhibited glutaminolysis results in decreased accumulation of αketoglutarate, the substrate of mutant IDH and a resultant slowed growth phenotype. In

Table 1.1 **Current and potential therapeutic targets based on non-canonical metabolic roles.**

Target	Agent	Mechanism to Target	References
Glutaminase 1	CB-839		NCT02071927 NCT02071888 NCT02071862
GRM1	Riluzole	Pro-tumorigenic glutamate signaling	NCT00903214 NCT01018836 NCT01303341 NCT00866840
HDAC	Belinostat Panobinostat Vorinostat SB939 ACY-241	Catalytic activity resulting in hypoacetylation	NCT00993642 NCT01075308 NCT02635061 NCT01528501 NCT00274651
mIDH	AG-221 AG-120 AG-881 IDH305	Production of co-metabolite 2hydroxyglutarate	NCT01915498 NCT02073994 NCT02492737 NCT02987010
Methyltransferase	5-azacytidine, 5-aza- 2'deoxycytidine	Catalytic activity resulting in hypermethylation	NCT03019003 NCT03182894 NCT02159820 NCT00084981
ACLY	2,2difluorocitrate Sulfoximine	Catalytic activity producing Acetyl-CoA which induces hyperacetylation	(108,109)
AMF	ERI4P G6P	Pro-metastatic, autocrine and paracrine signaling via gp78.	(110)
SLC1A5 (ASCT2)	AABA Benzylserine Benzylcysteine V-9302	Import of glutamine, which is an important carbon source for a number of pro-metastatic processes.	(54–56,111)
SLC7A11 (xCT)	Sulfasalazine Erastin Sorafenib	Release of glutamate which can act as a pro-metastatic signaling molecule.	(112–114)
AMPAR	GYKI-52466 CFM-2	Glutamate signaling which can drive metastasis.	(115)
		Increased activity resulting in	

HAT	C646 PU139 PU141	hyperacetylation.	(116,117)
sPLA2	Varespladib PLIs	Secretion and binding to receptors that drive Wnt signaling.	(118,119)
GAPDH (Potential)	N/A	GAPDH binding to EMT transcription factor sp1	
PKM2 (Potential)	N/A		
PRDX4 (Potential)	N/A	PKM2 binding to HDAC3 an ɨ TGIF2.	
ACOT12 (Potential)	N/A	Secretion and function as an osteoclastogenic signaling factor.	

addition, gliomas with mutant IDH have been shown to be especially sensitive to treatment with inhibitors of DNA methyltransferases(120) since altered methylation is a key effect of mutant IDH. These examples depict novel targets induced by altered tumor metabolism that can be exploited for treatment.

Conclusion

Dysregulation of metabolism is a hallmark of cancer is commonly associated with metastasis. The increased consumption of carbon sources such as glucose, glutamine, and fatty acids commonly occur in multiple types of cancer leading to enhanced metabolic pathway activation. However, non-canonical functions of these metabolic pathways can influence metastatic progression in ways that diverge from their usual roles in regulating bioenergetics and biosynthesis. Here, we have particularly highlighted the ability of metabolic enzymes such as PHI to alter cancer cell signaling independent of their normal enzymatic functions, as well as the ability of metabolites to act as signaling molecules or change the epigenome of cancer cells. The challenge is to convert this knowledge of novel capabilities to new therapeutic approaches for patients with metastatic disease.

(Ⅱ) Metastatic Breast Cancer

Breast cancer is the most frequently diagnosed cancer type, and the second leading cause of cancer death amongst women(121). Metastatic disease is a particularly important issue in the treatment of breast cancer, as shown by the significant decrease in survival observed in patients diagnosed at later stages(122). Breast cancer is a heterogenous disease that can be divided into several clinically relevant subtypes. An important subtyping of breast cancer is based on expression of the hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), as well as expression of the human epidermal growth factor receptor 2 (HER2). The expression patterns of these various receptors enable breast cancer to be divided into Luminal A (ER positive, PR positive, HER2 negative), Luminal B (ER positive, PR negative, HER2 positive), HER2+ (ER negative, PR negative, HER2 positive), and triple-negative or

basal-like (ER negative, PR negative, HER2 negative). This subtyping of breast cancer guides clinical decisions, and can be used as a prognostic indicator(123). Among these subtypes, triple-negative breast cancer is considered to have the worst survival(124) in line with its higher rates of recurrence and metastasis compared with other subtypes(125,126). Along with frequency of metastasis, the molecular subtype of breast cancer correlates with the location of metastases. Triple-negative and HER2+ subtypes have elevated rates of metastasis to the brain, lung, and liver(125,127,128). Brain metastases are correlated with lower survival(124), making the elevated rates of central nervous system metastasis in triple-negative and HER2+ breast cancer contribute to their worse clinical outcomes. Another indicator of the importance of molecular subtype to the metastatic process is the conversion of breast cancer subtype between primary tumor and metastases. The subtype observed in lymph node(129), and other metastatic sites(130), more strongly correlates with outcomes than the subtype of the primary tumor. As would be anticipated based on the associations for primary tumors, metastases with HER2+ and triple-negative status have worse outcomes. While gene expression between primary tumors and metastases is largely conserved, including expression of ER, PR, and HER2(131,132),luminal breast cancers frequently undergo subtype conversion becoming triple-negative or HER2+ subtype in the process(130,132), with luminal A showing the highest rates of subtype conversion(131). This subtype conversion between primary tumors and metastases also seems to be correlated with the location of metastases as indicated by higher rates of conversion observed in brain metastases(133). Effective ways of targeting metastasis in breast cancer would greatly improve clinical outcomes. Targeting IL4 signaling both in immune cells(134), and in breast cancer cells(135) has been shown to attenuate metastasis. In the coming chapters I will discuss mechanisms that allow IL4 signaling to contribute to metastatic progression of triple-negative breast cancer (TNBC) and provide rationale for targeting type Ⅱ signaling.

(Ⅲ) Introduction to IL4 receptors and IL4/IL13 Signaling Axis

Interleukin 4 (IL4), a Th2 cytokine, was initially discovered as a B lymphocyte proliferative factor secreted from T lymphocytes(136). IL4 signaling plays a number of

roles in immune cell function including promotion of Th2 differentiation of Tlymphocytes(137), regulating alternative polarization of macrophages(138), and supporting apoptosis resistance in lymphocytes(139). IL4 signals through IL4Rα(140), which then forms heterodimers with other receptor subunits in order to form two distinct receptor complexes. The type Ⅰ IL4 receptor consists of the IL4Rα and IL2RG (γc) subunits, and is primarily found in hematopoietic cells. The type Ⅱ IL4 receptor is made up of IL4Rα and IL13Rα1(141), and is expressed by a number of non-hematopoietic cells. This includes a variety of cancers such as colorectal, gastric, breast, and glioblastoma(142–144). In addition to IL4, IL13 is also a cognate ligand for the type Ⅱ IL4 receptor, although it has a lower affinity than IL4(145). In addition to the type Ⅰ and Ⅱ IL4 receptor, another receptor known as IL13Rα2, can also be expressed. It was originally thought to function solely as a decoy receptor(146) but stimulation of IL13Rα2 has since been demonstrated to have signaling consequences of its own. IL4 and IL13 are expressed by a number of cell types including macrophages, dendritic cells, mast cells, natural killer T-cells, natural killer cells, basophils, eosinophils, and T lymphocytes(147).

Signaling Downstream of IL4/IL13 Receptors

IL4 and IL13 can activate multiple signaling pathways including STAT6, IRS, and MAPK signaling (Fig 1.5). These pathways were originally thought to function independently of one another, with IRS signaling being responsible for effector functions of IL4 such as proliferation(148), and STAT6 signaling primarily mediating IL4-induced gene expression. However, it has recently been accepted that multiple signaling pathways downstream of IL4 and IL13 cooperate in order to induce gene expression(149,150). Both type Ⅰ and type Ⅱ IL4 receptor have distinct signaling, defined by domains in the receptor chain that associate with particular kinases(151). One of the primary signaling pathways downstream of IL4 and IL13 signaling is STAT6. After binding of IL4 to IL4Rα, JAK1 associates with IL4Rα and is phosphorylated and activated, which leads to subsequent phosphorylation of STAT6, its dimerization, and localization to nucleus to drive gene expression(152). Phosphorylation of STAT6 can

occur independently of SH2 binding motifs in the IL4Rα, however association with JAK1 is required for activation of STAT6 signaling(153). JAK3 association with the γc chain

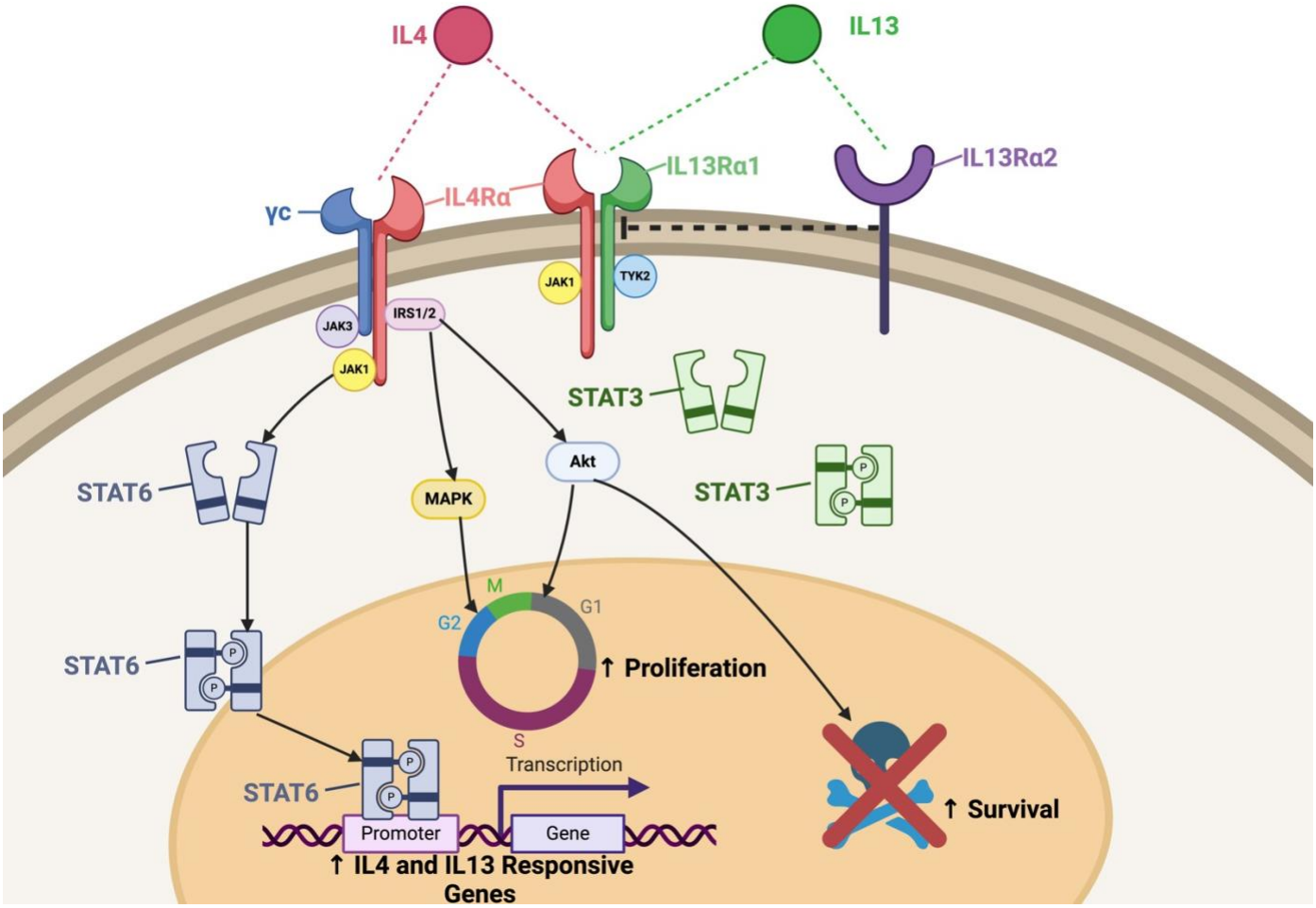


Figure 1.5: IL4/IL13 Signaling Axis. IL4 and IL13 can activate multiple signaling pathways through the type I and type II receptors. One of the primary signaling cascades is activation of STAT6 resulting in its dimerization and induction of IL4 and IL13 responsive genes. IL4 and IL13 can also activate IRS resulting in phosphorylation and activation of MAPK signaling which leads to increased proliferation, as well as activation of Akt which contributes to increased survival and proliferation. IL13Rα2 can antagonize signaling through the type Ⅱ receptor by having higher affinity for IL13. Created using biorender.

can also induce phosphorylation of pyk2 and STAT6 in response to IL4(154). Regulation of STAT6 target genes is dependent on changes in the epigenome. For example, expression of 15-lipoxygenase requires the demethylation of the promoter region prior to its IL4-mediated induction(155). IL4-induced gene expression is also dependent on histone acetylation mediated by STAT6 association with the histone acetyltransferase p300/CBP, with overexpression of p300 leading to increased STAT6 target genes(156). Although both the type Ⅰ and type Ⅱ IL4 receptors can activate STAT6 signaling, the STAT6-target genes induced vary significantly by cell type. In a study investigating STAT6-target genes in Ramos, a B lymphocyte derived cell line, and BEAS2B, a lung epithelium derived cell line, researchers found that there was little overlap in the genes induced by STAT6 between cell lines(157). This suggests that the outcome of STAT6 signaling is dependent on the existing chromatin landscape and the distinct transcription factors expressed by the cell.

Another important signaling axis downstream of the IL4 receptor is insulin receptor substrate (IRS) signaling. IL4 can induce phosphorylation and activation of IRS-1 through, recruitment of IRS-1 to a binding motif located between amino acids 437-557 in IL4Rα known as the insulin/IL-4R (I4R) motif(148). This activation of IRS-1 is essential for the mitogenic effects of IL4 and IL13 in both lymphocytes, and breast epithelial tissue(148,158). In addition, IL4 and IL13 can also induce activation of IRS-2, and this signaling has been shown to cooperate with STAT6 in order to induce proliferation in T lymphocytes, and maximal expression of Th2 cytokines(150). Activation of IRS-2 occurs primarily through the type Ⅰ IL4 receptor, with IL13 being unable to activate IRS-2 signaling as potently as IL4 in macrophages(159). This difference in IRS-2 activation correlates with expression of select M2 macrophages hallmark genes such as *arginase1, FIZZ1,* and *Ym1*, with type Ⅰ IL4 receptor stimulation leading to significantly higher induction of these genes compared to type Ⅱ IL4 receptor. This difference in signaling between the type Ⅰ and type Ⅱ IL4 receptor seems to be dictated by the extracellular and transmembrane regions of the IL13RA1, and γc subunits, as demonstrated by the inability of a chimera containing the intracellular domain of the γc subunit, and the extracellular and intramembrane domains of the IL13Rα1 subunit to

induce IRS-2 signaling at a similar level to the wildtype γc(151). In the same study the inverse chimera was able to induce IRS-2 at a similar level to wildtype γc. IRS signaling can also activate other signaling pathways such as PI3K/Akt signaling. PI3K/Akt activation has been shown to be vital to IL4-induced polarization of macrophages mediated by subsequent phosphorylation of ATP-citrate lyase, and its importance in epigenetic regulation of M2 macrophage genes(79,160). Another possible signaling consequence of IL4 receptor is activation of MAPK signaling(161), although that appears to be largely dependent on cell type(162).

In addition to the type Ⅱ IL4 receptor, IL13 is also a cognate ligand for IL13Rα2, which was originally thought to function solely as a decoy receptor for IL13(146). However, it has more recently been appreciated that IL13Rα2 serves a dual role as either a decoy receptor that inhibits type Ⅱ IL4 receptor signaling due to its higher affinity for IL13(163), or as a receptor that mediates signaling itself(164). In inflammatory disease IL13Rα2 appears to be involved in pathology through its role as a decoy receptor. In the context of inflammatory bowel disease loss of IL13Rα2 results in attenuated Th1 and Th17 inflammation(165). Signaling through IL13Rα2 appears to support tumor progression in several ways. In colorectal cancer IL13Rα2 stimulation activates PI3K signaling in a manner dependent on the scaffolding protein FAM120A(166), and silencing IL13Rα2 resulted in loss of Akt signaling and suppressed metastasis and liver homing in *in vivo* models(167). A peptide designed to inhibit binding of IL13 to IL13Rα2 also succeeded in reducing metastasis of colorectal cancer(168). Conversely in hepatocellular carcinoma silencing IL13Rα2 results in activation of ERK signaling and partial epithelial to mesenchymal transition, suggesting that IL13Rα2 expression can also be antagonistic to tumor progression.

Targeting the IL4/IL13 Signaling Axis

The IL4/IL13 signaling axis has largely been investigated as a therapeutic target in cancer through its effects on the immune system(169). It appears that limiting M2 polarization of macrophages inhibits their ability to support tumor progression(170), and treatment resistance(171). There are several trials investigating drugs designed to target

IL4 and IL13 and their potential benefit as cancer therapeutics. One such trial is an investigation of dupilumab, an IL4R inhibitor used in treatment of diseases such as allergic asthma and eczema, as a complement to immune checkpoint inhibition in treatment of non-small cell lung cancer(172). While there appears to be promise in targeting IL4 receptor signaling for treating cancer, the varying biology depending on cell type, and receptor subunit expression indicate a need for thorough understanding of the signaling occurring, and specificity in targeting it. This is well illustrated by glioblastoma where high expression of IL13Rα2 is associated with poor prognosis and tumor progression, but much of the effect of silencing IL13Rα2 is seen in type Ⅱ IL4 receptor inducing apoptosis of glioblastoma cells(144). Here, an inhibitor such as dupilumab would be counterproductive as the signaling through the type Ⅱ IL4R is necessary for tumor cell death.

Chapter Ⅱ: Type Ⅱ IL4 Signaling Activation in Basal Breast Cancer Promotes Tumor Progression via Metabolic and Epigenetic Regulation

(previously published as Demond Williams et al., “Type II Interleukin-4 Receptor Activation in Basal Breast Cancer Cells Promotes Tumor Progression via Metabolic and Epigenetic Modulation,” *International Journal of Molecular Sciences* 25, no. 9 (April 24, 2024): 4647, https://doi.org/10.3390/ijms25094647.)

Introduction

Breast cancer is the most frequently diagnosed cancer amongst women, and the second leading cause of cancer death(173). A particular concern in the treatment of breast cancer is the elevated mortality rate in metastatic disease(122). Triple-negative breast cancer (TNBC) is a subset of breast cancer that lacks expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. Patients diagnosed with TNBC have a worse prognosis than other subtypes of breast cancer, with a 5 year survival rate of 12%, for those diagnosed with metastatic disease. This compares with a 5 year survival rate of 32% for those diagnosed with metastatic breast cancer of other subtypes[1]. Additionally, TNBC shows increased rates of relapse and metastasis(128,174,175). This is likely owing to a lack of targeted therapies for triple-negative breast cancer, in contrast to the various options available for other subtypes. While some targeted therapies particularly immunotherapies, including immune checkpoint blockade, and adoptive T cell transfer, have found success in early trials for the treatment of TNBC, there remains a distinct difference in outcomes(176). Therefore, identification of avenues for efficacious targeted therapy in the TNBC setting is critical to improve patient outcomes.

Cytokines such as interleukins are associated with breast cancer progression and metastasis(177). Several of these or their receptors, such as the IL6 receptor, have been investigated as therapeutic targets(178). Here, we focus on the receptor for the Th2 cytokines IL4 and IL13. Levels of the type II IL4 receptor, the version

predominantly expressed by non-hematopoietic cells, are upregulated in breast cancer(179). We have previously shown that IL4 receptor signaling contributes to metastasis in mouse models of breast cancer (135). Additionally, IL4 signaling has been demonstrated to contribute to progression in prostate(180), colorectal(181), and hepatocellular carcinoma(182). Both IL4 and IL13 can act as ligands for the type II IL4 receptor and lead to the activation of STAT6, PI3K/Akt and MAPK pathways with downstream effects on multiple cellular processes(183).

To understand how IL4 signaling supports the metastatic progression of breast cancer, it is important to understand the downstream effects of IL4R activation in this context. In previous work, we identified metabolic changes including increased glucose uptake in breast cancer cells stimulated with IL4(143). A similar phenotype has also been described in pancreatic cancer(184). Additionally, IL4 signaling in macrophages was linked to epigenetic alterations responsible for the activation and function of macrophages(79). Here, we assess how these previously identified metabolic and epigenetic effects of IL4 signaling are active in epithelial TNBC cells. We explore how these effects are linked and can result in enhanced proliferative and survival signaling that could promote breast cancer metastasis.

Results

Type II IL4R is associated with reduced survival of basal breast cancer patients

In order to establish the relevance of type II IL4 signaling in breast cancer we investigated the correlation of the different receptor subunits expression to survival in breast cancer patients using publicly available datasets(185). Of the two components of the type II IL4 receptor (IL4R and IL13RA1), IL13RA1 is unique to this receptor, while IL4R is also found extensively on leukocytes as part of the type I IL4 receptor complex(186). Therefore, we used expression of IL13RA1 as a proxy for type II IL4R levels. IL13RA1 expression was not correlated with survival across all breast cancer

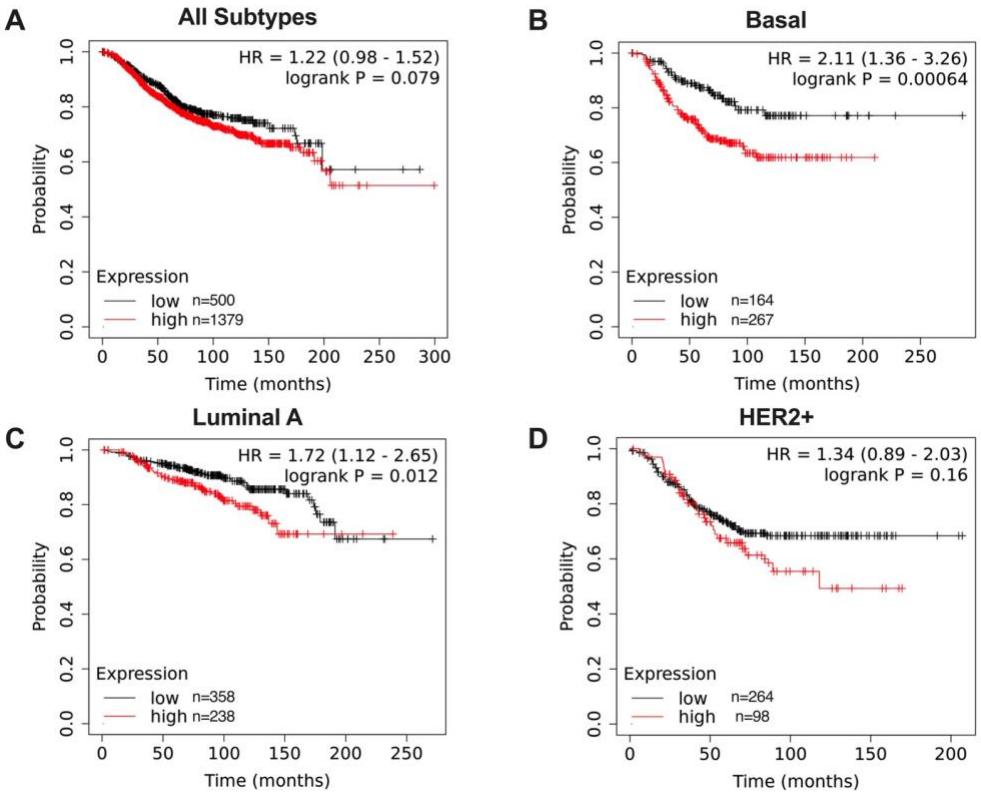


Figure 2.1 IL13RA1 expression and overall survival from KM plotter (www.kmplot.com) using publicly available data as described [17]. (A) In patients of all subtypes (n=1879) overall survival is not statistically different between patients whose tumors have high versus low IL13RA1 expression. (B) High IL13RA1 expression in tumors from patients with basal subtype breast cancer (n=431) have significantly reduced survival. (C) IL13RA1 expression in tumors from patients with the Luminal A subtype (n = 596) moderately impacts survival. (D) In HER2+ patients (n=362), IL13RA1 expression does not correlate with survival.

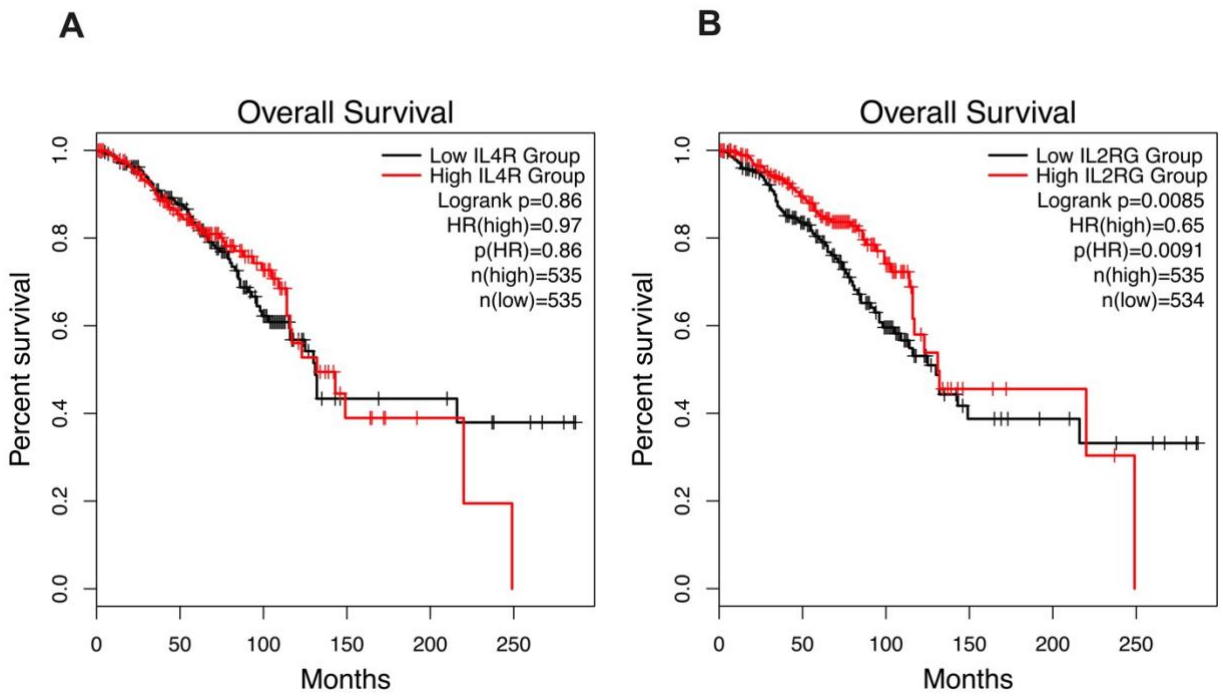


Figure 2.2 Type Ⅰ receptor expression and overall survival from KM plotter (A) In patients of all subtypes (n=1070) expression of IL4Rα does not correlate with overall survival. (B) Expression of IL2RG does not correlate with survival in all available breast cancer patients (n=1069).

subtypes (Fig 2.1A); however, there is a significant difference in survival observed particularly in the basal subtype (Fig 2.1B) and to a lesser extent in the luminal A subtype (Fig 2.1C). The HER2+ subtype showed no significant correlation between IL13RA1 expression and decreased survival (Fig 2.1D). Conversely, high expression of type I IL4 receptor subunits (IL4R, IL2RG) show a slight association with better survival in the same dataset (Fig 2.2), which may reflect a link between immune cell infiltration and outcome. Taken together, the data suggest that the type II IL4 receptor is a poor prognostic indicator, especially in the context of TNBC or basal breast cancer.

IL4 increases glucose uptake and metabolism

Type II IL4 receptor signaling has been previously associated with elevated glycolysis in breast cancer(143), and other cancer types(184). These IL4-induced changes in metabolism have been associated with increased metastasis. To assess these metabolic effects in triple-negative breast cancer *in vitro*, we treated two cell lines capable of spontaneous metastasis, human (MDA-MB-231) and murine (4T1) cell lines with IL4 and quantified glucose uptake using the fluorescent glucose analog 2-NBDG. Using this assay, we found that IL4 increased glucose uptake in both cell lines (Fig 2.3A,B). Additionally, we have previously shown that IL4 increases expression of glucose transporter GLUT1 in breast cancer cell lines(143). Using RNA-mediated knockdown of GLUT1, we found that IL4-induced glucose uptake was dependent on GLUT1 expression (Fig 2.3B).

To assess the potential impact of type II IL4 signaling on energy production, we used a Seahorse ATP rate assay to measure the glucose consumption rate of MDA-MB231 cells pre-treated with IL4 for 24 hours. Interestingly, we found that both of the ligands for the type II Il4 receptor - IL4 and IL13 - consistently increased the oxygen consumption rate (OCR) of these cells as compared to untreated controls (Fig 2.3C). However, changes in extracellular acidification rate were not consistent across replicate assays. Additionally, total ATP production rates were only marginally increased in cells treated with these cytokines (Fig 2.3D). Similar results were seen with the BT549, another human triple-negative breast cancer cell line (data not shown). Overall, the data

suggest that IL4 increases glucose uptake, but only leads to small increases in lactate production or ATP

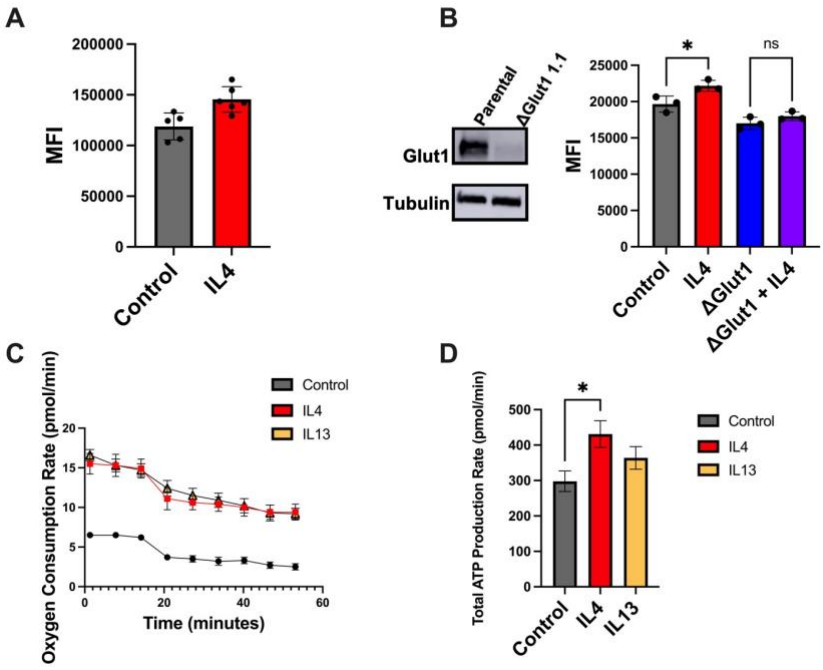


Figure 2.3 IL4 increases glucose uptake and metabolism in TNBC cell lines. (A) Treatment with 10ng IL4 in 4T1 murine mammary cancer cells increases glucose uptake assessed by NBDG assay. **(B)** Glut1 protein is undetectable in ΔGlut1 MDA-MB-231 cells generated using CRISPR/Cas9, and treatment with 20ng IL4 shows that an IL4induced increase in glucose uptake is dependent on GLUT1 expression. **(C)** Pretreatment (24 hr) with 20ng IL4 or IL13 of MDA-MB-231 cells results in increased oxygen consumption rates as measured by Seahorse ATP rate assay. **(D)** Pre-treatment (24 hr) with 20ng IL4 or IL13 results in increased total ATP production in MDA-MB-231 as measured by Seahorse ATP rate assay. Representative images from experiments that were repeated at least 3 times. *p<0.05.

production in the time frames examined. For this reason, we next considered other possible fates of glucose.

The pAKT/pACLY signaling axis is activated by IL4 signaling

Glucose can be used to produce acetyl-CoA, which among other roles is a substrate for histone acetylation(187). Histone acetylation is well established to be dependent on the abundance of acetyl-CoA(188). IL4 can regulate Akt activation which in turn results in serine 455 phosphorylation and increased activity of ATP-citratelyase(ACLY), an enzyme that produces acetyl-CoA from the TCA cycle intermediate citrate(79). In macrophages, IL4 increases glucose consumption, and ACLY activity necessary for the regulation of gene expression and polarization(160).

Since we observed increased glucose uptake with IL4 treatment, we investigated whether the pAKT/pACLY signaling axis was also affected by IL4 signaling in the context of breast cancer. An important precursor to the phosphorylation of ACLY is the phosphorylation of AKT at serine 473. We found that IL4 induced phosphorylation of AKT and ACLY in both human and murine mammary cancer cell lines (Fig 2.4A-C). Together, this suggests that type II receptor signaling may affect the utilization of glucose to support IL4-induced histone acetylation and gene expression in triplenegative breast cancer.

Histone acetylation leading to gene expression changes is altered by IL4 signaling

After confirming activation of ACLY, we next investigated whether IL4 impacts histone acetylation in TNBC. We first examined whether there were alterations in overall histone acetyltransferase (HAT) activity in nuclear lysates from TNBC cell lines with or without IL4 exposure. We found that IL4 increased HAT activity as demonstrated by a fluorescent HAT activity assay (Fig 2.5A).

Next, we were interested in whether IL4 induces global changes in histone acetylation. We first enriched for acetyl lysine proteins using immunoprecipitation, and then assessed their association with histone H3 by immunoblotting. Our results

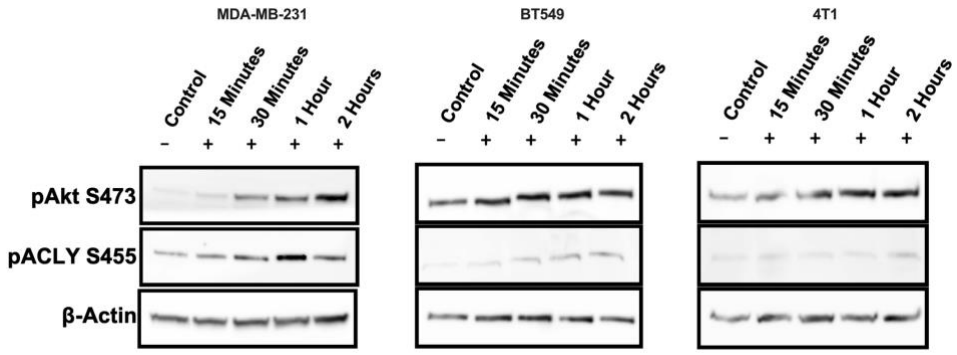


Figure 2.4 IL4 treatment induces phosphorylation of Akt and ATP citrate lyase (ACLY). Treatment with 20ng species-specific IL4 results in increased phosphorylation of AKT at serine 473 and ACLY at serine 455 over time in human MDA-MB-231, human BT549, and murine 4T1 cells. Representative images from at least 2 repeats per cell line.

indicated that lysates from IL4-treated cells were enriched for in acetyl lysine-positive histone H3 (Fig 2.5B). We then investigated specific acetylation marks that have been associated with IL4-mediated gene regulation(189). Histones that were isolated from IL4-stimulated cell lines showed small increases in global acetylation of H3K9 and H3K27 acetylation compared with controls (Fig 2.5C).

Due to the moderate increases in global histone acetylation, we focused on the role of IL4 in the modulation of acetylation at the gene level. IL4-mediated gene expression is dependent on the association of IL4 transcription factors such as STAT6 with cofactors such as the histone acetyltransferase p300(190). Thus, large changes in acetylation across the genome are less likely than more precise regulation of STAT6target genes. To investigate this, we performed CUT&RUN (cleavage under targets and release using nuclease) sequencing of MDA-MB-231 cell line to identify STAT6 and H3K9ac target genes that are regulated by IL4. In tandem, we investigated which genes are altered in response to IL4 treatment in MDA-MB-231 cells using RNASeq. Due to our interest in histone acetylation, which is a permissive epigenetic marker, our focus was largely on genes that showed increased expression with IL4 stimulation.

From the intersection of the datasets generated by these experiments (Fig 2.6), we developed a list of candidate genes whose expression was mediated by IL4 and showed dependence on histone acetylation. Within that gene set, we focused on those that are known oncogenes or have been associated with metastatic progression in other ways in the literature. Five genes satisfied these criteria and were subject to further validation – BCL2, CCND2, TNC, ST8SIA1, and EP300. Fig 2.7A, B shows examples of overlap in location of the H3K9 acetylation and STAT6 binding signals within 2 of these genes. We examined expression of genes of interest in cells treated with or without IL4 in the presence or absence of C646, a small molecule inhibitor of the STAT6 associated HAT p300(191) (Fig 2.7A-E). For all transcripts examined, C646 alone did not decrease the expression of these genes below baseline. However, in cells exposed to IL4, C646 attenuated the IL4-induced expression of CCND2, BCL2, TNC, ST8SIA1, and EP300.

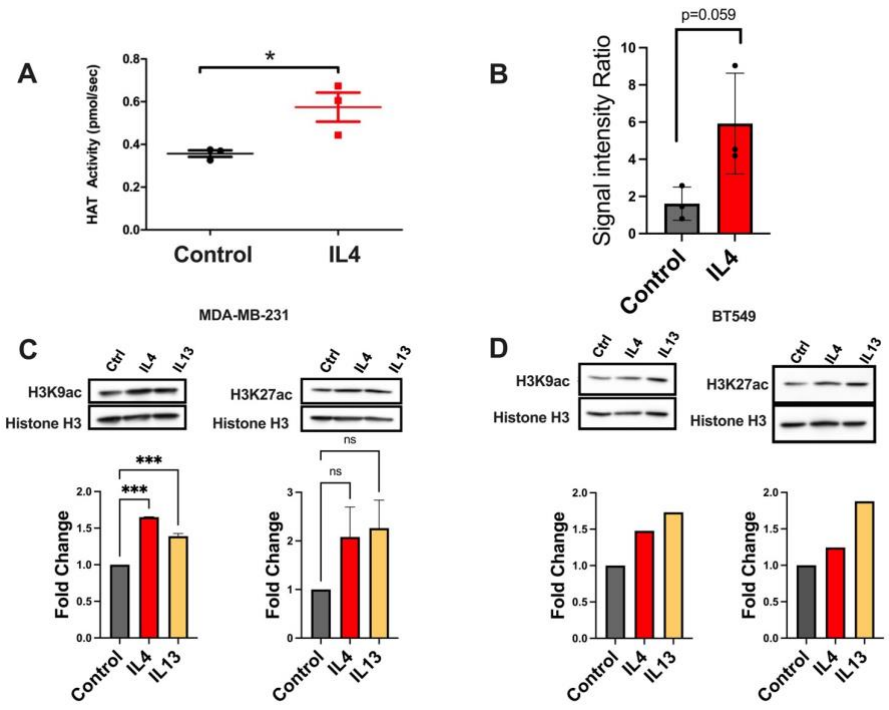


Figure 2.5 Stimulation of type II IL4 receptor increases histone acetylation in triple-negative breast cancer. (A) HAT activity assessed by fluorometric assay in nuclear lysates from MDA-MB-231 cells shows increased HAT activity with IL4 treatment. **(B)** Acetyl lysine pull down was performed on total lysates from MDA-MB231. Western blots for total histone H3 were performed on enriched lysates showing increased presence of histone H3 after IL4 treatment. Western blots for H3K27ac and H3K9ac in MDA-MB-231 **(C)** and BT549 **(D)** showed moderate increases in global acetylation at these marks of interest. Quantification of blots is shown below (n = 2 for MDA-MB-231, n = 1 for BT549).* $p<0.05$; ***$p<0.001$.

Both p300 and glucose uptake contribute to type II IL4R induced proliferation and survival

Several of the genes of interest identified in our sequencing experiments and confirmed by qPCR are known to be associated with cancer progression. CCND2 is a cell cycle protein that has been associated with progression in colorectal cancer(192). BCL2 is a known target of IL4 signaling(193), and an important regulator of apoptosis that has become a target of interest in several cancers(194). We have previously shown that IL4 stimulation causes increased proliferation and clonogenicity, which are *in vitro* surrogates for metastatic phenotypes closely associated with these genes(143). Due to the impact of C646 on the expression of these genes, we next investigated whether C646 could ablate the IL4-induced phenotypes.

In regard to cell proliferation, we found that treatment with IL4 increased cell number as measured by Cyquant assay, and C646 ablated the IL4 induced proliferation in BT549 (Fig 2.8A). We also performed clonogenic assays with both BT549 and 4T1 cell lines to assess the impact of IL4 and C646 on cell proliferation and clonogenicity. MDA-MB-231 cells were not used for these assays as they are highly migratory and do not form cohesive colonies. While IL4 did not impact the number of colonies, average colony size was significantly increased with IL4 treatment (Fig 2.8B,C), supporting proliferation as an IL4 induced phenotype. C646 treatment alone did not affect the size of the colonies formed however, it did ablate the effect of IL4 on increased colony size.

Using Glut1-deficient MDA-MB-231 cells, we also investigated whether IL4 treatment increased proliferation in a manner dependent on glucose uptake. As shown in Fig 2.8D, treatment of the parental MDA-MB-231 cells with IL4 increased proliferation as determined by Cyquant assay. However, the stimulatory effect of the cytokine was no longer observed in the cells lacking Glut1. Together these data support the idea that type II IL4 receptor signaling leads to increased survival and proliferation of TNBC cells via a mechanism involving glucose uptake and histone acetylation.

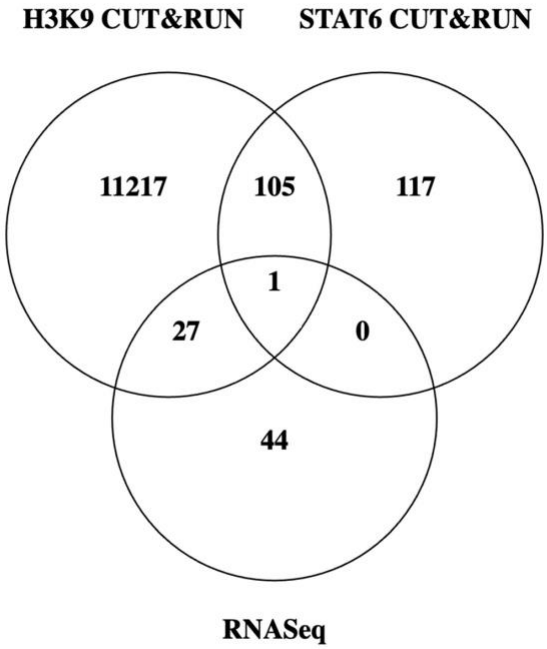


Figure 2.6 Overlap in genes identified by sequencing experiments. Gene lists from RNASeq experiments that showed increased expression, and H3K9ac and STAT6 target genes found using CUT&RUN were intersected in order to identify IL4-induced genes that are regulated by histone acetylation.

Discussion

Here we show that type II IL4 receptor signaling can contribute to metastatic phenotypes including proliferation and clonogenic survival in triple-negative breast cancer. Our data indicate that activation of the type II receptor by either of the cognate ligands – IL4 or IL13 -leads to increased glucose uptake, which is associated with enhanced proliferation. Additionally, a direct result of receptor activation is phosphorylation of Akt and ACLY, which has been linked with increased availability of acetyl-CoA, a substrate for histone acetyltransferases. Histone acetyltransferase activity is increased in IL4-treated cells, as are levels of lysines- 9 and -27 acetylation on histone 3 albeit only moderately on a global level. Specific IL4-target genes of relevance in breast cancer metastasis were identified by RNASeq and CUT&RUN experiments and show dependence on P300 histone acetyltransferase activity for their IL4-driven expression. Together these *in vitro* findings enhance our previous *in vivo* data[10], which showed significantly attenuated metastasis when the IL4 receptor was ablated in cancer cells. The current data suggest that changes to glucose uptake and histone acetylation are potentially key effectors of IL4 signaling in metastatic breast cancer cells.

Many of the IL4-mediated phenotypes investigated have been observed in various immune cells where IL4 generally signals through the type I IL4 receptor. For example, gene expression associated with M2 polarization of macrophages is dependent on IL4-driven glucose metabolism (195), and blocking glycolysis using 2DG impairs macrophage polarization(196). In addition to altered gene expression, 2DG treatment also impacts phagocytosis(197). In B-lymphocytes, IL4 has an anti-apoptotic effect that is also dependent on glycolysis(198), while activation of T-lymphocytes driven by autocrine IL4 signaling is similarly dependent on glycolysis(199). Other metabolic pathways modulated by IL4 have also been associated with macrophage function. IL4driven M2 polarization appears to be dependent on a certain energy threshold, with glutaminolysis and oxidative phosphorylation acting as compensatory metabolic pathways if glycolysis is inhibited(200). Serine biosynthesis pathways have additionally been implicated, with genetic ablation of PHGDH resulting in attenuated induction of M2 polarization by IL4 *in vivo*(201). We have also previously shown using pharmacological

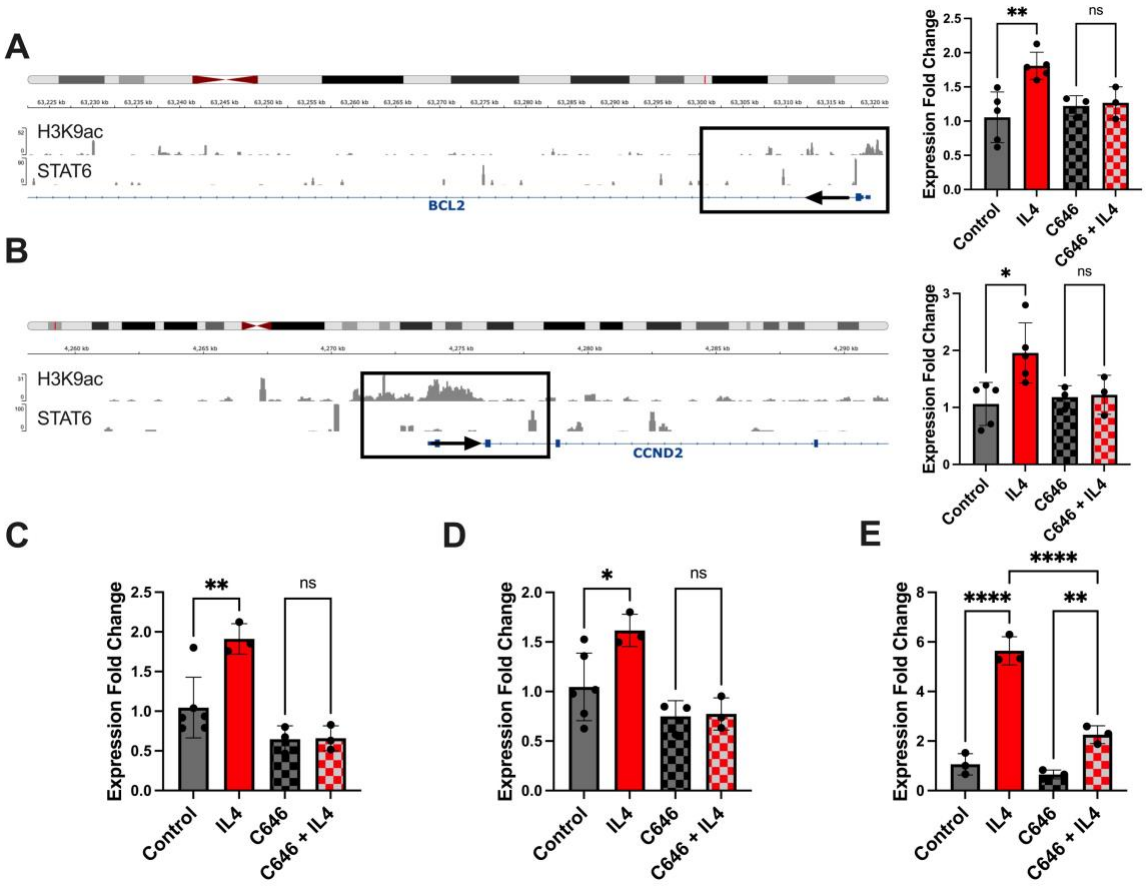


Figure 2.7 IL4 induces expression of target genes in a histone acetyltransferase dependent manner. (A, B) Integrative genome viewer views of signal from H3K9ac and STAT6 CUT&RUN experiments overlapping with BCL2 and CCND2 promoter region (boxed). Results of quantitative RT-PCR analysis of expression of the transcripts are shown to the right. **(C, D, E)** Quantitative RT-PCR for TNC, EP300, and ST8SIA1, respectively in MDA-MB-231 cells. For all qRTPCR experiments, cells were treated with 20ng recombinant IL4 +/- 20μM C646 for 24 hrs before RNA collection. Shown are results from three biological replicates. *p<0.05; **p<0.01; **** p<0.0001.

inhibitors, that glutaminolysis in addition to glycolysis are tied to IL4-induced proliferation in breast cancer(143). We extend that finding here using CRISPR-mediated deletion of GLUT1, which ablates IL4-induced proliferation in TNBC. Overall, the mechanisms by which IL4 signaling drives proliferation of breast cancer cells align well with those observed in other cell types. Of note, based on the data shown here, we can expand that model to include signaling through the type II Il4 receptor.

The Akt/ACLY signaling axis has been implicated as an important mediator in IL4-induced polarization of murine macrophages(79). This signaling links both glycolysis and mitochondrial metabolism to gene expression in M2 polarized macrophages(160). In human macrophages, while full polarization is not dependent on ACLY, some IL-4 induced gene expression is still reliant on ACLY activity as indicated by pharmacological inhibition of ACLY(80). ACLY is a substrate of Akt and we noted increased Akt activation in the cells treated with either IL4 or IL13. This is in line with data that we published previously showing that IL4-regulated metastatic colonization in mouse models of breast cancer was linked with increased Akt signaling, and IL4-enhanced colony formation *in vitro* was attenuated by Akt inhibition(135). We acknowledge that the Akt/ACLY signaling axis may play several other roles in TNBC tumor progression, for example in DNA repair (202); however, here we chose to focus on histone acetylation as a potentially relevant downstream effect.

Previous evidence suggests ACLY phosphorylation and activity regulates gene expression via histone acetylation in macrophages(79) and osteoclasts(203) Moreover, IL4- driven histone acetylation is an important regulator of gene expression in Blymphocytes(189) and macrophages(204,205), and the histone acetyltransferase P300/CBP is well recognized as an important mediator of IL4-stimulated gene regulation (156,206). In our hands, IL4 treatment induced relatively small increases in total histone acetylation. However, using more targeted methods, we could identify IL4target genes where histone acetylation was active. Other epigenetic modifications are known to be modulated by IL4 in immune cells, such as histone deacetylation(207,208), histone demethylation(209–211), and DNA demethylation(212). In this study, we did not examine the contribution of these modifications; however, it is likely that they play a role.

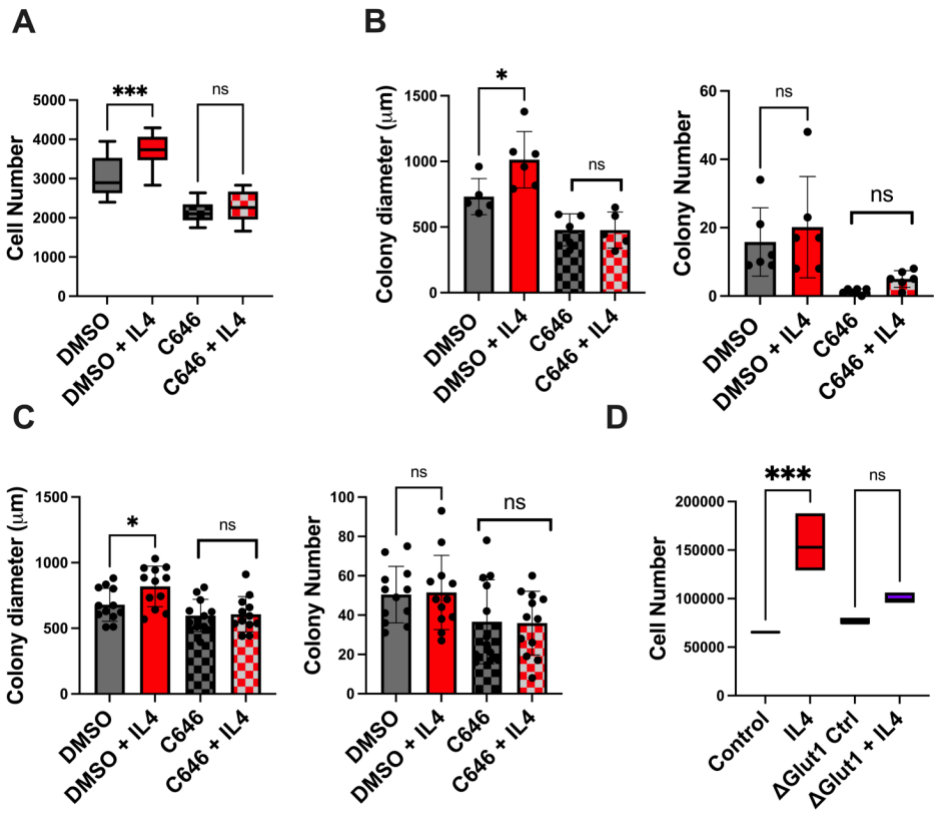


Figure 2.8 IL4 induced in vitro phenotypes are dependent on p300 activity and glucose uptake. (A) Cyquant proliferation assay showing increased cell number in BT549 cells treated with 10ng IL4 for 24hrs. Inclusion of 10µM C646 ablates the cell IL4-induced increase. (B) Clonogenic assay of BT549 cells show increased colony size in cells treated with 10ng IL4, while inclusion of10µM C646 ablates the effect of IL4 on colony size. Cells were treated every 2 days for a total of 14 days in culture. (C) Murine mammary cancer 4T1 cells show increased colony size after treatment with 10ng IL4, which is prevented by inclusion of 10µM C646. Colony number was unaffected by IL4. Cells were treated every 2 days for a total of 6 days in culture. (D) Cyquant assay in MDAMB-231 parental and Glut1 knockout cell lines show that IL4-mediated proliferation is also dependent on glucose uptake.* $p<0.05$; *** $p<0.001$.

A future goal is to systematically characterize epigenetic changes that occur downstream of IL4/IL13 signaling in cancer cells.

C646 has been previously investigated as a potential drug for pancreatic cancer(213), hepatocellular carcinoma(214), and prostate cancer(117). Here we show that inhibition of the histone acetyltransferase p300 by C646 blocks IL4-driven gene expression in TNBC cell lines. Specifically, we have identified CCND2, BCL2, TNC, ST8SIA1, and EP300 as genes upregulated by IL4 in a p300-dependent manner. Expression of CCND2 has been shown to be correlated with persistence of colorectal cancer(192). BCL-2 family proteins have been identified as targets for cancer therapy(194), and their expression has been shown to be induced by IL4 in lymphocytes(193). The expression of both genes is in line with IL4-induced proliferation and survival phenotypes observed in this study. Tenascin C (TNC) expression has been associated with multiple cancer processes including enhanced proliferation, migration(215) and immunosuppression(216). ST8SIA has previously been shown to play a role in chemoresistance in triple-negative breast cancer by modulating Wnt/βcatenin signaling(217). EP300 has been associated with metastatic capacity in triplenegative breast cancer(218). In addition to effects on gene expression, C646 treatment attenuates metastatic phenotypes in IL4-treated TNBC cell lines *in vitro*.

The work presented here is conducted *in vitro*, and there is a need for further investigation using *in vivo* models to provide a better understanding of the varied roles of IL4 signaling, and its contribution to cancer progression. In the context of immune cells IL4 signaling has been characterized as largely immunosuppressive, and protumorigenic, mediated by the previously discussed role it plays in polarizing tumorassociated macrophages to the M2 phenotype(171). However, there is some evidence in the literature to suggest IL4 signaling acting through CD8 lymphocytes expressing the type I IL4 receptor can boost anti-tumor immunity in the context of immune checkpoint blockade(219). Here we demonstrate that IL4 signaling acting on breast cancer cells through the type II receptor is mitogenic and supports expression of pro-survival genes. These context-dependent effects of IL4 signaling highlight the importance of specificity in targeting the IL4 signaling axis *in vivo*. Experiments using

specific inhibitors of type II IL4 receptor(220) vs type I IL4 receptor in immune competent mouse models of breast cancer would be necessary to elucidate the how the two receptors may contribute to tumor progression. In addition, it would be necessary to characterize immune cells in the TME and how they respond to modulation of the IL4 receptors, as well as the signaling consequences of IL4 targeting in cancer cells themselves.

As shown by the drastic decrease in five-year survival rates seen in patients diagnosed at later stages of breast cancer (122), metastatic disease remains a particularly significant clinical challenge. In addition, triple-negative breast cancer is often aggressive and there exist relatively few targeted therapies for this subtype. Therefore, identification of additional vulnerabilities of these tumors could greatly benefit patient care. In the context of cancer, IL4 signaling has largely been studied for its effects on immune cells that drive cancer progression. IL4-driven polarization of macrophages has been associated with immunosuppression in breast cancer(171). In addition, macrophages polarized by IL4 signaling secrete factors that promote invasion and migration in breast cancer(170). Repurposing of dupilumab, an IL4R targeted antibody, has shown promising synergy with immune checkpoint inhibitors in the treatment of non-small cell lung cancer(172). The data presented here support a direct effect of IL4 signaling in triple-negative breast cancer mediated through the type II IL4 receptor. This is in addition to the better studied role of IL4 signaling in immune cells driving immunosuppression and tumor progression indirectly.

Chapter Ⅲ: Additional Experiments to Investigate IL4R-driven Metabolic Adaptations in TNBC

Introduction

Previously, in chapter 2, I characterized the response of triple-negative breast cancer to IL4 and IL13 *in vitro*. I showed that IL4 increases glucose uptake by triple negative cell cancer (TNBC) cell lines but did not appear to lead to large increases in energy production *in vitro*. I also showed that IL4 activates signaling associated with acetyl-CoA production and induces histone acetylation. Cooperation of STAT6 with the histone acetyltransferase activity of p300 appeared to be vital for gene regulation by type II IL4 receptor signaling. *In vitro* phenotypes such as IL4-induced proliferation of TNBC cell lines was dependent on both the glucose uptake and histone acetylation mediated by p300. Here I expand further on the metabolic phenotypes driven by IL4R, to include a temporal aspect as well as a more detailed analysis of the ACLY/acetylCoA/epigenetic axis.

Results

Temporal Regulation of Metabolism by IL4

A consistent observation in my work as well as that done by previous lab members is that IL4 increases glucose uptake in mammary cancer cell lines *in vitro*. Regulation of glucose uptake and downstream metabolic pathways by IL4 appear to be controlled by substrate availability and duration of IL4 stimulation. In the BT549 TNBC cell line I observed increased uptake of the fluorescent glucose analog NBDG in cells stimulated by IL4 in 5mM glucose media, however this effect on glucose uptake was ablated when treatment occurred in glucose-free media (Fig 3.1 A). The duration of IL4 stimulation in these experiments was in line with previous work from the lab, showing that 24 hours of treatment induced increased glucose uptake. However, I have also

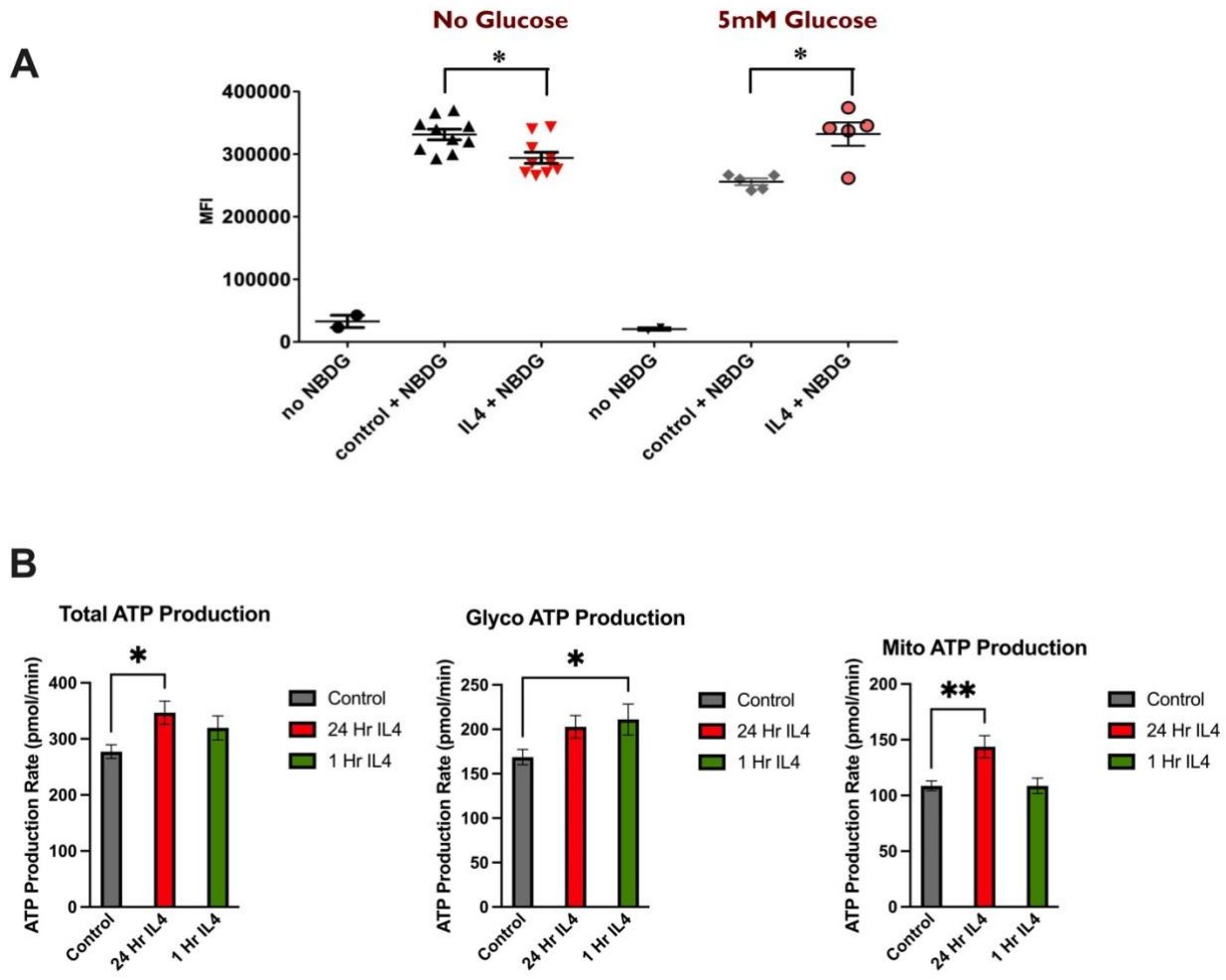


Figure 3.1 IL4 regulates glucose uptake and metabolism. (A) Treatment of BT549 with 10ng of IL4 increases glucose uptake when cytokine stimulation occurs in media with 5mM glucose. IL4 decreases glucose uptake with cytokine treatment is performed in 0mM glucose media. **(B)** MDA-MB-231 cells were pre-treated with 20ng IL4 for 24 hours or 1 hour, and ATP production was measured by Seahorse ATP rate assay. 24 hours of treatment resulted in significantly increased total ATP and mitochondrial ATP production. 1 hour of IL4 treatment resulted in significantly increased glycolytic ATP production. * $p < 0.05$; ** $p < 0.01$.

found that both shorter- and longer-term treatment with IL4 affects the response of TNBC cell lines. In a seahorse ATP Rate assay where MDA-MB-231 cells were treated with IL4 for 1 hour or 24 hours I found that only 24 hours of stimulation resulted in increased total ATP production (Fig 3.1B). This appears to be largely driven by increased production of ATP from mitochondrial respiration. In contrast, at the 1-hour time point mitochondrial ATP production was not increased, but glycolytic ATP production was.

Another aspect of IL4-mediated regulation of metabolism that I have investigated is production of downstream metabolites. Initially I was interested in the glycolysis product, lactate. I conducted experiments where MDA-MB-231 and BT549 cell lines were treated with IL4, with additional IL4 added every 48 hours, samples of media were collected every other day to quantify extracellular concentration of metabolites. At earlier time points (2,4, and 6 days), there was no evident difference in lactate production from either cell line, but at 192 hours of IL4 treatment there was increased lactate secreted by both cell lines (Fig 3.2). Previously, the lab showed that IL4 increases glutamine uptake in mammary cancer cell lines(143) thus we also measured glutamine metabolism over the same time-frame. We show that IL4 increases glutamate production in MDA-MB-231 at the 8-day time point but does not increase extracellular glutamate from the BT549 cell line (Fig 3.2). This suggests that the production of glutamate induced by IL4 is not a general phenotype, but dependent on the context of specific cell lines, whereas lactate production is more consistent across cell lines. It is worth noting that the concentration of metabolites in these experiments are not normalized to cell number.

Regulation of Acetyl-CoA Metabolism

In chapter 2 I showed that IL4 increases histone acetylation and activates signaling associated with increased acetyl-CoA production in TNBC cell lines. This activation was mediated by phosphorylation of ACLY at serine 455. It has been demonstrated that the nuclear localization of ACLY increases histone acetylation(203). I therefore analyzed the localization of phosphorylated ACLY using a cellular fractionation approach, using western blotting to identify phosphorylated ACLY in the different

fractions. In MDA-MB-231 cells treated with IL4, phosphorylation of ACLY in nuclear lysates was increased (Fig 3.3A). Since

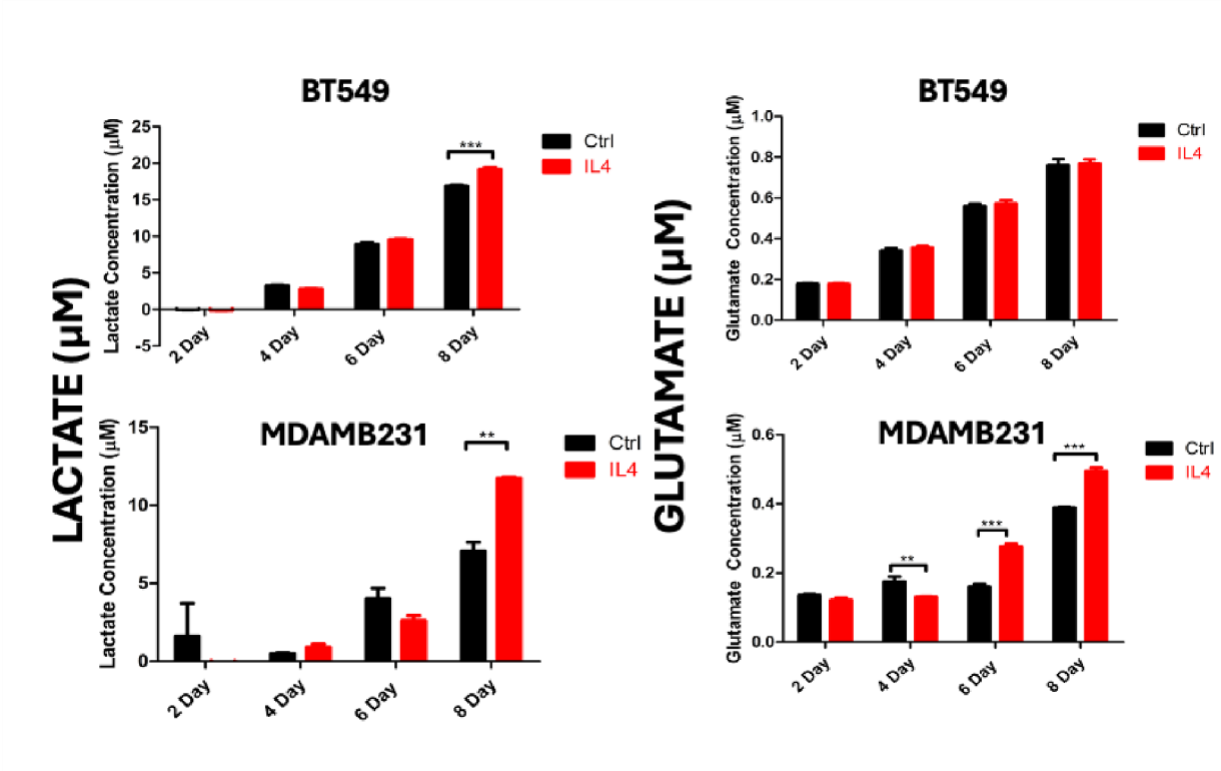


Figure 3.2 IL4 increases production of extracellular metabolites. MDAMB231 and BT549 cells were treated with 10ng IL4 every two days. Samples of media were collected at 2, 4, 6, and 8 days. Media was diluted with PBS, and concentration of lactate and glutamate was measured using chemiluminescent assay. ** $p < 0.01$; **** $p < 0.0001$

the concentration of acetyl-CoA is closely tied to regulation of histone acetylation(188), I next attempted assess how IL4 affects the production of acetyl-CoA in the MDA-MB231 cells. I had previously observed that 4-hour stimulation of TNBC cell lines by IL4 resulted in increased histone acetylation (as shown in Chapter 2, Fig 2.4). Following up on that result I used a fluorescent assay to measure the concentration of acetyl-CoA in lysates from MDA-MB-231 cells that had been treated or not with IL4. Despite seeing the activation of ACLY, the concentration of acetyl-CoA in the IL4 treated cells was lower than in the control samples (Fig 3.3B). It is worth noting that this result does not necessarily reflect the impact of IL4 on production of acetyl-CoA in TNBC, as the decreased concentration may be reflective of the utilization of acetyl-CoA for acetylation. Despite the decrease in acetyl-CoA in IL4-treated cells, inhibition of ACLY using BMS303141 ablated IL4-induced proliferation in MDA-MB-231 cells (Fig 3.3C). Together, these data indicate that activation of the nuclear pool of ACLY may be relevant to the pro-tumorigenic effects of IL4.

Genetic Regulation of Metabolism by IL4

Due to our interest in IL4-mediated epigenetic regulation, particularly histone acetylation, we wanted to assess how IL4 affected chromatin accessibility. Since we had previously observed that 4 hours of IL4 treatment was enough to increase histone acetylation in TNBC cell lines, we performed a brief time course experiment with MDAMB-231 cells treated for 4, 8 or 18 hours. These cells were then used in assay for transposase-accessible chromatin with sequencing (ATACseq). Although we see increased histone acetylation within 4 hours of IL4 treatment, the principal component analysis suggests that there are not substantial changes in chromatin accessibility until the 18-hour time point (Fig 3.4A). However, despite there being a clear difference by the PCA analysis, evaluation of accessibility across the genome showed minimal changes even at that 18-hour timepoint (Fig3. 4B,C). A short list of genes increased accessibility in the promoter region with differential accessibility showed little overlap with H3K9/STAT6 CUT&RUNseq or RNAseq experiments. It is worth noting that other sequencing experiments (particularly the CUT&RUN for H3K9) were performed in cells

that had been stimulated with IL4 for 24 hours, thus it is difficult to interpret whether these results are complementary or not.

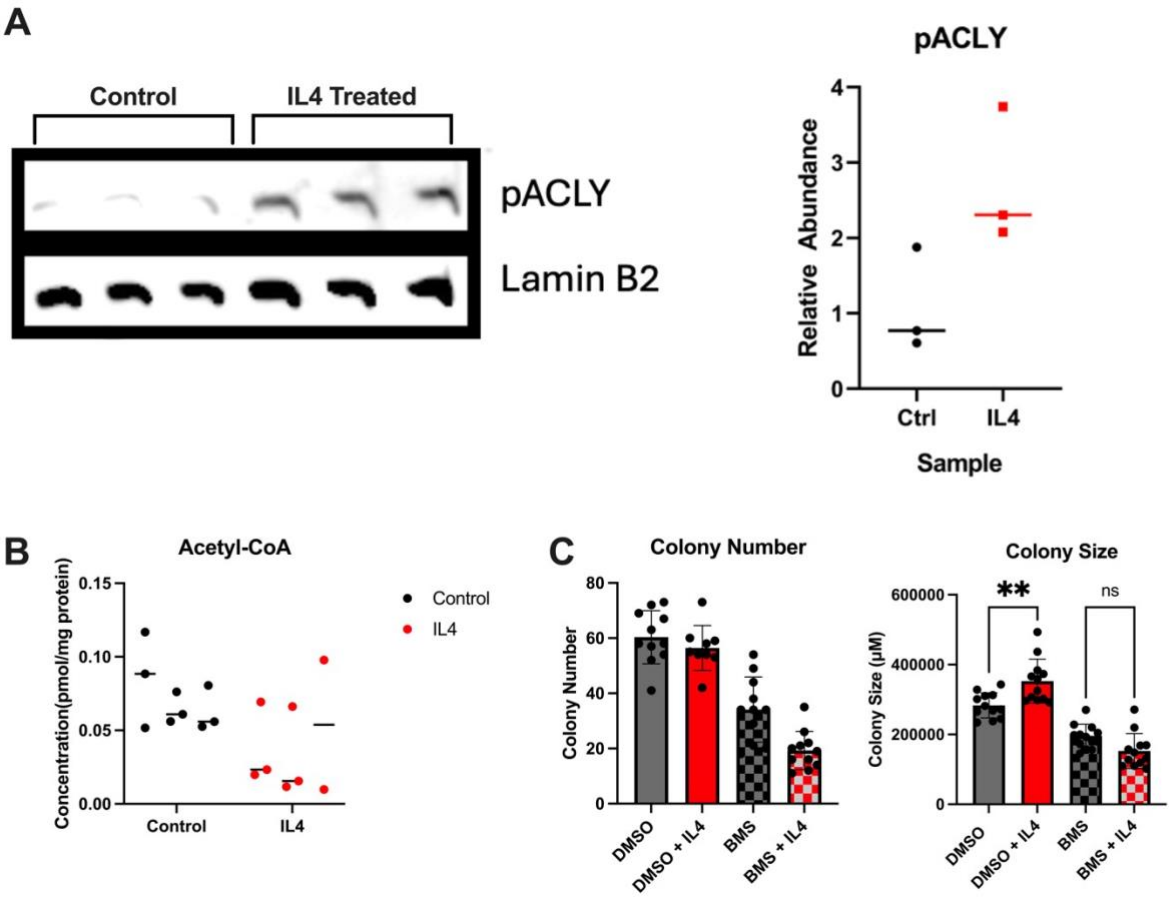


Figure 3.3 IL4 regulates acetyl-CoA metabolism in TNBC. (A) MDAMB231 cells were treated for 2 hours with 10ng IL4. Cells were fractionated and western blots (left) were performed on nuclear lysates for pACLY S455 showing increased phosphorylation. Quantification of band intensity (right) **(B)** MDAMB231 cells were treated for 4 hours with 10ng IL4, and total lysates were deprotenated, and acetyl-CoA concentration was measured using fluorometric assay. **(C)** Clonogenic assay shows that ACLY inhibitor BMS303141 inhibits IL4-induced increase in colony size. ** $p < 0.01$

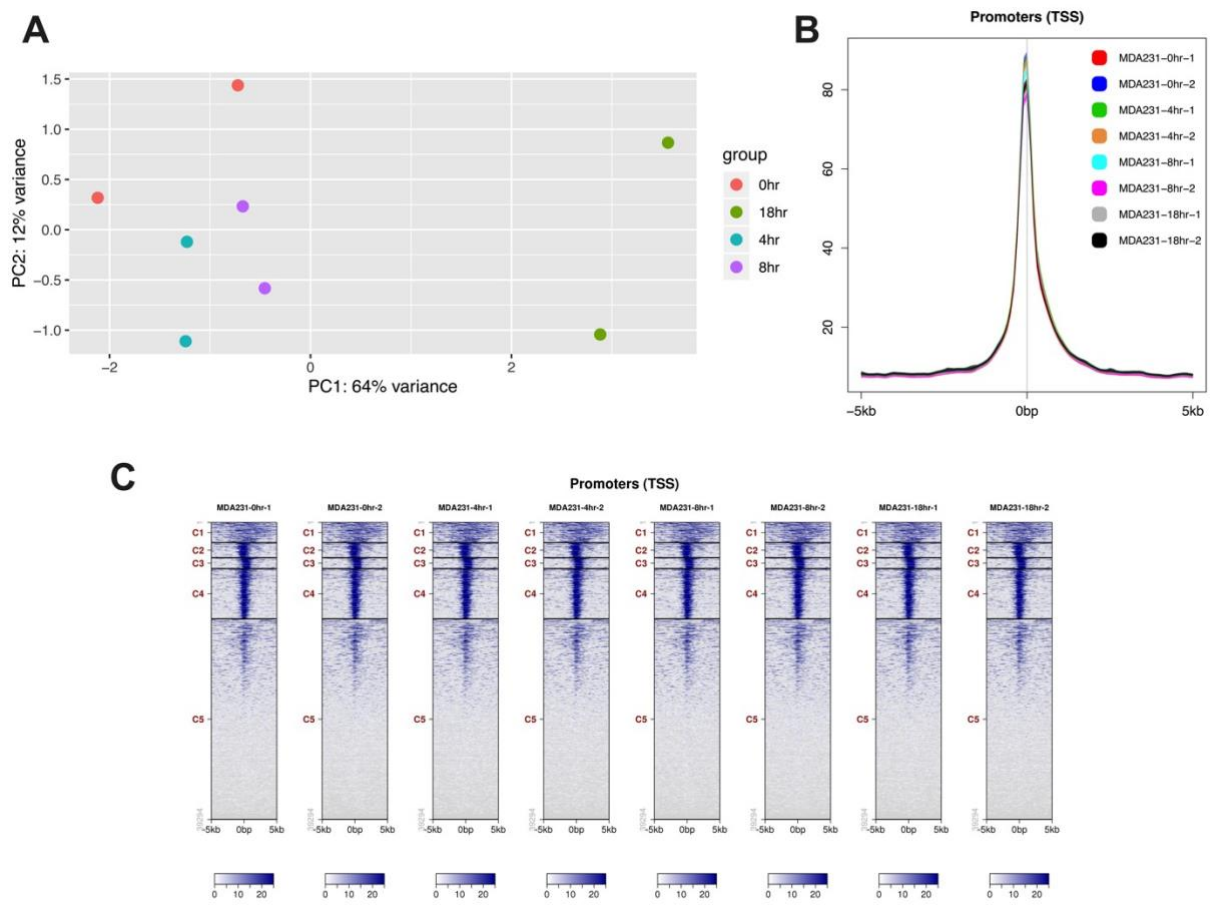


Figure 3.4 IL4 induces changes in chromatin accessibility in TNBC (A) Principal component analysis of ATACseq shows that 18 hours of IL4 treatment is required to induce changes in chromatin accessibility. **(B)** Average plot of tag distribution across promoter regions of ATACseq show that 18 hours of IL4 stimulation induces minimal changes. **(C)** Heatmap of tag distribution across promoter regions of genes.

Overall, the ATACseq findings were not informative and suggest that optimization is needed for this type of experimental approach.

While many of the studies that I have described focused on the role of metabolism in controlling epigenetic regulation downstream of IL4, I was also interested if IL4-initiated signaling could regulate metabolic programs transcriptionally. To analyze this in a focused but relatively unbiased manner, I made use of a commercially available qPCR array that included 90 gene targets all related to glucose metabolism. I treated the murine mammary TNBC cell line E0771 with IL4 for 24 hours and collected total RNA. After reverse transcription, the cDNA was added to the array plate and processed for qPCR. The results showed that the IL4-treated cells upregulated several glycolytic genes such as HK2 or G6PC3. Interestingly, others in the lab had previously conducted a very similar experiment in the murine colorectal cancer cell line MC38 and found an almost identical pattern of up-regulated glucose metabolism genes (Table 3.1). Together with my other data, these results indicate that IL4 signaling regulates metabolic behavior in multiple different ways, including at the level of transcription of relevant target genes.

Discussion

The data presented here show that IL4 affects the glucose metabolism of TNBC cell lines in a time dependent manner. Longer duration of IL4 stimulation resulted in production of extracellular metabolites like lactate and glutamate. I also show that IL4 regulates ACLY signaling associated with nuclear acetyl-CoA production and histone acetylation. However, the concentration of acetyl-CoA is reduced in IL4 treated samples. Despite this decrease in acetyl-CoA, inhibiting ACLY resulted in ablation of IL4-induced proliferation in TNBC cell line. While this would suggest chromatin modification, an ATACseq analysis showed that eighteen hours of IL4 stimulation induces only very modest changes in chromatin accessibility in the MDA-MB-231 cell line. Those changes did not align with what I observed in H3K9 CUT&RUN seq experiments discussed in the previous chapter, again reinforcing that there is a temporal aspect to IL4-induced changes. Lastly, I show that a subset of glucose metabolism transcripts including hexokinase2, are significantly and reproducibly upregulated following IL4 treatment.

Gene Name	Fold Change in MC38 Array	Fold Change in E0771 Array
Aldolase C, fructose-bisphosphate	3.24	16.56
Dihydrolipoamide S-succinyltransferase	4.54	N/A
Glucose 6 phosphatase, catalytic, 3	2.62	1.87
Hexokinase 2	2.75	2.38
Phosphoenolpyruvate carboxykinase 2	3.96	N/A
Phosphofructokinase, liver, B-type	5.09	2.53
Phosphoglycerate mutase 2	3.36	2.58
Phosphoglycerate kinase 1	5.28	N/A
Phosphoglucomutase 3	5.15	N/A
Ribulose-5-phosphate-3-epimerase	4.10	N/A
Hexose-6-phosphate dehydrogenase (glucose 1dehydrogenase)	4.56	2.44

Table 3.1 Overlap in upregulation of glucose metabolism genes in microarray. Murine colorectal cancer cell line (MC38) and murine mammary carcinoma cell line (E0771) were stimulated with recombinant murine IL4 for 24 hours, and RNA collected was used in a commercially available glucose metabolism microarray. Genes with >2 fold upregulation in the microarray are reported here.

My data suggests that the IL4-dependent increase in glucose uptake in TNBC cell lines is affected by substrate availability during IL4 stimulation. When TNBC cells are stimulated with IL4 in media with no glucose, they do not demonstrate increased NBDG uptake *in vitro*. When stimulated in media containing 5mM glucose, IL4-induced increased NBDG uptake compared to control. In chapter 2 we discuss how IL4-induced glucose uptake is dependent on expression of GLUT1. IL4 could either increase expression of GLUT1 or affect its trafficking to mediate increased uptake. Our lab has previously shown that IL4 can induce expression of GLUT1(143). Glucose deprivation has been shown to increase expression of GLUT1(221), so initially it might be expected that cytokine stimulation in glucose-free media may potentiate IL4-mediated increased glucose uptake due to further induction of GLUT1 expression. Activation of PI3K/Akt (also activated by IL4 receptor stimulation) signaling has been shown to increase GLUT1 activity by affecting its trafficking(222). Determining whether IL4-mediated glucose uptake is dependent on changes in expression of GLUT1 or its trafficking would provide insight into why it is regulated by glucose availability. In chapter 2, I demonstrated that IL4-induced proliferation of TNBC cell lines is dependent on glucose uptake, so there is also a question whether abundance of glucose affects phenotypes of mammary cancer cells downstream of IL4 signaling. One hour of IL4 stimulated resulted in higher ATP production from glycolytic metabolism, while 24 hours of stimulation resulted in higher total and mitochondrial ATP production. At 8 days of IL4 stimulation we saw changes in production of extracellular lactate and glutamate. One question is whether there are changes in energy production at later time points where we see increased extracellular lactate and glutamate production.

The secretion of extracellular lactate by cancer cells can contribute to progression of breast cancer(223). Extracellular lactate contributes to an immunosuppressive tumor microenvironment. Lactate reduces the number of Th1 CD4+ T cells, and increase number of Treg cells in a model of prostate cancer(71). Lactate also contributes to the M2 polarization of macrophages, in an ACLY dependent manner, that showed increased histone acetylation and expression of M2 marker genes(160). Histone lactylation is a novel epigenetic modification that links glucose metabolism to genetic regulation(224) via uptake of extracellular lactate, and this

mechanism can drive immunosuppression (225). These effects of extracellular lactate on immune cells suggest that the effects of IL4 on lactate within a tumor microenvironment may be an important aspect of metabolic regulation by IL4 *in vivo*. I observed that the effect of IL4 on glutamate secretion appears to be cell line dependent. Targeting glutamine metabolism using glutaminase inhibitor CB-839 has been shown to attenuate progression of triple-negative breast cancer(106). Additionally, extracellular glutamate signaling through the metabotropic glutamate receptor 1 has been shown to promote angiogenesis(226), and inflammation(227) in breast cancer. Thus, it is likely that at least for some breast cancers, IL4-induced increases in extracellular lactate and glutamate could promote tumor aggressiveness.

Subcellular localization of ACLY and its subsequent acetyl-CoA production have been demonstrated to play an important role in its phenotypic effects(228). I show that IL4 increases phosphorylation of nuclear ACLY at serine 455. Translocation of ACLY to the nucleus has been shown to acetylate transcription factors, leading to full activation of signaling cascades(229). This ACLY driven acetylation of transcription factors may be relevant to IL4 signaling due to the fact that lysine acetylation of STAT6 – the main transcription factor associated with IL4R activation - has been shown to be both a positive(206), and negative regulator(230) of gene expression. ACLY has also been closely tied to histone acetylation to control both DNA repair(202), and gene expression in macrophages(79). Additionally, ACLY enzyme activity has been associated with metastatic progression in hepatocellular carcinoma(231). In my hands, targeting ACLY using the inhibitor BMS-303141 ablated IL4-induced proliferation in breast cancer cells. This result suggests that ACLY signaling is important for tumor outgrowth, and it would be useful to assess whether loss or neutralization of IL4 signaling is sufficient to decrease ACLY phosphorylation *in vivo*. I did not observe consistent changes in acetylCoA concentration, the product of ACLY activity, in IL4-treated TNBC cell lines. These measurements of acetyl-CoA concentration were obtained using a fluorescent assay reflective of a steady state levels which may not be reflective of the impact of IL4 signaling on acetyl-CoA production. One way to circumvent this limitation would be to utilize a mass spectrometry approach to perform metabolic flux analysis(232), and

assess incorporation of carbon from glucose into acetyl-coA and ultimately histone post translational modifications(233).

In chapter 2, I showed that acetylation of histone H3 at both lysine 9 and lysine 27 was increased after IL4 treatment of breast cancer cell lines. In an attempt to determine how much this impacted chromatin accessibility, I sent samples of MDA-MB231 cells treated with IL4 for different amounts of time for ATACseq. The results indicated that 18 hours of IL4 treatment was needed to see any changes in chromatin accessibility. However, even at this time point, there were relatively few genes with significant changes. It has been shown that NGS experiments such as ChIPSeq for activating histone marks can be integrated with regions from ATACseq data showing increased chromatin accessibility(234). There were some peak regions in genes showing significantly increased chromatin accessibility after 18-hour treatment that aligned with ENCODE tracks for H3K27ac (Fig 3.5), however the ENCODE data was not from IL4-treated cells. I compared the list of genes with increased chromatin accessibility from the ATACseq with the list of enriched genes from the previously described H3K9 Cut&Run experiment (chapter 2) but did not find any overlapping genes. This may be due to the differences in treatment in the two experiments. In the CUT&RUN experiment cells were treated for 24 hours with IL4, while the ATACseq was treated for 18 hours. The longer duration of treatment may be necessary to induce the acetylation identified in the CUT&RUN experiment. The few changes in chromatin accessibility seen in the shorter time point could also be due to other epigenetic changes. Although I focus on histone acetylation in these studies, removal of methylation is an important prerequisite for IL4 induced gene expression(155). Loss of repressive epigenetic regulation by removal of methylation could also be responsible for the ATACseq identified targets and may precede histone acetylation observed at the later time point used in CUT&RUN experiments. In the future, optimizing treatment duration and conditions will be necessary for a more useful ATACseq analysis.

In addition to the potential targets identified by ATACseq, I also did a more direct analysis of transcripts that could be potentially be upregulated following IL4 treatments using a qPCR array. Based on previous data from the lab that (i) showed Glut 1 protein

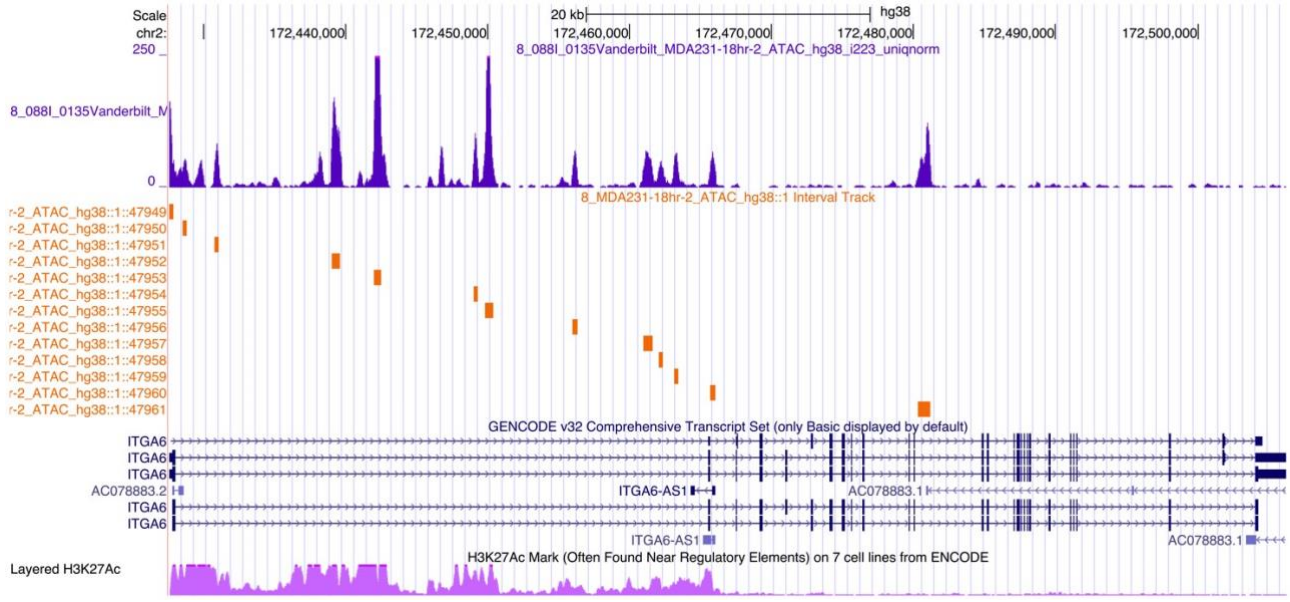


Figure 3.5 ATACseq peaks overlap with histone acetylation signal. Signal from ATACseq indicating increased histone accessibility overlap with H3K27 acetylation signal from ENCODE.

expression was increased after IL4 treatment(143), and (ii) that murine colorectal cancer cells responded to IL4 treatment by increasing levels of some glucose metabolism gene transcripts (unpublished data). I examined whether there were similar increases in those transcripts in a murine TNBC cell line. Interestingly, almost the same list of transcripts was upregulated in the murine mammary cell lines as in the murine colorectal line. However, it should be acknowledged that these genes were not identified by RNAseq analysis of IL4-treated and untreated human MDA-MB-231 cells (discussed in chapter 2). This may be because of the difference in methodology – or a difference between human and mouse. However, the increase in Glut1 protein that was part of the reason for this analysis was observed in human (MDA-MB-231) and murine (4T1) TNBC cell lines. One open question that could support a feed-forward mechanism is whether histone acetylation, resulting from enhanced glucose uptake is necessary for IL4 to increase expression of these glucose metabolism-related transcripts.

Altogether, the data presented here gives further insight in the regulation of metabolism in TNBC by IL4. Increased glucose uptake, and downstream metabolism appears to be modulated both by duration of IL4 stimulation, and availability of substrate. Getting a better understanding of how IL4 induces glucose uptake, and how longer term IL4 stimulation affects TNBC cell metabolism would elucidate other ways that type Ⅱ signaling may contribute to tumor progression.

Chapter Ⅳ: Inhibition of type Ⅱ IL4 Signaling in Triple-Negative Breast Cancer

Introduction

As discussed in the first chapter there are two receptors for interleukin 4. The type I receptor consists of IL4Rα and γc receptor subunits, which is primarily expressed by hematopoietic cells. The type II IL4 receptor consists of IL4Rα and IL13Rα1 and is expressed predominantly by non-hematopoietic cells including epithelial cells. Although there is some overlap between the two receptors, they also have distinct functions due to their different subunits(235). Signaling through both receptors has been demonstrated to play roles in cancer progression. In the context of cancer, IL4 is largely considered an immunosuppressive cytokine, and signaling through the type I receptor has been shown to support tumor progression by reducing anti-tumor immunity(236). In addition to the immunomodulatory effects mediated by the type I receptor, signaling intrinsic to cancer cells expressing type II Il4 receptor can also contribute to tumor progression. Type II signaling in epithelial cells has been shown to drive proliferation(158,237), and tumor initiating characteristics(180) of epithelial cells.

Our lab has previously demonstrated via genetic manipulation that loss of type II IL4 receptor signaling in breast cancer cells attenuates metastatic colonization(135). Inhibitors of IL4 signaling under clinical investigation, such as dupulimab affect both type I and type II receptor(172). To further confirm the findings of our previous studies and investigate the viability of type II signaling as a therapeutic target, a more specific inhibitor that only affects the type II receptor is necessary. Fortunately, the Garcia lab at Stanford identified a dominant negative mutant of IL13 (DNIL13) that was licensed to a biotech company called Medicenna and renamed MDNA413. This dominant negative mutant of IL13 was discovered in a study designed to explore the role of affinity between IL13 and the IL13Rα1 subunit(220) in modulating the kinetics of downstream signaling. Using a yeast surface display of IL13Rα1, and a random mutagenesis library of IL13-derived peptides focused on mutations in “site III” (the domain of the protein

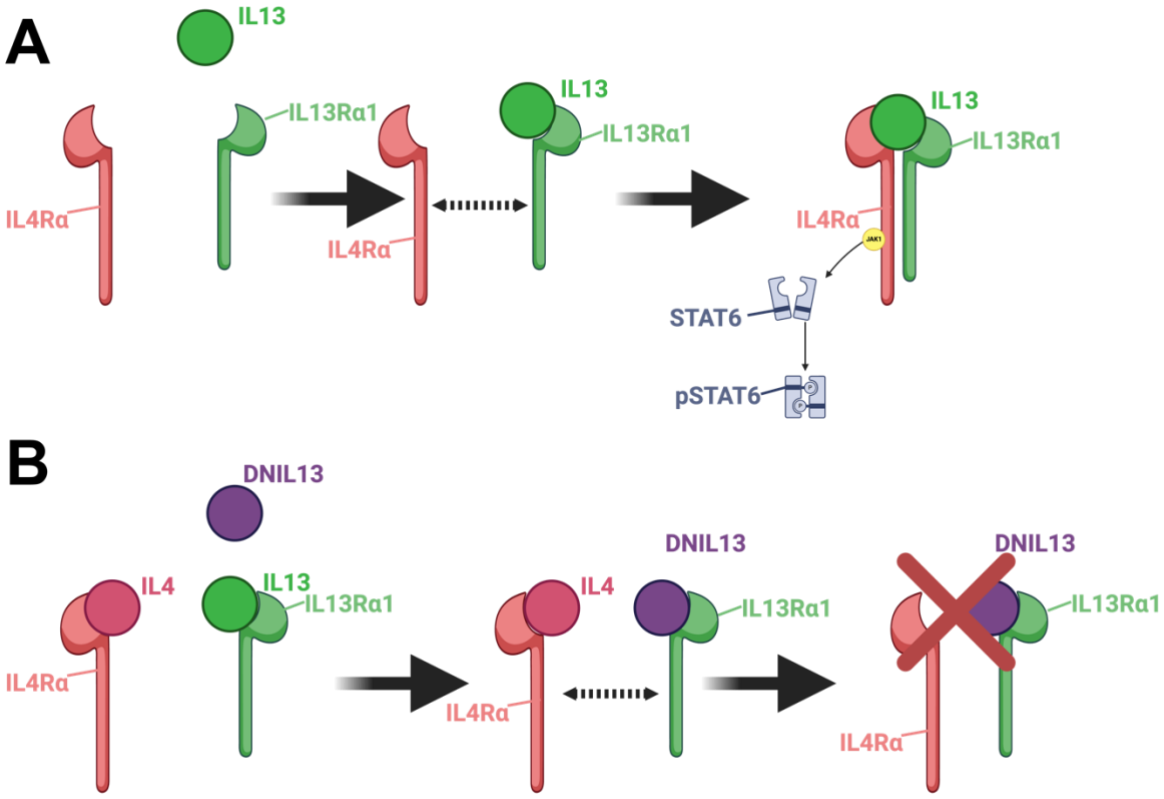


Figure 4.1 Mechanism of DNIL13. (A) In normal signaling, ligand binding to a receptor subunit allows for the dimerization, and activation of signaling pathways. **(B)** DNIL13 inhibits dimerization of type Ⅱ receptor in the presence of IL4 or IL13. Due to its higher affinity DNIL13 outcompetes IL13 and binds to IL13Rα1 and prevents the subsequent dimerization and activation of downstream signaling. Created using biorender.

largely responsible for interaction with IL13Rα1), the authors identified mutants with altered affinity for the receptor subunit. DNIL13 was one of the mutants from the library that displayed increased affinity for the IL13Rα1 subunit. DNIL13 also demonstrated decreased affinity for IL4Rα and did not bind to this receptor subunit. This decreased affinity for IL4Rα allowed DN to bind IL13Rα1 and prevent the subsequent dimerization necessary for signaling through the type II Il4 receptor(Fig 4.1)(238).

Due to its increased affinity for IL13Rα1 compared to wild type IL13, and inability to stimulate type II Il4 signaling, Medicenna are exploring DNIL13/MDNA413 in preclinical investigations as a competitive inhibitor of type II IL4 signaling in cancer. We were fortunate to obtain a baculoviral construct for DNIL13 directly from the Garcia lab, which our lab expressed and purified for use in initial studies. We also obtained pharmaceutical grade purified protein from Medicenna to enable our analysis of whether specific blockade of the type II IL4R could impact tumor progression.

Results

In Vitro Inhibition of Type II Signaling in Triple-Negative Breast Cancer Cell Line

We first investigated the *in vitro* effects of DNIL13 on signaling in the triplenegative breast cancer cell line MDA-MB-231. Since the type II IL4 receptor consists of IL13Rα1 and IL4Rα, both IL4 and IL13 are cognate ligands for the receptor. Cells were pre-treated with varying concentrations of DNIL13 for 10 minutes. Cells were then treated with 20ng of either human recombinant IL13 or IL4. In these experiments we used the phosphorylation of STAT6 as an indicator of IL4 signaling activity. We found that DNIL13 decreased STAT6 activation induced by IL4 and IL13 in a dose dependent manner (Fig 4.2).

Effects of DNIL13 on Metastatic Progression

To confirm the importance of type II signaling in metastatic progression of TNBC we started work with experimental metastasis models in vivo. In other models, the predominant anti-tumor effect of generally neutralizing IL4 signaling appeared to be

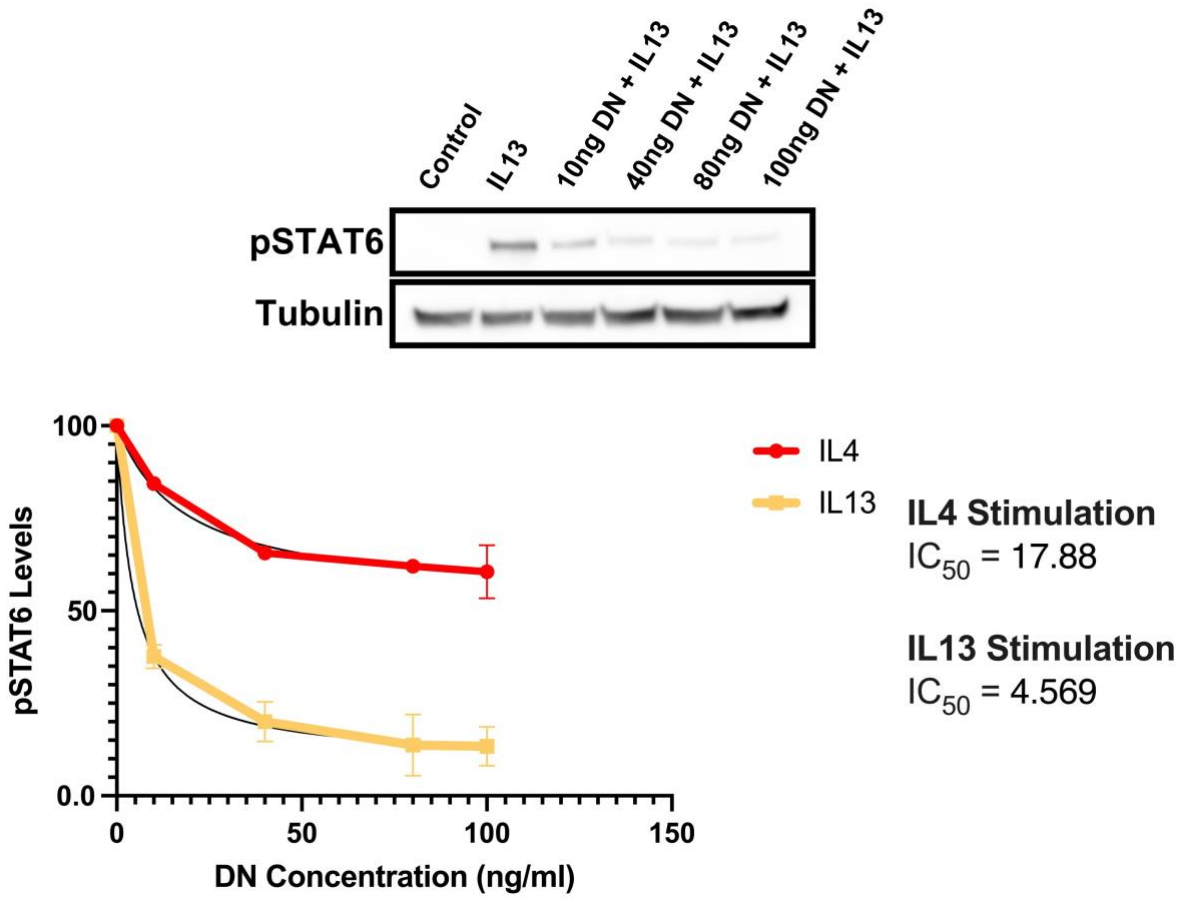


Figure 4.2 DNIL13 inhibits *in vitro* signaling in TNBC. MDAMB231 cells were treated with 20ng IL4 or IL13 + or – 10ng, 40ng, 80ng or 100ng DNIL13. Lysates were collected 10 minutes following cytokine stimulation. Western blots were performed on total lysates from these cells for pSTAT6. pSTAT6 levels were used to calculate the IC_{50} of DNIL13 in blocking STAT6 activation of IL4 and IL13 in MDAMB231 cells.

dependent on certain immune cell subsets(134,236). Additionally, the main producers of IL4 in vivo are lymphocytes, thus these cells should be present and secreting cytokine in order to fully assess the effect of blocking the type II IL4 receptor. Since we are most interested in effects of this inhibition in human disease, we chose a model of BT549 human TNBC cells injected into humanized Nod/Scid/Gamma (NSG) mice (239). The mice had been engrafted with cord blood-derived hematopoietic stem cells and were designated as huNSG mice. We obtained the huNSG mice from Jackson Labs 20

weeks after engraftment. At this point, all mice had a minimum human hematopoietic percentage of 27%, with an average across all mice of 56%. Tail vein injection of BT549 was performed to establish pulmonary metastases. Mice were treated with intravenous injection of either DNIL13, or an IgG control, twice a week for 3 weeks at a dosage of 1 mg per animal per treatment, based on suggested dosing from Medicenna. IgG was used as the control since the DN1L13 construct included the Fc domain from human IgG.

Effects of DNIL13 on *In Vivo* Metabolism

As discussed in chapter 2, our previous studies indicated that type II IL4 signaling increases glucose uptake in triple-negative breast cancer, and IL4-induced proliferation is dependent on GLUT1 mediated glucose uptake. Our lab has also previously shown that IL4 increases glutamine uptake and that inhibition of glutaminase using inhibitor 968 ablates IL4-induced proliferation(143).To assess whether blockade of the type II IL4 receptor would attenuate glucose or glutamine uptake in vivo, we used positron emission tomography (PET) imaging of a subgroup of the treated mice immediately prior to the termination of the experiment. In collaboration with the Vanderbilt University Institute of Imaging Science (VUIIS) small animal imaging group, mice were injected with ^{18}F-fluorodeoxyglucose or ^{18}F-glutamine and underwent PET along with a computed tomography (CT) scan to assess glucose uptake in regions of interest in the lung. Mice treated with DNIL13 had lower glucose uptake in lung metastases compared to mice receiving the IgG control (Fig 4.3). This *in vivo* decrease in glucose uptake in DNIL13 treated mice may be reflective of the IL4-induced glucose uptake observed *in vitro*, in TNBC cell lines. It is worth noting that tumor infiltrating

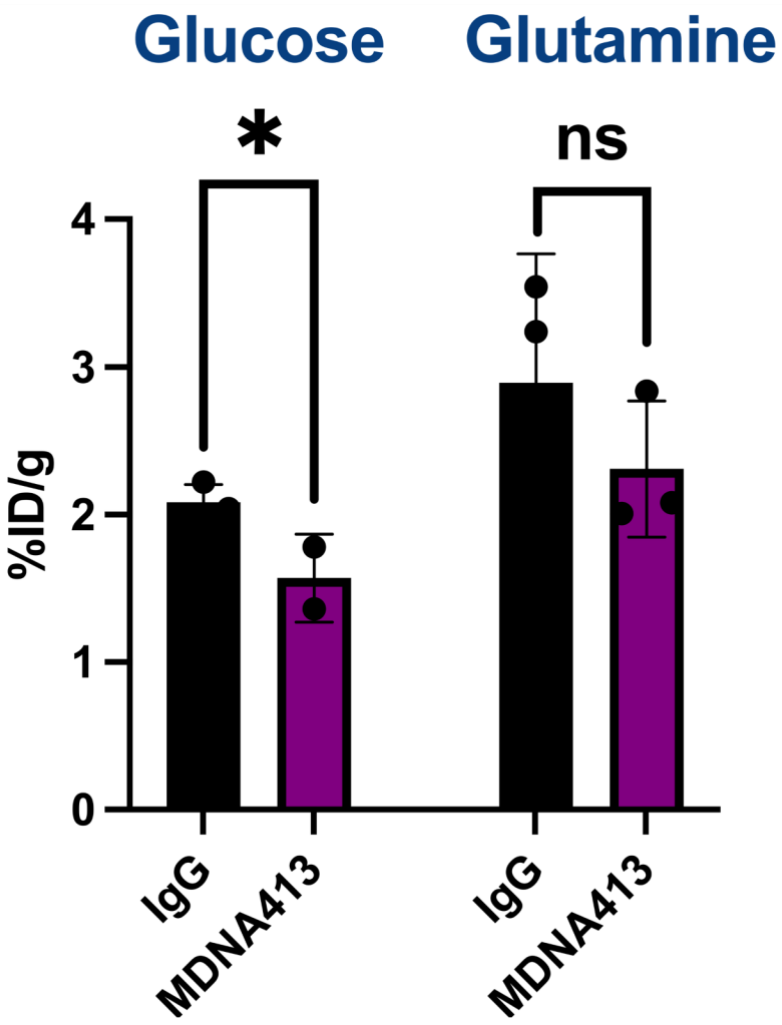


Figure 4.3 DNIL13 decreases glucose uptake. PET imaging of lungs of HuNSGSGM3 mice bearing BT549 tumors 20 minutes after injection with either ^{18}Ffluorodeoxyglucose (FDG; left) or ^{18}F-glutamine (right). Signal is represented as percent of the injected dose detected per gram. Mice receiving DNIL13 demonstrated lower signal. *p<0.05.

immune cells uptake a significant amount of glucose available in the tumor microenvironment(240), and that differences in FDG signal may be reflective of changes in glucose uptake in immune cells rather than cancer cells themselves.

One day post imaging, after all of the isotope had cleared, the mice were euthanized and the lungs were resected, fixed, and paraffin embedded. Tumor burden was assessed in hematoxylin and eosin-stained sections of the lungs. Analysis of pulmonary metastases area showed that mice treated with DNIL13 had significantly lower tumor burden in the lungs (Fig 4.4). This data indicates that pharmacological inhibition of type II IL4 receptor attenuates metastatic colonization of triple-negative breast cancer.

Effects of DNIL13 on Tumor Infiltrating Immune Cells

IL4 is largely studied as an immunosuppressive cytokine in cancer, however varied roles of IL4 in supporting antitumor immunity have also been elucidated(241). One of the more well studied functions of IL4 signaling in antitumor immunity is the role of polarizing macrophages to the M2 phenotype, and downregulating antitumor immunity(242). To determine if type II receptor inhibition affects polarization of macrophages, we performed a preliminary experiment in the previously discussed *in vivo* model using DNIL13. Tumor infiltrating macrophages from lung metastases were stained for the M2 macrophage marker mannose receptor. In this initial study macrophages from mice treated with DNIL13 appeared to show lower expression of mannose receptor (Fig 4.5A). Another important aspect of antitumor immunity is the number of infiltrating immune cells(243). An important subset is CD8+ cytotoxic T cells, which can be an important prognostic indicator in various cancers(244). In mice treated with DNIL13 there was no significant difference in the number of tumor-infiltrating CD8+ T cells (Fig 4.5B). This data indicates that type II signaling inhibition may be sufficient to attenuate the immunosuppressive polarization of macrophages similar to what is observed with inhibitors of both type I and type II signaling(171,236). However, the role of type I signaling in modulating antitumor immunity in this context requires further investigation, and characterization of other immune cell subsets.

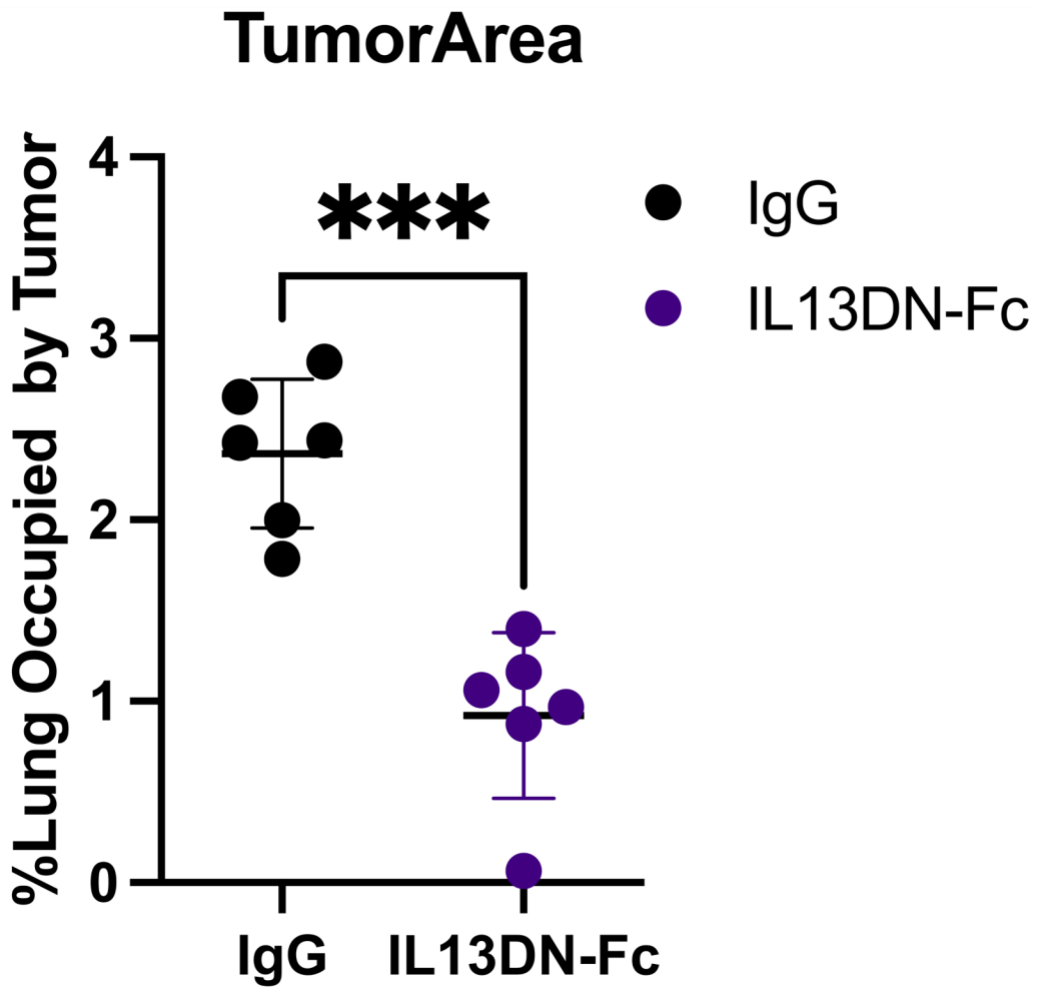


Figure 4.4 DNIL13 reduces lung tumor burden. Pulmonary tumor burden in HuNSGSGM3 mouse experimental metastasis model was measured. Mice treated with the type Ⅱ IL4 receptor targeting drug DNIL13 showed decreased tumor burden. ***p<0.001

Discussion

Here we demonstrate the viability of pharmacological targeting of type II IL4 receptor signaling to attenuate metastatic progression of TNBC. We show that DNIL13 is a potent *in* vitro inhibitor of both IL4 and IL13 signaling through the type II receptor in TNBC cell line. Treatment with DNIL13 in experimental metastasis models show that type II inhibition decreases pulmonary metastasis, and FDG uptake *in vivo*. In preliminary experiments tumor-associated macrophages from tumors in DNIL13 treated mice appeared to show lower numbers of mannose receptor-expressing macrophages suggesting reduced M2 polarization There was no significant difference in the number of tumor-infiltrating CD8+ lymphocytes in treated mice.

Although this initial work is promising there is still further work needed to fully elucidate the impact of type II inhibition on tumor progression. We show that DNIL13 inhibits both IL4 and IL13 induced STAT6 activation through the type II receptor in a TNBC cell line. Since the ligands for both receptor subunits are blocked equally well, as shown by the similar IC50 values, the mechanism of action via prevention of subunit heterodimerization is supported. Further characterization of the effects of DNIL13 *in vitro* would be a necessary step to understand its *in vivo* effects. Here we show that DNIL13 inhibits the activation of STAT6 signaling downstream of the type Ⅱ receptor. Characterizing how it impacts activation of other signaling pathways such as Akt/ACLY, MAPK, or IRS signaling *in vitro* would give insight into what phenotypes such as proliferation, survival, acetylation, or glucose uptake are affected by DNIL13 in TNBC cells *in vivo*. Understanding how DNIL13 affects cells with differing levels of expression of IL4/IL13 receptor subunits might provide an indicator of what patients would benefit most from targeting IL4/IL13 signaling.

When used in an experimental metastasis model in vivo, we found that DNIL13 resulted in decreased metastatic burden in the lung. However, it is unclear whether this decrease in metastasis is because of DNIL13 acting directly on cancer cells expressing type II receptor, and/or effects on the immune system. The experiments shown here were

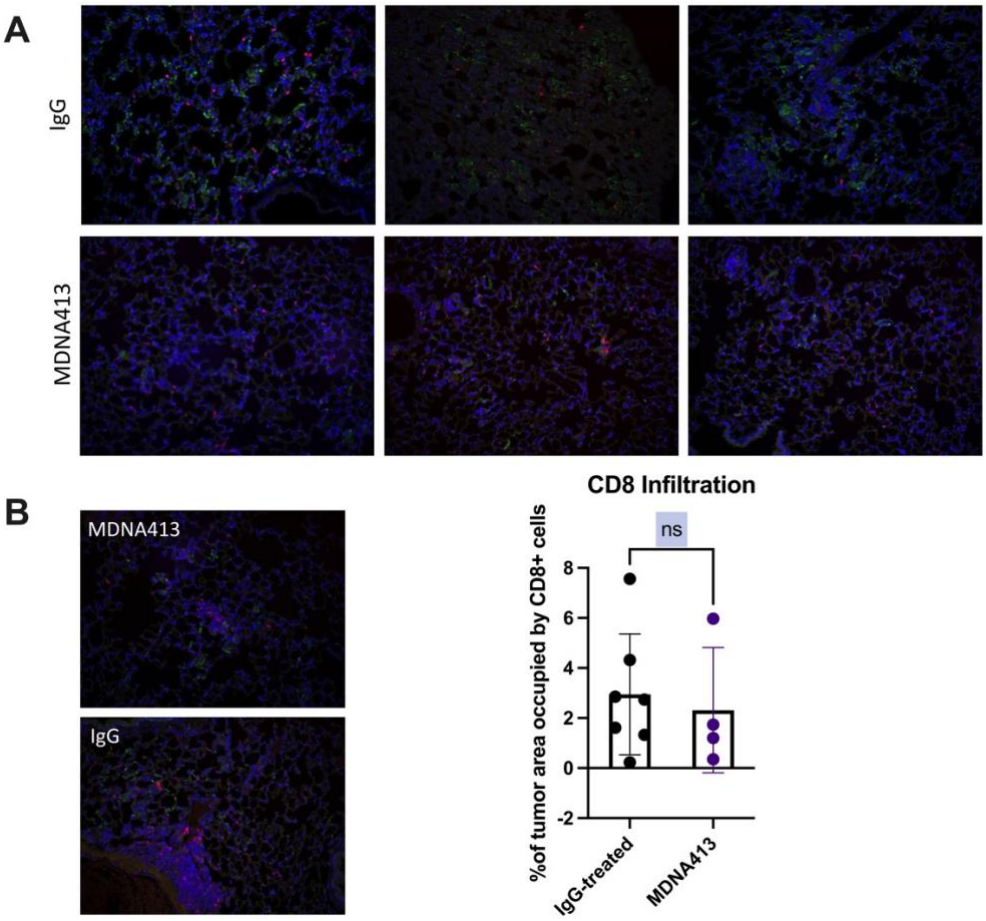


Figure 4.5 Impact of DNIL13 on immune cell infiltration. (A) M2 macrophage infiltration measured by mannose receptor staining (green fluorescence) in lung parenchyma of BT549 tumor bearing HuNSG-SGM3 mice. MDNA413 mice appear to have fewer mannose receptor-expressing cells. **(B)** Measurement of CD8+ T lymphocytes (red fluorescence) infiltrating lung tumors in BT549 tumor bearing HuNSDSGM3 mice. MDNA413 did not appear to reduce the number of infiltrating CD8+ T cells. performed in humanized mice. Immune cells can act as both regulators of tumor development(170) or as sources of IL4(242). These experiments could be performed in fully immunocompromised mice so that any effect of DNIL13 on tumor development could be limited to effects on tumor cells. A potential concern is that relatively little IL4 would be available without immune cells to produce it. This makes it difficult to discern

the contribution of DNIL13 action of tumor cells versus immune cells. Our lab has previously characterized the impact of type II signaling on TNBC *in vitro*. In that work we demonstrated that IL4-induced proliferation is dependent on multiple signaling pathways(135), increased glucose uptake, and histone acetylation(245). Assessing how DNIL13 impacts these aspects of cancer cells *in vivo* is important to determine the relevance of cancer cell type II signaling in tumor progression. We would expect that cancer cells from mice treated with DNIL13 would show downregulation of pathways such as Akt/ACLY, STAT6, and MAPK signaling *in vivo*, and decreased signal for proliferation markers such as Ki67 or phospho-histone H3.

Using FDG we show that DNIL13 decreased glucose uptake of lung metastases *in vivo*. This is in line with *in vitro* observations that IL4 signaling increases glucose uptake of mammary cancer cell lines. We have shown that glucose uptake is vital for IL4-induced proliferation of mammary cancer cell lines *in vitro*, indicating that decreased FDG uptake in DNIL13 treated tumors may be an important mechanism for attenuating metastatic progression. It is worth noting that while we observe lower glucose uptake *in vivo*, this change may be attributed to tumor resident immune cells. Myeloid cells in particular have been shown to be the subset of immune cells that utilize the most glucose in the tumor microenvironment(240), and glycolysis has been shown to modulate IL4 stimulated macrophage effector functions(195). Thus, the reduced glucose uptake that we observed may be reflective of reduced macrophage infiltration rather than a change in the levels of glucose being consumed by the tumor cells. An alternative explanation is that the tumors are smaller in the DN-treated mice, which could also explain the reduced PET signal. However, it is worth noting that a glutamine tracer showed no difference in signal between the IgG and DN-treated mice.

We also investigated the impact of type II signaling inhibition on immune cells. A preliminary analysis suggested that there were fewer tumor-associated macrophages expressing the M2 marker mannose receptor in mice treated with DNIL13. This could indicate that inhibition of type II Il4 signaling alone may prevent the M2 polarization of tumor-associated macrophages. This is similar to what is observed in models where both type I and type II receptor signaling is inhibited in breast cancer(236,246). In these

cases, depletion of M2 macrophages in the tumor microenvironment results in increased number of activated CD8+ T lymphocytes, and reduced the number of regulatory T cells(246). In addition to effects on antitumor immunity, M2 polarized tumorassociated macrophages can secrete paracrine factors that support tumor progression. These include EGF, which has been shown to drive invasion of breast cancer cells in an EGFR dependent manner(134), and cathepsin protease which can induce angiogenesis and invasion *in vivo*(247).

An important distinction of targeting type II signaling is that IL4 may still affect antitumor immune response by signaling through the type I receptor expressed predominantly by hematopoietic cells. Although we do not see any change in the number of CD8+ tumor infiltrating lymphocytes in mice treated with DNIL13, that does not exclude that IL4 signaling may affect the effector functioning of CD8+ T cells. Higher concentration of circulating IL4 has been associated with decreased T cell response in non-small cell lung cancer patients(248). However, IL4 has been shown to have varied roles in antitumor immunity depending on the context. On one hand IL4 has been shown to induce loss of CD8 expression in lymphocytes(249), and leads to poor cytolytic functioning(250). This loss of CD8 expression and cytolytic function has been shown to be long lasting and can impact antitumor immunity *in vivo*(251). In contrast IL4 has been shown to induce proliferation of CD8+ T cells(252). Stimulation of CD8+ T cells by IL4 can also reduce expression of CD39 and inhibit adenosine-mediated immunosuppression(253). These contradictory effects of IL4 signaling on antitumor immunity can also be observed in clinical and preclinical models of immunotherapy. In one example treatment with dupulimab, an IL4R-blocking antibody that acts as an inhibitor of both type I and type II IL4 signaling, demonstrated synergy and improved response to anti-PD1 therapy in non-small cell lung cancer(172). In a model of colon cancer, activity of anti-PD1 antibody was dependent on IL4 expression(219).

In total we show that inhibition of type II signaling attenuates metastatic outgrowth in a mouse model of triple-negative breast cancer. Our initial studies reveal changes in the tumor microenvironment, such as decreased glucose uptake, and infiltration of M2 macrophages. There is still much work needed to understand the

mechanics driving loss of tumor progression, and to distinguish between the effects of loss of type II signaling in cancer cells, vs immune cells in the TME.

Chapter Ⅴ: Implications and Future Directions

Dissertation Summary

Interleukin 4 signaling in the context of breast cancer has largely been investigated for its impact on alternative polarization of macrophages(254). Our lab has previously shown that IL4 contributes to metastatic progression of breast cancer as a result of signaling through the type II receptor expressed by breast cancer cells(135) in addition to its effects on immune cells. The data presented in this dissertation show mechanisms of how type II IL4 signaling in TNBC may contribute to disease progression and provide preliminary evidence that pharmacological targeting of type II receptor ablates metastatic colonization. In chapters 2 and 3, I show that IL4 increases glucose uptake, and histone acetylation in TNBC. Increased glucose uptake was dependent on the expression of GLUT1, and IL4-induced proliferation was ablated with knockdown of GLUT1. Although there was a significant increase in glucose uptake, there were not large changes in ATP production. The effects of IL4 on TNBC metabolism vary depending on time of exposure. IL4-induced gene expression was shown to be dependent on histone acetyltransferase activity of p300, an important cofactor of STAT6. Inhibition of p300 by C646 also prevented proliferation of TNBC cell lines stimulated by IL4. In chapter 4 we investigate use of DNIL13, an inhibitor of specifically type II IL4 signaling, and show that it blocks signaling downstream of IL4 and IL13. Using *in vivo* models of humanized mice, we show that DNIL13 prevents pulmonary metastasis, and appears to reduce the number of M2 polarized macrophages infiltrating lung metastases. These findings are summarized in Fig 5.1.

Role of Type II IL4 Signaling in Other Breast Cancer Clinical Subtypes

Although we focus on the effects of IL4 signaling in TNBC, there is a possibility type II signaling is relevant in other clinical subtypes. We focus on TNBC because of its worse clinical outcomes(124). The publicly available microarray data shown in chapter 2 suggests that the association of IL13Rα1 expression with lower survival is largely

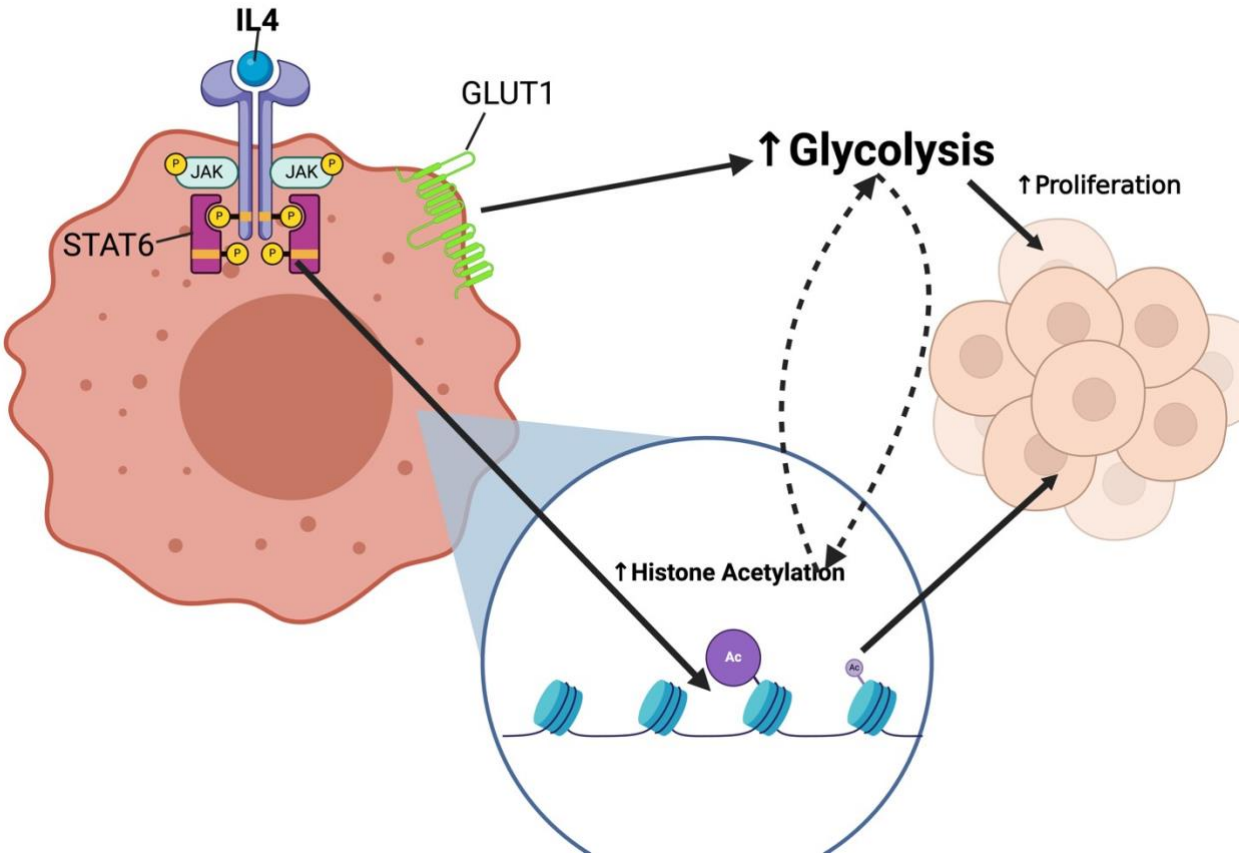


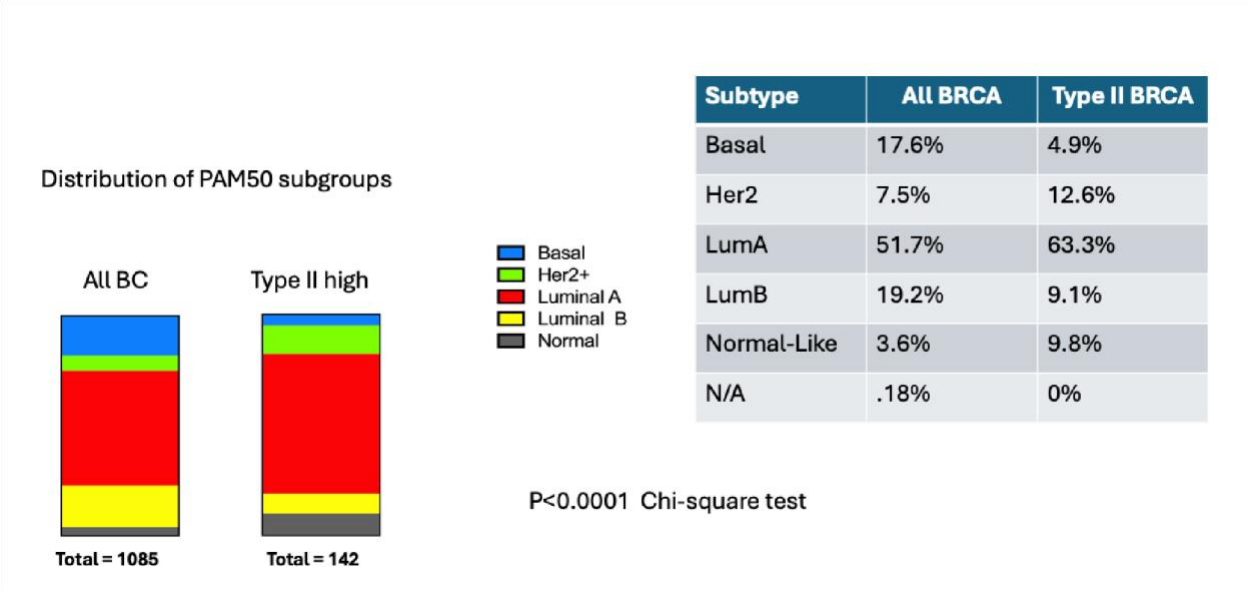
Figure 5.1 Summary figure of working model. IL4 induces increased glucose uptake, and histone acetylation in TNBC cell lines. Type Ⅱ signaling-induced proliferation is dependent on IL4-induced glucose uptake and histone acetylation. Created using biorender.

carried by TNBC with other subtypes showing no correlation. The expression of single subunits is not necessarily reflective of type II signaling, as expression of IL13Rα1 without simultaneous expression of IL4Rα would not allow type II signaling. TCGA data was used to determine whether there was a correlation between clinical subtype and expression of type II IL4 receptor. Patients in the upper tertile of expression for both IL13Rα1 and IL4Rα, were defined as type II receptor expressing. The distribution of clinical subtypes between all available breast cancer patients, and type II receptor expressing patients was compared (Fig 5.2). The number of HER2+ patients among the type II receptor expressing cohort was increased compared to all breast cancer patients. Akin to TNBC, HER2+ breast cancer patients have higher rates of metastasis to brain,

lung, and liver compared to luminal subtypes(126). Targeting IL4 signaling in HER2+ breast cancer may reduce metastasis similarly to what we have shown here in TNBC. Studies using HER2+ cell lines, and with transgenic mouse models of HER2+ mammary cancer would be a valuable extension of the studies already done.

Further Investigation of DNIL13

The data shown in chapter 4 demonstrate that DNIL13 inhibits activation of STAT6 signaling downstream of IL4 and Il13 in TNBC cell lines. There is a need for further characterization of the impact of DNIL13 on IL4-induced signaling in TNBC. Of particular interest is activation of Akt, and phosphorylation of ACLY, which I have shown it essential for IL4-induced proliferation *in vitro*. In addition to effects on signaling, determining whether DNIL13 can also inhibit increased glucose uptake and histone acetylation *in vitro* would be important to do. Our *in vivo* data show that DNIL13 decreases apparent M2 tumor infiltrating macrophages as determined by their expression of the hallmark protein mannose receptor (CD206). To fully characterize the impact of DNIL13 on tumor-associated macrophages the expression of additional M2 markers such as Arg1, Retnla, CCL22, and IL10 should also be evaluated. M2 polarized macrophages have been shown to secrete paracrine factors that drive metastasis, invasion, and angiogenesis such as EGF(134), and cathepsin proteases(247). Assessing how DNIL13 impacts the expression of these paracrine factors in tumor infiltrating



Subtype	All BRCA	Type II BRCA
Basal	17.6%	4.9%
Her2	7.5%	12.6%
LumA	51.7%	63.3%
LumB	19.2%	9.1%
Normal-Like	3.6%	9.8%
N/A	.18%	0%

Fig 5.2 Distribution of clinical subtypes in type Ⅱ receptor expressing breast cancer. Breast cancer patients in the upper tertitle for expression of both IL13Rα1 and IL4Rα were defined as type Ⅱ expressing. The clinical subtypes of all breast cancer patients, and type Ⅱ expressing patients was obtained and a chi square analysis shows that there is a significant difference in the distribution between all breast cancer patients and type Ⅱ patients.

macrophages would provide further insight into how targeting type II IL4 signaling can attenuate tumor progression.

DNIL13 specifically inhibits type II IL4 signaling while leaving type I signaling active. Drugs currently under investigation for clinical use typically inhibit both type I and type II IL4 signaling. To determine whether targeting type II signaling via DNIL13 has comparable efficacy to targeting both type I and type II signaling in TNBC, a head-tohead comparison of DNIL13 to a drug like dupulimab, which blocks IL4R, would be required. In animal models where both type I and type II signaling is inhibited, the number of activated CD8+ T cells is increased and the number of T regulatory cells is decreased(246). Although the number of CD8+ tumor infiltrating T cells was not changed in DNIL13 treated mice (Fig 4.5), that does not preclude that DNIL13 may affect antitumor immunity in other ways. Type I IL4 signaling can either promote or inhibit antitumor immunity depending on the context. IL4 signaling can decrease

expression of CD8 in T cells resulting in ablated cytolytic function(251). In contrast IL4 can also reduce CD39 expression in CD8+ T cells resulting in attenuated adenosinemediated immunosuppression in response to melanoma(253). Assessing CD8 expression, and CD39 expression in models of treatment with DNIL13, or inhibition of both type I and type II signaling would allow us to assess whether type I IL4 signaling is of benefit to the effector function of CD8+ T cells in the context of TNBC. Type I IL4 signaling has also been shown to play a role in regulation of Tregs, decreasing expression of Treg marker Foxp3(255), and also inhibiting expression of CTLA-4 in regulatory T cells(256). Assessing expression of CTLA4 and Foxp3 in CD4+ T cells would provide further insight into the effects of type I IL4 signaling in TNBC. IL4 also enhances expression of activating receptor NKG2D in natural killer cells, and improves cytotoxic response to melanoma(257). Assessing the expression of these markers of CD8+ effector function, regulatory T cells markers, and natural killer cell activation in DNIL13 treated mice, as well as contrasting them with treatment with therapeutics such as dupulimab would elucidate the contributions of type I versus type II IL4 signaling in regulating antitumor immunity.

Inhibition of IL4 signaling is also under investigation for synergy with other therapies. Whether inhibition of type II signaling alone is adequate to induce synergistic response with other therapies is not known. Combination with immune checkpoint blockade is one of the ways that IL4 targeting therapies are being investigated. In models of non-small cell lung cancer, treatment with IL4R targeting drug dupulimab resulted in stronger response to anti-PD1 therapy leading to decreased tumor burden, and stronger cytotoxic CD8+ T cell response(172). Further, in a small clinical trial based on this data, patients with progressive disease that had already received anti-PD-L1 therapy, showed that administration of dupulimab resulted in decreased tumor burden, with one patient showing a near-complete clinical response on PET scan. This data indicates that inhibition of type I and type II IL4 signaling may enhance response to ICB therapy. On the other hand data from pre-clinical models of colorectal (MC38), and lymphoma (EG7) suggest that activation rather than blockade of IL4 signaling actually enhances response to anti-PD1 therapy(219). This suggests that the impact of IL4 on ICB may be context dependent, particularly concerning disease type. ICB can improve

clinical response in TNBC(258), and determining whether type II signaling inhibition with DNIL13 can improve response is an intriguing avenue for further research. In addition, IL4 signaling has been shown to improve resistance to chemotherapy and radiotherapy in breast(171), and rectal cancer models(259). In breast cancer this resistance is shown to be mediated by the presence of M2 polarized macrophages, which my preliminary data suggests are reduced by DNIL13. Thus, synergy of DNIL13 with chemotherapy, or radiotherapy is another interest for investigating therapeutic efficacy of targeting type II IL4 signaling.

Further Investigation of Epigenetic Regulation

The experiments in chapters 2 and 3 show that IL4 induces histone acetylation in TNBC cell lines. I focus on histone acetylation because IL4 activates signaling associated with acetyl-CoA production and the transcription factor STAT6 associated with histone acetyltransferase p300(260). There are other epigenetic modifications associated with IL4 signaling that may be relevant to TNBC. DNA demethylation is required for expression of some STAT6 target genes(155). IL4-induced DNA demethylation in dendritic cells and macrophages is dependent on TET2. IL4 is also known to induce expression of the lysine demethylase KDM6B (also known as JMJD3) in macrophages(209), and IL4-induced expression of CCL17 is dependent on KDM6B expression in monocytes(261). Assessing how IL4 changes expression of TET2, and KDM6B in TNBC cell lines would give an indication of whether these enzymes regulate demethylation downstream of type II receptor signaling. To assess how IL4 affects DNA methylation in TNBC, bisulfite-seq may be used. To determine how IL4 impacts histone methylation, techniques similar to the acetylation analysis techniques from chapter 2 could be used. Of particular interest would be removal of repressive methylation marks at H3K9 and H3K27, which would than allow activating acetylation marks to be added. It is worth noting that the ATACseq experiment discussed in chapter 3 showed relatively few changes in chromatin accessibility. This may indicate that major changes in methylation should not be expected as a result of IL4 stimulation, or that optimization of conditions for ATACseq are necessary.

Further Investigation of IL4-Mediated Metabolism

The data presented in chapter 3 show that IL4 induces increased glucose uptake in TNBC cell lines. Using the seahorse ATP rate assay I found that 1 hour of IL4 stimulation increases glycolytic ATP production, however 24 hours of IL4 stimulation led to increased mitochondrial ATP production. At 8 days of IL4 stimulation there were changes in production of downstream metabolites lactate and glutamate. Whether energy production is also different at these later times needs to be assessed. Extracellular lactate has been associated with progression of breast cancer(223). Expression of MCT4, the transporter responsible for secretion of lactate, drives invasion of breast cancer cells(262,263). Our lab has previously shown that IL4 increases glutamine uptake by mammary cancer cells(143). I show that IL4 induces production of glutamate in MDA-MB-231 cell line. Metabotropic glutamate receptors are associated with angiogenesis in breast cancer(226). Characterization of how IL4 affects expression of these transporters in TNBC cell lines would give insight into the secretion and/or uptake of downstream metabolites. IL4 induces histone acetylation and phosphorylation of ACLY in TNBC. Although this signaling is associated with increased acetyl-CoA production, steady state measurements of acetyl-CoA concentration in IL4-treated TNBC cells show no significant difference in the concentration. Use of mass spec for metabolic flux may better elucidate the utilization of glucose for production of acetyl-CoA and incorporation into histone acetylation. I've performed an initial metabolic flux experiment in IL4 treated TNBC cell line in collaboration with the lab of Dr. Jamey Young. Although there are not large differences observed in these experiments which indicate that they may require further optimization as far as time point of IL4 treatment, the initial data from this pilot experiment corroborate the observation of increased lactate production because of IL4 treatment. (Fig 5.3)

In conclusion while my data have revealed some tumor intrinsic mechanistic roles of IL4-induced signaling, they also suggest some new directions for future research. Further understanding of IL4 and its impact on metabolism are needed to fully appreciate how it may contribute to tumor progression, particularly concerning production and secretion of metabolites into the TME. More investigation is needed to fully understand how type Ⅱ signaling in TNBC cells may contribute to tumor

progression, and to distinguish its impact from the effect of the IL4/IL13 signaling axis on immune cells. Further work using targeted inhibitors of type Ⅱ signaling such as DNIL13/MDNA413 will help to elucidate the role of TNBC intrinsic IL4 signaling.

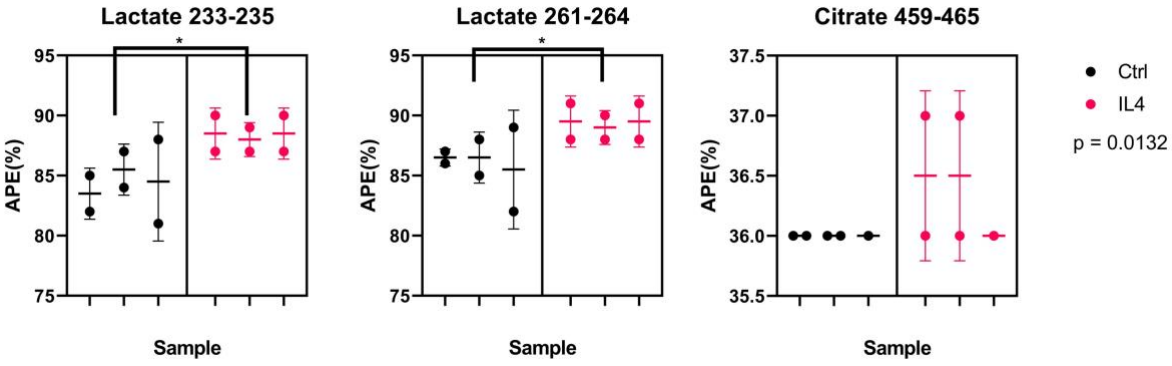


Figure 5.3 Small changes in flux of labeled carbons in lactate and citrate. The levels of isopotomers measured in control (black bar) versus following IL4 treatment (red bar).

Chapter VI:Methods & Materials

Reagents

Primary antibodies used in these studies include those targeting pAkt S473 (Cell Signaling Technology #4060S), Total Akt (Cell Signaling Technology #4691S), pACLY S455 (Cell Signaling Technology #4331S), Total ACLY (Cell Signaling Technology #13390S), B-Actin (Cell Signaling Technology #5125S), STAT6 (Santa Cruz Biotechnology sc-374021), H3K9ac (Active Motif #91103), H3K27ac (Active Motif #39133), and Histone H3 (Active Motif #39763). Control and secondary antibodies were Anti-Rabbit IgG, HRP-linked Antibody (Cell Signaling Technology #7074S), and CUTANA IgG Negative Control Antibody (Epicypher Cat# 13-0042). Other reagents used were CUTANA pAG-MNase for CHIC/CUT&RUN Workflows (Epicypher Cat# 151016), C646 (Selleck Cat#S7152), NBDG (Caymen Chemical #11046), Human IL4 (Peprotech #200-04), Human IL13 (Peprotech #200-13), Human IL4 (BD Biosciences #554605), Mouse IL4 (Peprotech #214-14), Mouse IL13 (Peprotech #210-13), Mouse IL4 (BD Biosciences #550067), Mouse IL13 (BD Biosciences #554599).

Cell culture

MDA-MB-231, BT549, and 4T1 cell lines were purchased from the American Type Cell Culture Collection ATCC). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Corning) containing 10% fetal bovine serum (FBS; Atlanta Biologicals) and supplemented with gentamicin (Corning) at 37^{o}C and 5% CO_2. Cell lines were used below passage 25.

Generation of GLUT1-deleted cells

MDA-MB-231 were transfected with ribonucleoprotein complexes formed between a mix of sgRNAs targeted to GLUT1 (Synthego) and Cas9 protein (Synthego) using lipofectamine CRISPRMAX reagent (Invitrogen) according to the Synthego standard protocol. 3 days-post transfection, cells were cloned out by limiting dilution. After

individual clones had sufficiently grown out, samples were processed for GLUT1 detection by western blotting. Clones with confirmed deletion were used for subsequent experiments.

Preparation of cell lysates and Quantification

Cells were washed in PBS and then scraped into RIPA buffer on ice and lysate transferred to Eppendorf tubes. Samples were spun at 10,000xg at 4^{o}C. Lysate were then transferred to new tubes and kept at -20^{o}C until use. Total protein concentration of lysates was determined using the BCA assay (Thermo Fisher).

Histone Isolation

Histones were extracted using an acid extraction method(264). After nuclear fractionation, the nuclear pellet was suspended in 0.4N H_2SO_4 overnight. The nuclear pellet in sulfuric acid was then centrifuged at 16000xg, and histones were precipitated in trichloroacetic acid.

Western blotting

Cells were treated with 20ng recombinant IL4 or recombinant IL13 prior to total lysate isolation. Cell lysates were run on SDS poly-acrylamide gels and subsequently transferred to nitrocellulose membrane and then blocked in 5% milk in TBS-T for 1 hour at room temperature. The membranes were incubated with the appropriate primary antibody in 5% milk in TBS-T at 4^{o}C overnight with shaking. This was followed by incubation with HRP-linked secondary antibodies for 1 hour at room temperature with shaking. Signal was detected using Clarity Western ECL Substrate (Biorad) with an Amersham Imager 600 instrument (GE Healthcare Life Sciences). Blots were stripped with Oneminute western blot stripping buffer (GM Biosciences) according to manufacturer’s recommendations and then reprobed with loading control antibodies (anti-b actin or anti-total histone H3, as appropriate).

Glucose Uptake Assays

NBDG uptake assay was performed as described previously(143).

Extracellular flux/ATP rate assays

MDA-MB-231 cells were plated at a density of 10,000 cells per well on XF Cell Culture Microplates (Agilent) at 37^{o}C and 5% CO_2. Cells were treated with 20ng human IL4 or 20ng human IL13 for 24 hours in DMEM. 1 hour prior to the assay medium was replaced with Agilent Seahorse XF DMEM pH 7.4. The plate was then placed in a CO_2free incubator for 1 hour at 37^{o}C. Experiments were run using a Seahorse XFe96 Analyzer. An ATP Rate assay kit (Agilent) was used following the manufacturer's protocol. Cells were stained with Hoechst solution, and ATP rate assay data were normalized to cell number counted using fluorescence.

HAT activity assay

MDA-MB-231 cell lines were treated with 10ng recombinant human IL4 (BD Biosciences) for 24 hours. After treatment nuclear extracts were collect using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). HAT activity was measured using the Histone Acetyltransferase Activity Assay Kit (Abcam) following manufacturer's protocol. The protein concentration of nuclear extracts was measured using BCA assay. HAT activity was normalized to the protein concentration of each sample.

Acetyl-Lysine immunoprecipitation

Cells were treated with 10ng recombinant human IL4 for 24 hrs. After being washed with PBS, cells were immunoprecipitated with the Signal Seeker Acetyl-Lysine Detection Kit (Cytoskeleton #BK163) following manufacturer's instructions.

CUT& RUN

MDA-MB-231 cells were plated at a density of 500,000 cells per plate in 60mm dishes and treated for 48 hours with 20ng recombinant human IL4 in DMEM. CUT&RUN was performed following CUTANA (Epicypher) protocol using anti-STAT6, and anti-H3K9ac, and CUTANA negative control antibodies. DNA fragments were generated into libraries using the NEB Next Ultra II DNA library Preparation kit. Sequencing was done via the NovaSeq6000 (PE150) with an average of 10-15M reads/sample. Library preparation and sequencing was performed by the Vanderbilt University Medical Center VANTAGE core. Raw data was provided as FASTQ files. Trimmomatic was used to remove adapters and low quality bases from reads(265). Trimmed reads were then aligned to GRCg38 – hg38 genome using bowtie 2(266) with parameters --local, --very-sensitive, --no-mixed, --no-discordant, --dovetail . Aligned SAM files were converted to sorted BAM files using SAMtools(267) sort function. PICARD was used to mark duplicate reads in sorter BAM files (https://broadinstitute.github.io/picard). Duplicated and low-quality reads, based on bowtie2 score, were removed using SAMtools view with parameters -f 2 -F 1024 -q 30. Peaks were called using MACS2(268) over individual replicates using IgG samples as controls. Peak lists were labeled with closest gene name using ChIPpeakAnno(269). Biological replicates BAM files were merged using SAMtools merge function. These merged BAM files were converted into BedGraph files normalized to show 1x genome coverage using Deeptools(270) bamcoverage function. These BedGraph files were used for visualization in integrative genome viewer (IGV)(271) over genes of interest.

RNA extraction and qRT-PCR

Cells were treated in serum free medium. Following treatment cells were washed with PBS and lysed in TRI-zol reagent (Invitrogen). RNA was collected using Quick-RNA Miniprep Kit (Zymo Research). cDNA was made using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). qPCR was performed using Power SYBR Green PCR Master Mix (Thermo Fisher) following manufacturer instructions. qPCR was run on QuantStudio 3 instrument (ThermoFisher). Quantification was done using the delta

delta cT method, using expression of 18S as the reference gene for normalization of samples.

RNASeq

MDAMB231 cells were treated in 10% serum DMEM with 10ng human IL4 for 24 or 48 hours, and RNA was collected as above. cDNA library preparation was performed using NEBNext Poly(A) selection library kit. Library quality control was performed using Qubit and bioanalyzer. Sequencing was then performed on the Illumina NovaSeq 6000 paired-end 150bp, for an average of 50M reads per sample. Analysis was carried through Illumina's Dragen RNASeq pipeline. Library preparation, quality control, sequencing, and analysis was performed by VANTAGE.

Colony-forming assays

4T1 or BT549 cells were plated at 200 cells per well in 12-well plates in 1 ml of medium containing DMSO or 10μm C646 with or without 20 ng/ml species-specific IL4. The medium was aspirated and replaced with fresh medium containing the same additives every 2 days. At day 6-7 for 4T1 cells or day 14 for BT549 cells, the plates were aspirated, rinsed with PBS and stained by covering wells with 1 ml 0.5% crystal violet containing 6% glutaraldehyde for 30 minutes at room temperature. The stain was removed, and plates washed by repeated submersion in distilled water before drying overnight. Colony imaging and quantification was performed on the GelCount System (Oxford Optronix) in the Digital Histology Shared Resource at Vanderbilt University Medical Center.

Proliferation assays

MDA-MB-231 and BT549 cells were seeded at 5,000-10,000 cells per well onto 96 well plates and treated for 24 hours with 20ng IL4 and/or other drugs as noted in figure

legends. Cell number was then measured using a Cyquant Direct Cell Proliferation Assay (Thermo Fisher) and following manufacturer's instructions.

Prognostic analysis of IL4 receptor subunits in breast cancer

We accessed the KM plotter web-based tool(185) (www.kmplot.com) to assess the prognostic potential of IL4 receptor subunits, and how their expression correlated to overall survival. The publicly available datasets used for this analysis is described in the paper describing the algorithim [17]. All available patient samples were used with no selection for treatment. The probe set used was IL13RA1 201888, with 'overall survival' and auto-select cutoff checked. Selections of the different subtypes were made using the PAM50 designations.

Statistical Analysis

Analysis was performed using Graphpad Prism version 10.2.0. Students t test was used for comparison between two conditions and one way ANOVA for experiments with 3 or more conditions. $P<0.05$ was considered statistically significant.

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Appendix 1

Measurement of metabolites from migrating cells(272)

Running Title: Migration and metabolism

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Abstract

Metastasis is a multi-step process that involves responses to extrinsic and intrinsic signals at every step. It is thus only truly appreciated in the context of a whole organism. Nevertheless *in vitro* studies can be used to facilitate understanding of the possible factors contributing to any phenotype that is associated with metastatic competence. The use of migration assays – where monolayers of cells migrate to cover gaps or 'wounds' – has been described for decades to identify signaling pathways that

regulate motile competence and to screen for ways of interfering with this ability. Here we depict the combination of such an assay with assessment of indicators of carbon metabolism using commercially-available assays. This enables identification of changes in cellular metabolism associated with actively migrating cells.

Key Words

Metastasis; migration; scratch assay; glucose consumption; lactate production;

Introduction

The multiple steps of metastasis have been well-described (273) and include both tumor cell autonomous behaviors and microenviromental interactions. Thus, studying cell cultures *in vitro* cannot give a complete assessment of metastatic ability. Nevertheless, reductionist approaches enable evaluation of complex changes within cells under specific conditions. Here we use a straightforward 2-dimensional (2D) cell migration assay as a means of assessing metabolic changes within tumor cells. We chose migration as one of the quintessential behaviors linked to metastatic potential and used an assay protocol that slows us to separate the metabolic changes associated with this single behavior. In the method described below, we describe the use of commercially available cell culture inserts. These enable the creation of a space for cells to migrate into without having to “wound” a cell monolayer. This is considered better than the traditional scratch assay where a pipet tip is used to literally scratch through the monolayer, thus creating an empty space (274). In creating the scratch, cells are damaged and can release factors that may impact cell behavior (275). Additionally, use of inserts allows direct comparison of the same number of cells since removal of inserts

should not significantly reduce cell numbers and reproducibility is improved (276). In contrast, the creation of scratches or wounds directly removes cells that were part of the monolayer. Nevertheless, the method described below can be easily adapted for a scratch assay format, as indicated.

For assaying metabolic activity, there are a number of different commercially available assays and many of them are appropriate for use in the method described below. The optimal method, which we describe, is to assay the medium thus allowing multiple timepoints from the same set of specimens. It is also possible to set up multiple parallel sets of specimens that can be used at different timepoints if cell lysates are needed for the assays chosen.

Materials

2.1 Materials for migration assays

- Actively growing culture of cells of interest
- Trypsin (0.25% with EDTA) or whatever is standard for dissociation and collection of the cells used
- Growth medium with glucose concentration of up to 5mM, preferably serum free (**Note 1**)
- Sterile PBS
- Trypan blue
- Cell counter and necessary slides or hemocytometer
- 24-well cell culture plates

- Silicone 2- or 3-well migration inserts, available from Ibidi
- Sterile forceps
- Inverted microscope with camera that can capture phase contrast images
- ImageJ software - freely available from NIH (https://imagej.nih.gov)

2.2. Materials for metabolic assays

- Promega-Glo assay kits for glucose uptake, lactate production, glutamine uptake and glutamine production or other equivalent biochemical assay kits
- Microfuge tubes
- Micro-centrifuge
- -20^{o}C freezer
- 96-well non-cell culture treated assay plates
- Microplate luminometer (**Note 2**)
- Optional if assaying cell lysates: Deproteinization kit such as Biovision Deproteinization Sample Preparation kit
- Optional if preparing lysates for assay of total protein content: Radioimmunoprecipitation assay (RIPA) buffer for protein lysis (0.1% Sodium dodecyl sulfate, 0.5% Sodium Deoxycholate, 1% Triton X, 10 mM Tris pH 7.5, 150 mM NaCl)
- Optional if conducting total protein assay for normalization: Bicinchoninic acid assay or other standard assay for determination of total protein content

Methods

3.1 Migration assay and sample collection

1. If using migration inserts, use sterile forceps to transfer a 2 or 3-well migration chamber into multiple wells of a 24-well plate. Ensure the inserts adhere to the bottom of the well by gently tapping all around the top with the forceps and checking that they do not slip when the plate is moved. (**Note 3**)
2. Prepare cells by trypsinizing, counting and diluting to ~200,000/ml of viable cells (trypan blue-excluding) (**Note 4**).
3. Place 1ml of the cell suspension into each well, making sure it is as homogenous as possible before pipetting. If using a treatment, prepare it a 2X final concentration in the same medium as the cells are in and also make cell suspension at double cell concentration e.g. 4 x 10^5/ml, then mix together before adding to the appropriate wells. Use pipette tip to mix and ensure cells are evenly dispersed within insert. (**Note 5**)
4. Incubate 15-24 hours under standard culture conditions (**Note 6**).
5. After incubation, observe under microscope and ensure cells within insert and well are confluent.
6. If using migration inserts, use sterile forceps to gently remove inserts from each well to be used for migration assay; pull up so as not to disturb edges of cell layers. If using scratch assay, carefully scratch a single line down the center of the well with a sterile pipet tip (**Note 7**).

7. Gently rinse each well with 1 ml of sterile PBS – add to side of well and rock plate carefully before aspirating from side of well. Repeat. This will remove any accidentally dislodged cells.
8. Gently add 0.6 ml of medium to well so that cell layer is completely covered. If using a treatment, add it to this medium. Save 30 l samples as the t0 medium for metabolism assays. The medium samples should be stored at -20^{o}C for later analysis (**Note 8**).
9. Under phase contrast and 4X magnification, take time zero (t0) pictures along length of gap between cell sheets. At least 4 images should be taken for each gap. Ensure that all images show the cells on either side of the gap (example in Figure 1A). Save each image as a tif file.
10. Return plate to incubator and remove to image as above at 2 – 3 timepoints over 24 hours (**Note 9**). Continuing the assay for longer than 24 hours is not recommended since proliferation of cells could then play a significant role in the apparent filling of the gap.
11. At each timepoint of interest, collect 30 µL of medium from each well, spin at 10,000 rpm to remove any cellular debris and snap-freeze the supernatant at 20°C until ready to use for metabolism assays. Make sure to freeze in aliquots of 5-10 µl if anticipating needing to perform multiple assays at different times so that individual samples do not undergo multiple rounds of freeze/thaw.
12. Analysis of the ability of cells to fill the gap can be done using ImageJ. Use a freehand tool to outline the area in each picture that has no cells – this should get smaller over time. The percent coverage is calculated as [(Area at time 0 – Area

at time x) / Area at time 0] x 100. The average for each time point can be plotted against time, and the slope gives the rate of migration (area covered per unit time). See Figure 1 for an example.

3.2 Metabolism Assays

1. Optional: If cell lysates will not be prepared for the metabolic assays, a standard lysis buffer such as RIPA buffer can be used to collect lysates from each well from the migration assay to enable normalization between wells based on protein concentration. This is recommended if performing a scratch assay where cell numbers may be different between migrating and non-migrating wells, of if cell proliferation is possible.
2. Gently thaw all the medium samples to be analyzed on ice.
3. Dilute media collected with assay buffer (provided in assay kit) in order to bring concentration of metabolites of interest within the linear range of the assay being used (**Note 10**). For the samples shown in Figure 2, the cells were in medium containing 5mM glucose and the dilution used was 1:200.

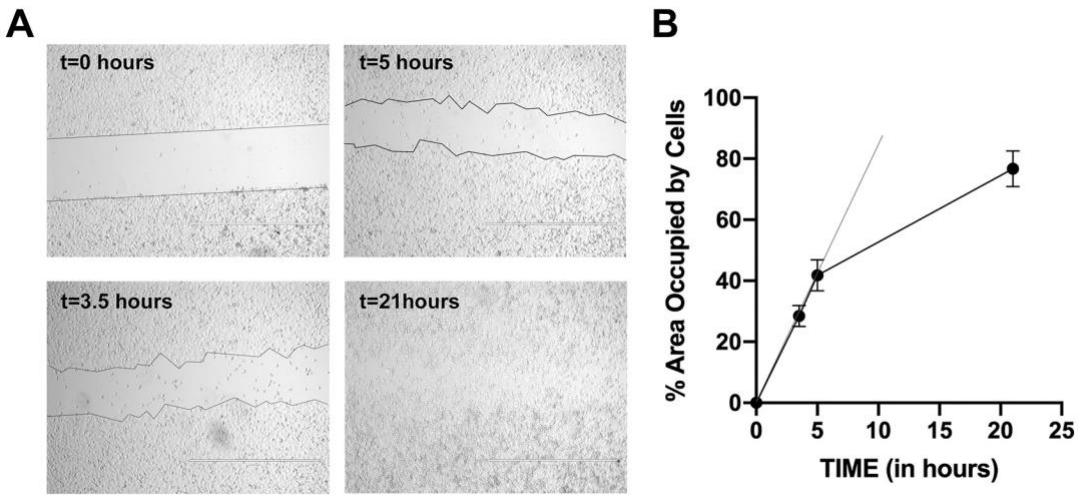

Figure App.1: Migration assay using 4T1 mammary cancer cells. (a) Sample images of a gap at times 0, 3.5, 5 and 22 hours post-plating with area of gap outlined. Note no obvious gap remains at the final timepoint. (b) Graph showing area covered by cells over time. There is a clear change in the kinetics of migration between the 5 and 22 hour time points, thus the initial rate of migration was calculated from the slope of the line for the 0, 3.5 and 5 hour timepoints and is shown as a grey line.

4. Optional: If analyzing intracellular concentration of metabolites, harvest cells and wash with PBS before resuspending cells in assay buffer and homogenizing by trituration with a pipet.
5. Optional: If using lysates, deproteinize using a standard deproteinization protocol such as perchloric acid precipitation (**Note 11**). Do not deproteinize the entire lysate so that a protein assay can be performed on a fraction to enable normalization between samples.

6. Follow specific assay instructions given with each kit to determine the amount of metabolic substrate or product in medium or lysate sample at each time point (**Note 12**).
7. To calculate substrate consumption, use the standard curve to determine the amount of glucose in each sample at time x and at time 0, and correct for any dilution factors. Glucose consumption = concentration at time 0- concentration at time x. Levels of metabolic products present in medium e.g. lactate, can be determined directly from the standard curve with correction for any dilution factors. Example values from the migration assay shown in Fig 1 are shown in Fig 2. If lysates were analyzed for total protein content, the levels of substrate or product can be further divided by the protein concentration from that well to report the metabolite level per mg of total cellular protein.

Notes:

1. When analyzing metabolism in cell culture the concentration of metabolic substrates in the medium is an important consideration. For example, the concentration of glucose in most cell culture media is 25 mM, far out of the detection range of commercially available assays. Many assays will suggest using media with reduced concentration, for example DMEM with 5mM glucose as opposed to normal formulation, in order to limit how much samples must be diluted before they can be processed. Additionally, serum contains both metabolic substrates and products from the donor animal, thus minimizing the serum level is advantageous. We use serum-free or 2% serum (depending on cell line) for our assays. Since the medium is changed at the start of the

migration assay, it is possible to use different media for initial plating and for the actual assay. This maybe especially important for cells that are sensitive to reduced serum levels

2. For the Glo assays used here, the signal is luminescence and a luminometer is needed, however other biochemical assays may require a fluorimeter or spectrophotometer.
3. The numbers of wells for each condition should be a minimum of 3 to enable statistical testing, but more may be needed. With practice, variability amongst wells should be minimal. To compare migrating versus non-migrating cells, can either use wells with no inserts, or use wells in which inserts will not be removed the next day.
4. The optimal cell concentration can change depending on the cell line and will need to be empirically determined. The aim is to have a confluent layer after 15-24 hours of plating in the insert. Try 0.75-4 x 10^5/ml.
5. It is absolutely key that the cells are evenly distributed both within and around the migration inserts, and the well in general. The plate can be swirled gently to aid in achieving a homogenous layer of cells.
6. The length of time for incubation is based on obtaining an even monolayer within each well. Another factor is the timepoints for the ensuing migration assay. In our experience, we typically plate in the late afternoon on one day, and start the migration assay in the morning of the following day after ~16 hrs of incubation.
7. For making a scratch, we have found a smaller size tip (for a 1-20 l pipettor) to be easiest to control. Hold directly with fingers rather than with forceps. While it is possible to make multiples scratches in each well to obtain multiple data points

for the migration easy, this will impact the interpretation of any metabolic assays since each scratch removes cells from the well thus reducing overall cell number.

8. At this point, the medium can be changed from that used for the initial plating as indicated in Note 1. If any wells are receiving different treatments, e.g. addition of a growth factor, or a drug or vehicle, separate samples must be collected for each of these as such additives could potentially affect the metabolic assays.
9. This is very cell -line dependent. Highly motile cells should be imaged at shorter timepoints e.g. 2, 4 and 6 hours; while others may only show movement beginning at 12-14 hours.
10. We often perform serial dilutions with new samples when processing new cell lines or assaying for different metabolites. While the kit instructions make suggestions regarding dilutions, there is still cell line dependence and thus the optimal dilution for each timepoint may have to be determined empirically. To minimize kit usage, we suggest that for dilution testing, only two standards (low and high concentration) be used. We aim for a dilution that puts the samples in the middle of the detection range for the assay being used in order to account for the natural variability in metabolism that can occur between replicate samples.
11. Protein in samples can often interfere with assays for small molecules such as metabolites. In order to prevent this interference samples such lysates that may contain protein must be deproteinized, however different methods are possible. Some methods such as perchloric acid precipitation will not necessarily be compatible with every commercially available metabolism assay. As such it is

important to test compatibility and consider alternative methods of deproteinizing samples such as trichloroacetic acid protocols.

12. We typically perform 3 technical replicates for each sample to be assayed, however since there will be multiple biological replicates if 3 or more wells were plated for each condition, it is acceptable to do a single assay well for each of the samples.

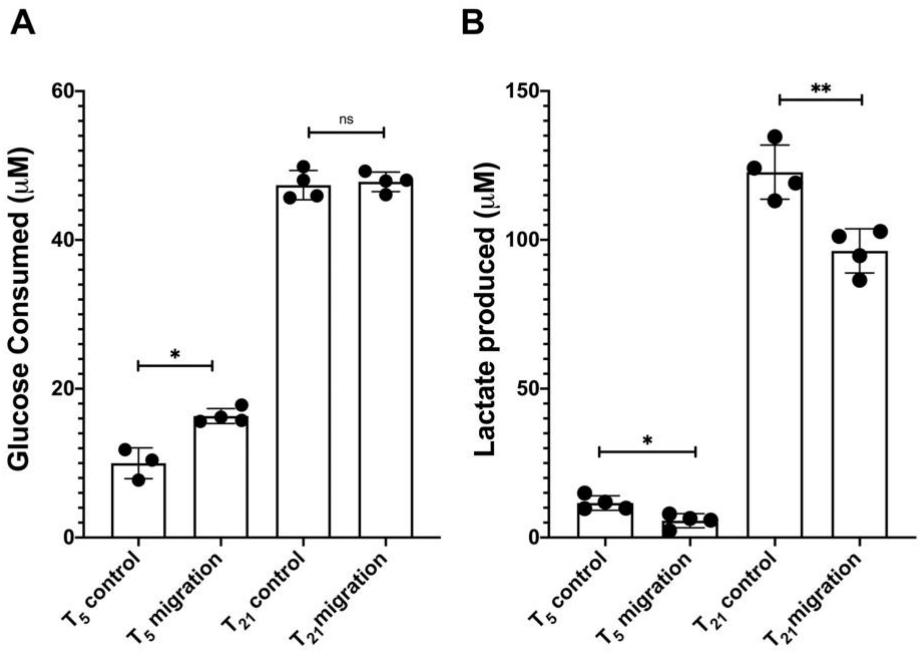

Figure App.2: Changes in (a) glucose consumption, and (b) lactate production between wells with actively migrating cells versus intact monolayers at the 5 and 21hour timepoints from the cells shown in Figure 1. The migrating cells show increased glucose consumption that is not matched by increased lactate production

suggesting that the glucose is used for oxidative phosphorylation rather than glycolysis.

Appendix 2

Table A_1: Genes with significant changes in expression following 24 hours of IL4 treatment in MDA-MB-231 assessed by RNASeq

Feature_gene_name	padj	FoldChange
LOX	8.33E-47	2.873543584
CCL26	4.14E-30	26.04142301
COL17A1	1.11E-25	3.728487043
KAL1	1.04E-24	2.310124642
KRT8	5.38E-22	2.088754336
TNC	7.73E-20	2.773062558
RP11-554I8.2	4.98E-10	2.988699525
ST8SIA1	7.71E-09	6.236947022
ABCG2	7.03E-08	2.978145167
SLIT2	2.66E-07	2.013345626
PDCD1LG2	8.4E-05	2.007327105
PPIAP29	0.001700895	0.015884651
KRT8P36	0.002440778	2.308377671
HRASLS5	0.003144485	0.006013081
RP11-152N13.16	0.009976476	0.006720272
TRAPPC5	0.014477064	0.006882173
KRT81	0.021011331	0.401565694
LINC00862	0.024134854	0.007353334
BRSK1	0.024827554	78.52027302

AC092675.3	0.02513362	8.191761936
ANXA10	0.026560098	0.007892589
NOVA2	0.02869409	130.8355303
RP3-475N16.1	0.036913673	2.152962464
AC005932.1	0.036913673	0.008119121
ADCY5	0.046858857	22.67646228
DUX4L19	0.04737206	122.2792467
BATF	0.048098848	2.821616445

Table A_2: Genes with significant changes in expression following 48 hours of IL4 treatment in MDA-MB-231 cells assessed by RNASeq

Feature_gene_name	**padj**	**FoldChange**
SAA1	3.39965299518718E-41	0.173433073553865
LAMC2	2.99854247730618E-37	0.358088684048214
C3	2.99854247730618E-37	0.335361924321578
INSIG1	2.93142754563925E-28	3.30228674335297
SERPINA1	1.67585981320023E-26	0.389528778650374
SAA2	2.68492576352671E-25	0.151672647487273
SPINK4	9.82970024639222E-25	0.136778122490987
COL18A1	1.91249156802977E-21	0.499458226754447
HLA-DRA	3.66678729938117E-21	0.433264480185862

TGFBI	9.77507106571193E-21	0.493968048163958
TFF2	5.4113906903546E-20	0.138278459893647
MMP1	1.68937567005901E-19	0.347407756955426
TFF1	9.65053644664487E-19	0.112169840233029
LINC00472	4.70579519690563E-18	2.57323511231878
KIF20A	1.34978748249658E-16	2.03522318443174
BNIP3	2.52834792256192E-15	2.00192600266138
SCG2	6.28322319001003E-14	0.385324362336843
HLA-DPB1	9.15480445838308E-14	0.419705432928659
CCL26	1.63550377770063E-13	9.50338885575263
PCSK9	1.63821032786678E-12	2.45523670740073
C1S	3.1820553149495E-12	0.332479312352728
IFIT1	5.48467837272239E-12	2.14097150996809
RARRES3	7.66916208396507E-12	0.472655916715151
C1R	1.07684385450689E-11	0.41154008323485
COL17A1	1.08979935597282E-11	2.1506575309976
CHI3L2	1.81108969002838E-11	0.31420159725779
HIST1H2AC	1.8862635930516E-11	2.97055473117156
ARTN	2.17800846073243E-11	3.68225122516501
SLC7A11	2.20289381441267E-11	0.43312739916129
SNHG3	4.12315698726092E-11	2.16391560037239
HLA-DPA1	4.96266036882922E-11	0.485474067592766
PORCN	1.01533608370463E-10	0.483564628266994
CPE	1.125533772717E-10	0.478670635144379
TMEM38A	3.79402691899837E-10	0.469384912710483
COL6A3	4.66584861906806E-10	0.474550197174655
TMEM71	5.53144982934162E-09	3.02010159481672
LCN2	6.12435006654489E-08	0.357339856098048
HLA-DRB1	6.23285819269362E-08	0.495414000055607
ST8SIA1	1.10625452699691E-07	4.12656741978846

TCN1	1.64671829827114E-07	0.352205479563333
SCG5	9.46807413856153E-07	0.33062264399872
GRIK2	1.64964613640782E-06	0.385407507848628
ADIRF-AS1	1.76510265527355E-06	3.16570989801464
MT-TP	3.36440527021978E-06	0.495696122563121
MIR210HG	4.57586752261531E-06	2.41632548525307
IL8	6.28169080801836E-06	0.336055440591999
CCNA1	6.2989054255438E-06	3.6923798669799
RP11-304L19.5	1.37950970970105E-05	3.26915594076146

SPAG4	1.45803918823829E-05	2.39807236382231
CLIC3	2.16160636549095E-05	2.26489750254991
ALDH1A3	2.95369778146108E-05	0.45781807672069
CHAC1	3.66070647315246E-05	0.377108249582885
ASS1	3.75558213315662E-05	0.461476259922587
LINC01003	4.00121535049236E-05	2.10620819500665
AC144652.1	5.82101262870053E-05	2.60696797962289
RP11-554I8.2	0.000115518045928758	2.03123632302166
COLEC12	0.000238811428227378	0.200197574920039
SNHG10	0.000238811428227378	2.32854107263758
ZNF674-AS1	0.000298872727109451	2.02469280219152
ADAM28	0.000300405741749772	0.358018342474679
PRSS1	0.000302078164296928	0.433189682927612
NMU	0.00043567031113784	2.12736242972291
S100A5	0.000659637617870411	2.36863906443039
AC092675.3	0.000680459409708448	6.74773373782692
RP6-109B7.3	0.000692670797134797	2.33866459336533
CLDN7	0.000729767047334735	2.02313063079148
ALPK1	0.000775765628102628	3.04186216733042
CTD-2368P22.1	0.000798583101928878	2.15080576685317
PRSS3P2	0.000847413587276278	0.417591455262489
TBKBP1	0.00103078263297319	2.0487414356218
CYP1A1	0.00113867839620157	0.378753536443603
APOE	0.00128902617953178	0.395648629181432
BPI	0.00136234083869773	0.0999186840477244
FAM117B	0.0016651512228505	2.016740387494
AK5	0.00280709140478897	0.360008609211118
PSG5	0.00335368887380452	0.365711261464174
PIK3C2B	0.00409394163124492	2.01346908527335
AC112721.2	0.00437080919909545	0.286834802742355
SPACA6P	0.00468120115620051	2.7529406185317
UFSP1	0.00535563174754289	2.87061221422999
IFITM10	0.00551055521864434	0.490117639581205
TCP11L2	0.00613161480359099	2.57418965054433
CA9	0.00625222778861991	2.03327953323051
C4B	0.00631251317645821	0.345620024355336

KMT2E-AS1	0.00870195767865133	2.90859566395272
VSIG10L	0.00893844229914752	0.478578546549459
CXorf24	0.0101547993724157	2.30307116274698

PIF1	0.0102590600280178	2.09856594098931
PPIAP29	0.0103148875201069	0.0303253234758062
RP11-199F11.2	0.0108863988477504	5.28982146822032
KRTCAP2	0.0111464482738477	3.53381024995679
KRTAP2-3	0.0113888777571277	2.356592991124
SLC38A5	0.0113888777571277	0.26248009927153
GGT1	0.0122556641050444	0.33848262909226
CTD-2263F21.1	0.0123503778354918	2.24909840996897
CPLX1	0.0124307621660681	0.178045876566097
AP000769.1	0.0128285282585887	2.48735703098049
A2M	0.0129398434220852	0.404116321788112
LINC00338	0.0140193134977605	2.13189898241859
ALPP	0.0145867582619275	0.451829661912228
SERPINA5	0.0150071510625523	0.198764495513453
TMEM37	0.015047963975136	0.444677510461533
NRCAM	0.015047963975136	0.251808908553363
HLA-DOA	0.0156175749309866	0.421751961569334
AC009404.2	0.0170285138337559	4.1558128666257
ABI3BP	0.0204110778442623	0.441401108001867
RP1-39G22.7	0.0205404596413603	2.06766216320428
CCND2	0.0205697036578735	3.15395235915759
RP4-555D20.4	0.0226335166290781	2.14354539331942
C1QTNF1	0.0234945059297604	0.412016972018103
SEPT3	0.0260931713363607	0.477109813318884
AC084018.1	0.0274973485378234	2.77650355944082
FAM179A	0.032307188763723	2.1754136559567
GPR87	0.0340720735792839	0.308545415548312
TLE2	0.0363296522044686	0.390727883024663
MNX1	0.0368850626914603	2.09198811725328
PNPLA3	0.038252015123645	2.07263316480226
SLC4A4	0.0398253180336222	0.384043806476766
AC005256.1	0.0402211992475451	2.93149812568869
RP11-265N7.1	0.0402766092016754	0.410140911088028
PRSS3P1	0.0414592056023897	0.451549807061811
RNF128	0.0419299957700216	0.456089490979521
BATF	0.0423952421257313	2.06666380880782
FGFR3	0.0434071743716401	0.488951999457882
RP3-416H24.1	0.0462593595584629	2.01975726284356
SPOCK3	0.0472316864749615	0.468577818451451
KCNQ5-IT1	0.0478680516707129	0.325463372675788

KCNC3	0.0499666130988182	2.35849805474897