Mammary Tumor Microenvironment Reprogramming in Response to Pyruvate Carboxylase Modulation

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Abstract

Background: Breast cancer is the deadliest cancer amongst women globally, with metastatic breast cancer being particularly deadly. Pyruvate carboxylase (PC) catalyzes the conversion of pyruvate to oxaloacetate for anaplerotic refilling of TCA cycle intermediates, feeding numerous energetic and biosynthetic pathways. Upregulation of PC is an important contributor to metabolic reprogramming and aggressiveness in metastatic breast cancer. Less understood, however, is the effects of PC expression on metabolic reprogramming and the TME in primary tumors. In this study, we investigate whether suppression of PC alters metabolism and drives microenvironmental adaptation in a primary tumor model of breast cancer.

Methods: C57Bl/6 mice were injected with M-Wnt cells transduced with doxycycline-inducible ShRNA targeting PC. Doxycycline treatment began once tumors were palpable. Tumors were harvested 4 weeks following injection. Tumor transcriptomic analysis was conducted via GSEA and enrichment mapping following Affymetrix microarray analysis. Digital cytometry using CIBORSORTx was conducted to determine tumor microenvironment composition. *In vitro* metabolic adaptation to PC suppression in breast cancer cell lines following knockdown of PC was analyzed. Perturbations of mitochondrial metabolism and respiration were assessed by extracellular flux analysis. Assays of extracellular lactate and glucose concentrations determined changes in the production and utilization of carbon sources in the context of loss of PC.

Results: In vivo suppression of PC resulted in increased tumor mass and volume relative to control. Gene expression data from PC knockdown tumors revealed distinct transcriptomic profiles between groups. GSEA analysis further showed profound suppression of immunological pathways following loss of PC, indicating that PC knockdown resulted in a diminished immune response. Digital cytometry supported this finding with PC suppression resulting in decreased proportions of critical innate and adaptive immune cell populations. Metabolic assays revealed that cells with PC knockdown export more lactate into their environment and respire less efficiently, without consuming additional glucose. This indicates potential mitochondrial dysfunction with loss of PC-derived anaplerosis

Conclusion: PC knockdown resulted in increased lactate production with a decrease in mitochondrial respiration, suggesting that diminished PC-mediated anaplerosis alters carbon utilization and contributes to metabolic reprogramming. Suppression of PC also resulted in tumors with distinct transcriptomic profiles versus control, with signatures of immune responses diminished in response to loss of PC. As lactate is a profound immunosuppressive signaling molecule in the TME, it may be a driver of TME immunosuppression in response to PC suppression. We conclude that PC knockdown promotes a metabolically altered tumor microenvironment associated with immunosuppression and tumor growth.

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Introduction

Breast Cancer Prevalence and Mortality

Breast cancer is the most commonly diagnosed cancer globally, representing 11.7% of total cases, and is the leading cause of cancer death in women[1]. In the United States alone, the American Cancer Society predicts 284,000 new breast cancer cases and 44,130 deaths in 2021[2]. Triple negative breast cancer (TNBC), defined as those cancers that are estrogen receptor-negative, progesterone receptor-negative, and HER2/neu negative, have poor prognoses relative to other subtypes, due in part to a lack of targeted therapies and increased incidence of metastatic spread[3]. While patients with localized TNBC have a five-year survival rate of 91%, TNBC patients with metastatic spread to distal organs have only a 12% five-year survival rate[2]. Metastatic breast cancer is particularly deadly due to limited surgical resection options, with the first-line treatment instead being palliative chemotherapy regimens[4]. The most common sites for metastatic spread from the breast are to the lungs, liver, and bone, with each site presenting unique challenges to treatment[5]. Research into the biological mechanisms underlying the metastatic processes to these sites is crucial to identify targeted therapies for successful interventions.

Metabolic Reprogramming in Cancer

A hallmark of cancer is the reprogramming of cellular metabolism to promote growth, proliferation, and survival[6]. Central to this reprogramming is increased glycolysis and fermentation of pyruvate to lactate in the presence of adequate oxygen concentrations, termed aerobic glycolysis[7]. This metabolic phenotype was first characterized in cancer over 90 years ago by Otto Warburg and is subsequently known as the Warburg effect[8]. Many theories have been proposed for why rapidly proliferating cells such as tumor cells engage in aerobic

glycolysis, as it is a less carbon efficient means by which to derive ATP from glucose[9]. Although early theories from Warburg suspected damaged mitochondria to be the cause of cancer and the mechanism driving the need for aerobic glycolysis[10], it's now understood that functioning mitochondria play an important role in cancer cell metabolism for energetic and biosynthetic needs[11]. Emerging evidence suggests that the need for faster NADH to NAD^+ turnover via lactate dehydrogenase rather than the electron transport chain promotes aerobic glycolysis in rapidly proliferating cancer cells, where the demand for oxidative equivalents exceeds the demand for ATP production[12].

In addition to intrinsic metabolic changes, cancer cells are often in a dynamic cooperation with non-transformed cells in the tumor microenvironment (TME), exchanging nutrients and growth factors with cancer-associated fibroblasts, immune cells, and epithelial cells among others[13-15]. In addition to the heterogeneous cell populations within the TME, zonal differences in hypoxia and nutrients are often present due to incomplete vascularization of the tumor[16]. These differences result in cell populations of similar origin possessing unique metabolic phenotypes depending on their access to oxygen, making it difficult to challenge tumors with metabolic inhibitors of only one enzyme or pathway[17]. To overcome their insufficient vasculature, tumors often overexpress proangiogenic factors, such as vascular endothelial growth factor (VEGF)[18]. Not only do these factors increase the growth potential of the tumor by supplying nutrients and oxygen, but are also important mediators of metastasis, as VEGF upregulation induces an invasive phenotype in multiple cancers[19, 20].

Metastasis from a primary tumor through the vasculature and to distal organs is a dynamic multi-step process, often referred to as the metastatic cascade[21]. This process can be divided into five steps: invasion of the basement membrane, intravasation into the vasculature or

lymphatic system, survival in the circulation, extravasation from vasculature to secondary tissue, and colonization of the secondary site[22]. In order for successful completion of the metastatic cascade, metastasized cells must metabolically adapt to both the circulatory system and the foreign microenvironment of the secondary site, with each possessing varying nutrient concentrations, oxygen levels, and nonmalignant cell populations[23, 24]. To overcome this challenge, many aggressive breast cancer cell lines possess greater metabolic flexibility than non-aggressive lines, with the ability to readily switch between glycolysis and oxidative phosphorylation in response to different extracellular environments[25]. Metabolic reprogramming in metastatic spread is further complicated by the heterogeneity of the secondary tissues, with distinct metabolic profiles observed in liver, lung, and bone metastases[26]. In the liver, increased HIF-1α activity and expression of the HIF-1α target pyruvate dehydrogenase kinase-1 (PDK1) drives a glycolytic phenotype with increased conversion of glucose-derived pyruvate into lactate and a reduction in oxidative phosphorylation[26]. In contrast, metastasis to the bone and lung is driven by enhanced oxidative phosphorylation marked by increased expression of the major mitochondrial biogenesis regulator PGC-1α[27]. Thus, the study of metabolic reprogramming and targeted interventions in breast cancer will benefit from considering the heterogeneity in environmental conditions seen within and between primary tumors as well as the unique demands of the various secondary sites.

Lactate and Immunosuppression of the TME

Metabolic reprogramming in cancer cells can result in a wide range of metabolic phenotypes; however, one consistent feature of most cancers is the enhanced production of lactate[28]. While lactate concentrations in the blood and healthy tissues range from 1.5-3.0 mM, tumors are capable of producing up to 40 times more lactate than normal tissues, resulting in a

TME with lactate concentrations as high as 30 mM[29, 30]. Long considered an inert waste product of glycolysis, lactate is now regarded as an important signaling molecule and carbon source in many tissues, including tumors[31]. Indeed, ^{13}C-labeled nutrient studies have shown that in most organs, the main route through which glucose is incorporated into TCA cycle intermediates is through circulating lactate[32]. This preferential conversion of circulating lactate to TCA intermediates has been replicated in human tumors[33]. Specifically, oxidative tumors cells near the vasculature preferentially utilize lactate exported into the TME from highly glycolytic cells without adequate access to oxygen, leaving glucose available in the TME for those hypoxic cells in a process termed metabolic symbiosis[34]. Considerable tumor cell heterogeneity within tumors is common, in part due to rapidly growing tumors constructing insufficient vasculature and the resulting spatial and temporal differences in hypoxia[35]. Thus, metabolic symbiosis is one process by which to enhance the metabolic flexibility of solid tumors with varied access to oxygen, yielding a survival advantage for cancer cells in an otherwise hostile TME.

Lactate is also recognized as a potent signaling molecule and a driver of immunosuppression in cancers[36]. Evasion of immune destruction is a hallmark of cancer and represents a significant barrier to successful elimination of tumors with standard treatments such as chemotherapy[37]. Immunotherapies, such as immune checkpoint inhibitors (ICIs), are being utilized in multiple cancers to diminish immunosuppressive pathways and enhance immunosurveillance of tumors[37, 38]. However, other TME characteristics such as impaired immune cell infiltration and nutrient competition are known to limit the efficacy of immunotherapies, highlighting the complexity of tumor immune evasion[39, 40].

Within the TME, the complex cross talk between immune and cancer cells can both inhibit or enhance tumor growth[41]. Tumors utilize several immunosuppressive pathways in both innate and adaptive immune cells to evade destruction, including direct tumor cell mechanisms, such as downregulation of MHC molecules on the cell surface, or through production of immunosuppressive cytokines or metabolites that impair immune cell function[42]. The TME suppresses the direct anti-tumor immune response mediated by $CD8^+$ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, as well as causes dysregulation of immune cell recruitment and activation via regulatory T cells (T_{reg}), $CD4^+$ T-cells, and antigen presenting cells (APCs)[43]. In order to effectively overcome these immunological limitations, therapies must address both factors of tumor immunosuppression, including eliminating immune suppressing factors and enhancing tumor-killing activities of CTLs or NK cells[44]. ICIs such as those targeting cytotoxic T-lymphocyte associated protein 4 (CTLA-4) or the programmed death receptor 1 (PD-1) have shown promise in the treatment of some solid tumors (e.g. melanoma and lung cancer), promoting antitumor immunity and resulting in extension of progression-free survival in a significant subset of patients[45]. However, other cancers, including breast, have only seen modest responses with ICI's, indicating an incomplete rescue of tumor immunosurveillance[46]. Hence, delineating how tumors continue to evade immune destruction is vital to expand the efficacy of immunotherapies.

Lactates role as an immunosuppressive metabolite is now well recognized and believed to play a role in the limited efficacy of immunotherapies[47]. LDHA expression is correlated with reduced immunosurveillance and poorer clinical outcomes, and is a strong predictor of ipilimumab (CTLA-4) efficacy in advanced melanoma[48, 49]. Lactate exerts its immunosuppressive effects on both innate and adaptive immune cells, causing anergy and

reduced function in T-cells, inhibition of antigen presentation by dendritic cells (DCs), and polarization of tumor associated macrophages (TAMs) toward their wound healing M2-like phenotype[50-52]. These cell specific effects suppress the overall anti-tumor immune response, as LDHA suppression in melanoma cells led to impaired growth of tumors in immune-competent C57BL/6 mice, yet had little effect in immunodeficient mice[48]. In addition to its role in immune cell signaling, lactate metabolism also plays a role in acidification of the TME, which further impedes immune cell function in a lactate-independent manner. Lactate is exported from the cell via the monocarboxylate transporter (MCT) family of H^+/lactate$^-$ symporters, resulting in a pH between 6.4-7.0 in the TME[53]. Low pH levels in solid tumors is associated with invasive growth and metastasis through a variety of mechanism, including increased angiogenic signaling through the release of VEGF, degradation of the extracellular matrix by proteinases, and immune evasion[54-56]. As aerobic glycolysis and its characteristic enhancement of lactate production is a common metabolic phenotype in cancer, the contribution of lactate to the energetics and immunosuppression of the TME is a vital component of future research studying the efficacy of metabolic or immune-based therapies.

Pyruvate Carboxylase in Breast Cancer

Upregulation of anabolic pathways that supply macromolecules for cellular division is a crucial part of the metabolic reprogramming of rapidly proliferating cancer cells[57]. Critical to this anabolic metabolism is the refilling of TCA cycle intermediates to support ATP and biomass production, a process termed anaplerosis[58]. In mammalian cells, adequate concentrations of TCA cycle intermediates are required for the biosynthesis of lipids, non-essential amino acids, nucleotides, glutathione, heme, and other cellular components, with many of these products recognized as crucial metabolites in cancer[59, 60]. The major anaplerotic pathways in the cell

are the pyruvate carboxylase (PC) mediated conversion of pyruvate to oxaloacetate and the conversion of glutamine to alpha-ketoglutarate via glutaminase (GLS) and glutamate dehydrogenase(GDH). PC serves two primary biosynthetic functions: production of oxaloacetate for phosphoenolpyruvate carboxykinase (PEPCK) to convert to phosphoenolpyruvate, allowing the bypass of pyruvate kinase in gluconeogenesis, and sustaining oxaloacetate and thus other TCA cycle intermediate levels in the mitochondria for biosynthetic and energetic purposes[61]. The relative contribution of PC and glutamine in anaplerosis varies by tissue and physiological condition[62]. In many cancers, glutamine is the primary anaplerotic precursor, contributing to up to 90% of oxaloacetate pools[63]. However, PC is the preferred anaplerotic enzyme in some glutamine-independent cell lines[64], indicating the ability of both pathways to support mitochondrial metabolism in cancer.

In instances of inherited PC deficiency, a rare autosomal recessive disorder, patients often present with severe lactic acidosis in the blood, indicating excess production and export of lactate[65]. Without PC-dependent anaplerosis, the pyruvate dehydrogenase (PDH)-catalyzed decarboxylation of pyruvate to acetyl-CoA is the primary reaction for glucose-derived carbon incorporation into the TCA cycle for mitochondrial metabolism[66]. However, the lactic acidosis seen in PC deficiency cases suggests that PDH cannot incorporate all PC-fated pyruvate into the TCA cycle, with the remaining pyruvate being shunted into lactate production. Without PC-derived oxaloacetate, less glucose-derived substrates are available for acetyl-CoA to condense with and form citrate, forcing increased reliance on other anaplerotic pathways fueled by amino acids or odd-chained fatty acids[67]. Insufficient availability of oxaloacetate will cause mitochondrial acetyl-CoA levels to rise, inhibiting PDH activity[68]. Thus, PC-dependent anaplerosis and PDH activity are both necessary for maximum incorporation of glucose-derived

carbons into the TCA cycle in cells that cannot meet their anaplerotic needs through amino acids or odd-chain fatty acids[69]. In conditions of PC deficiency, the PDH complex is unable to fully catalyze the increased available pyruvate, with cells instead increasing the flux through LDHA and lactate export.

While glutamine has been extensively studied as a contributor to cancer's anabolic metabolism and as a potential target for metabolic therapies[70], accumulating evidence now indicates PC as a significant driver of growth and progression in multiple cancers[62]. PC activity has been proven critical to the metabolism of non-small-cell lung cancers, with its knockdown resulting in diminished TCA cycle activity, inhibition of lipid biosynthesis, and decreased proliferation[71]. PC activity is also important for breast cancer metastasis to the lungs, with lung metastases showing higher PC-dependent anaplerosis than primary tumor cells[72]. Further, suppression of PC in breast cancer mouse models significantly reduces rates of metastasis to the lungs, indicating that pulmonary metastatic outgrowth is dependent on PC activity[73]. This observed PC-dependence in lung cancers and breast-cancer-derived lung metastases is most likely a product of the oxygenated lung microenvironment and the subsequent shift to an oxidative metabolism with increased oxidative stressors[73]. In contrast to the restricted, transient nature of oxygen concentrations in most tumors, well-oxygenated tumors in the lung may require PC-mediated aerobic utilization of pyruvate.

In breast cancer cell lines, PC expression is 2-3 fold higher in highly metastatic breast cancer lines relative to those with low metastatic potential[74]. Additionally, targeting PC *in vitro* reduces proliferation and migration of the metastatic cell line MDA-MB-231[75, 76]. These studies indicate that PC activity is important to breast cancer cell lines independent of its role in pulmonary metastases survival. Depletion of PC potently inhibited pulmonary metastasis in 4T1

cells, but did not have an effect on primary tumor growth in an orthotopic injection model[73], though the effect of PC suppression on tumor metabolism and remodeling of the TME at the primary site was not examined. Thus, although PC's role in breast cancer aggressiveness and lung metastasis is now well established, its role in the progression of the primary tumor is less understood. Additional studies are needed to further explain the roles of PC expression in primary tumor metabolism and in the TME. Due to the severe lactic acidosis seen in PC deficiency cases, we hypothesize that PC suppression in primary tumors will impact lactate metabolism and increase its concentration in the TME. As lactate is a potent signaling molecule and known immunosuppressive metabolite, its increased concentration may affect non-transformed cells in the TME such as innate and adaptive immune cells. This study thus aimed to delineate the effects of PC suppression on the metabolism of primary tumors and how the resulting metabolic reprogramming impacts the TME.

Methods

Cell Culture

M-Wnt cells transduced with lentiviral particles containing a doxycycline-inducible short hairpin RNA (ShRNA) targeting PC construct (Smartvector), herein referred to as ShPC M-Wnt cells, were a kind gift from Dr. D. Teegarden (Purdue University, Indiana). PC knockdown was confirmed via qPCR. Cells were cultured in Gibco RPMI Medium containing 10% Fetal Bovine Serum (FBS), 11mM glucose, 2mM glutamine, and penicillin/streptomycin unless otherwise noted.

Animal Study

The mouse study was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Mice were housed in a climate-controlled Division of Comparative Medicine facility with ad libitum access to water and diet. 10 female C57BL/6 mice (Charles River Labs, Wilmington, MA) were injected in the 4^{th} mammary fat pad with 50,000 ShPC M-Wnt cells. Mice were randomized to the control or treatment group (n=5), with the treatment group having ad libitum access to water supplemented with 150ug/mL of doxycycline. Doxycycline supplementation began once tumors were palpable. Mice were sacrificed 4 weeks following injection and excised tumors were measured and weighed ex vivo. Tumors were then flash frozen.

Microarray Data Analysis

Total RNA was extracted from excised tumors using the E.Z.N.A Total RNA isolation kit (Omega Bio-tek). Isolated RNA was labeled, hybridized to a Clariom S HT array (ThermoFisher), and processed by the UNC Functional Genomics Core. Gene expression data was analyzed via TAC4 software. Principle Component Analysis (PCA) plot of the most

differentially expressed genes (defined as two-fold expression difference and p-value < 0.05) was generated using the *FactoMineR (version 2.4)*, *factoextra (version 1.0.7), NbClust* (version 3.03) packages in R (version 3.6.2).

Gene Set Enrichment Analysis

Microarray gene expression data was utilized for pathway enrichment analysis via Gene Set Enrichment Analysis (GSEA)[77]. Mouse Gene Symbol Remapping to Human Orthologs (Version 7.2) was selected from GSEA-MSIGDB file servers. The Gene sets Hallmarks[78] and Gene Ontology (GO) Bioprocesses were used with gene set permutation type and 1,000 permutations. Leading edge analysis of gene sets enriched at the most conservative statistical cutoffs (FDR q-value ≤ 0.001) was performed in GSEA.

Cytoscope Enrichment Mapping

GSEA data from GO Bioprocesses gene set was exported into Cytoscape (version 3.8.2) with statistical cutoffs for significant enrichment at FDR-q value < 0.05 and a similarity index $=$ 0.35. The similarity coefficient was used at a cutoff of 0.5 to connect related GO Bioprocesses gene sets. Cytoscape plug-ins *EnrichmentMap (version 3.3), clusterMaker (version 3.0), clusterMaker Dimensionality Reduction (version 1.1), and AutoAnnotate* (*version 1.3*) were utilized to generate an enrichment map as previously described[79].

CIBORSORTx

Digital Cytometry via CIBERSORTx software was performed online (https://cibersortx.stanford.edu) using the "Impute Cell Fraction" module[80]. Seq-ImmuCC signatures[81] were utilized as the signature matrix file to identify mouse immune cell populations. Batch correction was enabled and quintile normalization was disabled. 1,000

permutations for significance analysis were performed to find significant differences (p-value < 0.05) in immune cell fractions between groups.

In Vitro Assays

ShPC M-Wnt cells were seeded in 6-cm plates for 24 hours with control media ± doxycycline. Culture media was then deproteinated with 3KD spin filters for 20 minutes at 16,000 RCF in 4°C. Lactate concentrations in media were quantified following 1:40 dilution of media in assay buffer with the Sigma-Aldrich Lactate Assay Kit. Glucose concentrations were quantified following 1:10 dilution of media in assay buffer with the ThermoFisher Glucose Colorimetric Detection Kit. Samples were incubated for 30 minutes. Absorbance was measured at 570 nm for lactate samples and 560 nm for glucose samples

Extracellular Flux Analysis

Mitochondrial metabolism alterations were evaluated with a Seahorse XFe96 Metabolic Analyzer (Agilent Technologies). ShPC M-Wnt cells were seeded in a 96-well Seahorse bioanalyzer plate for 24 hours with control media ± doxycycline. Control media was then removed and 100 μL of assay medium (XF base medium supplemented with 10mM D-glucose and 2mM glutamine or 10mM D-glucose, 2mM glutamine and 10mM L-lactate) was added to respective wells. The plate was incubated in a CO_2-free chamber at 37°C for 1 hour. Basal oxygen consumption rate (OCR) was measured with the Seahorse Mito Stress Test protocol. Following the assays, cells within each well were lysed and protein content was measured using a BCA Assay for normalization.

Results

I. Pyruvate carboxylase knockdown alters tumoral gene expression *in vivo*

Although PC has been associated with breast cancer metastasis to the lung, its roles in metabolic reprogramming and the TME at the primary site remain unclear. To study the effects of PC knockdown in a primary tumor model of TNBC, C57BL/6 mice were injected with M-Wnt cells transduced with a doxycycline-inducible ShRNA construct targeting PC (ShPC) and treated with or without doxycycline. This study revealed increased tumor mass and volume in the knockdown group (Dox) relative to control (Supplementary Figure 1A-C), a finding in contrast to previously described literature in which PC suppression led to decreased proliferation *in vitro* [75, 76] and no growth effect *in vivo*[73]. In order to investigate the mechanisms underlying the increased tumor growth, transcriptomic analysis of RNA isolated from dissected tumors was conducted to identify potential differences in gene expression caused by the loss of PC.

To determine whether the transcriptomic profile of each tumor clustered together within treatment groups, hierarchal clustering was conducted of the most variably expressed genes between Dox tumors and control. Indeed, strong clustering within relative to between groups was observed (Figure 1A). Heatmap visualization and dendrogram branching indicates that the samples within each group are related to one another more so than to the samples of the other group. This finding indicates that the PC knockdown tumors alter their transcriptomes relative to control in a similar fashion. Dimensionality-reduction via principle component analysis (PCA) of the data again shows strong clustering within groups (Figure 1B). All samples from each respective group are outside the 95% confidence interval of the other group, although they are not significantly different as there is overlap between the confidence intervals. This data suggests

that PC knockdown relative to control conditions resulted in distinct transcriptomic differences between tumors.

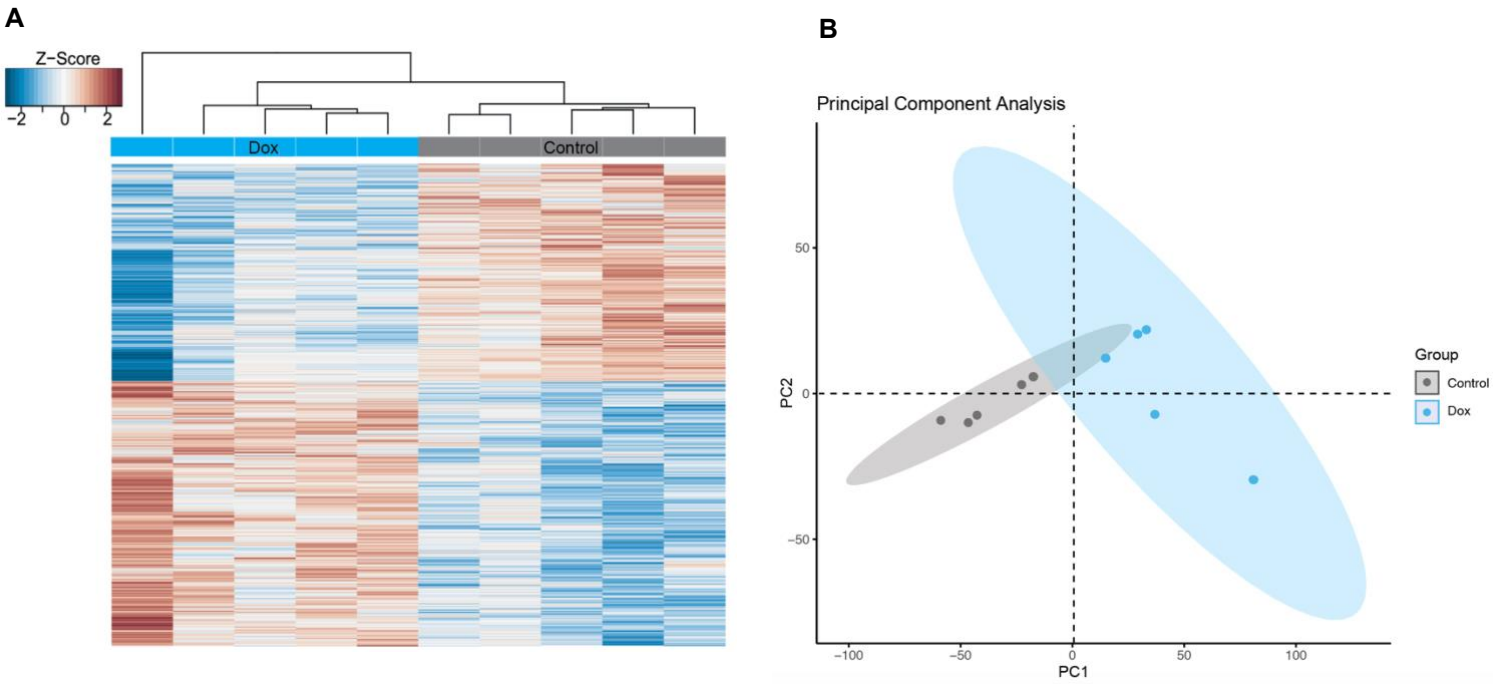

Figure 1. Clustering of most differentially expressed genes between PC knockdown tumors (Dox) versus control (p-value < 0.05 and 2-fold difference) (n = 5). Hierarchical clustering of heat map containing genes with significantly different expression levels reveals strong clustering within groups relative to between (**A**). Principle component analysis reveals strong clustering within groups, 95% confidence intervals indicated by ellipses (**B**).

II. Suppression of PC promotes a GSEA enrichment profile indicative of an immunosuppressive tumor microenvironment

Having demonstrated that loss of PC results in distinct transcriptomic changes in tumors, gene set enrichment analysis (GSEA) was utilized to identify major pathways present within the tumor derived gene lists. Following statistical comparisons of genome-wide expression data, the differential gene list yielded is often exceedingly long without any apparent biological themes. Attempting to analyze single genes from this list can be impractical due to size and complexity, but may also miss important effects of related genes along a pathway, as smaller increases in

expression of a related group of genes may be more biologically relevant than large increases in a single gene. An alternative to single-gene analysis is GSEA, which allows for the functional characterization of phenotypes based on curated gene lists that represent biological pathways. By ranking the genes within the gene list by their correlation with the two experimental conditions (PC knockdown versus control), it can then be determined whether a pathway is over-represented in one condition over the other. This analytical method allows for more biologically relevant analysis and identification of which pathways are driving transcriptomic differences.

The Hallmarks gene set contains refined gene lists that represent well-defined biological states with the aim of reducing noise and redundancy between gene lists[78]. GSEA using the Hallmarks gene set revealed 12 pathways that were significantly enriched (FDR q-value < 0.05) in the control condition and 5 pathways enriched in PC knockdown. GSEA calculates the normalized enrichment score (NES) by walking down the ranked list and calculating a running sum statistic that increases when a gene is present within a gene set and decreases when it is not. The NES is the greatest deviation from zero the running sum reaches normalized to gene set size, and represents the magnitude to which a gene set is over-represented at the top (positive NES) or bottom (negative NES) of the ranked gene list. The enrichment plot for interferon gamma response shows the gene set is over-represented at the bottom of the ranked gene list, with the black lines representing individual genes and their position along the gene list (Figure 2A). The negative NES corresponds to a negative correlation with the Dox group, indicating that suppression of PC downregulated this pathway. Of the 12 pathways downregulated in the Dox condition, 8 were related to immune processes, 2 are involved in lipid metabolism, and the remaining relate to common metabolic and cell cycle pathways (Figure 2B). The 8 immune gene sets (Table 1) are involved in multiple aspects of the immune system, affecting both innate and

adaptive leukocyte signaling and function, as well as inflammatory signaling. As these processes are critical towards effective anti-tumor immunity, the downregulation of these pathways in the Dox condition suggests that the host immune response to PC knockdown tumors is diminished relative to control. Heatmap visualization of the genes present in the immunological gene sets shows strong clustering of samples within groups versus between groups (Figure 2C), indicating that hierarchical clustering using solely immune-related genes segregates the two groups.

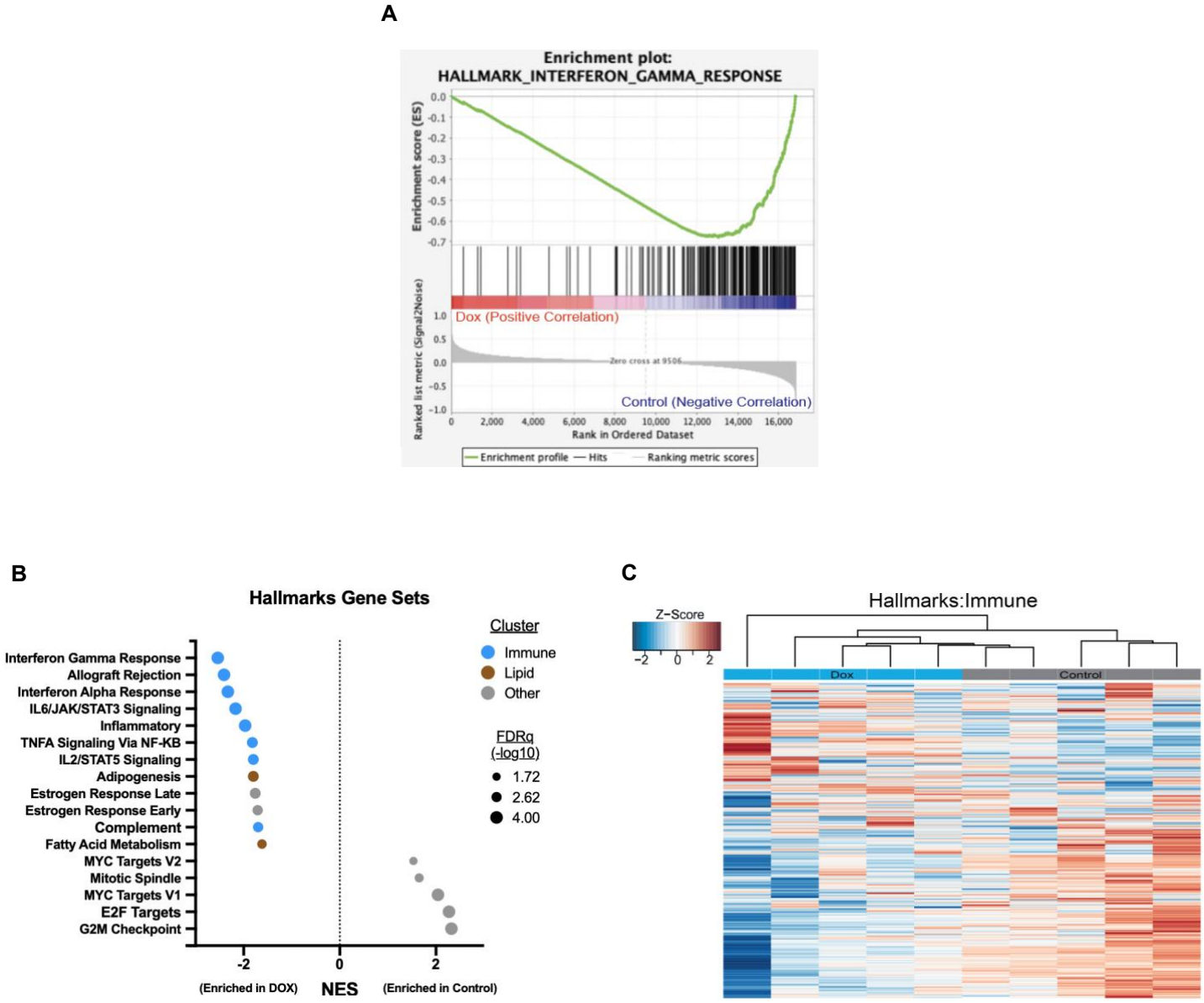

Figure 2. Gene set enrichment analysis (GSEA) with Hallmarks gene sets. Enrichment plot for the gene set Interferon Gamma Response shows the pathway is overrepresented in the control group (n = 5), indicating a positive correlation with the control condition (**A**). Hallmarks gene sets with statistically significant normalized enrichment scores (NES) (FDR q-value < 0.05) (**B**). Heat map of the immune related gene sets shows strong clustering within groups relative to between the two conditions (**C**).

Hallmarks	NES	FDR q-val
Interferon Gamma Response	-2.54	0
Allograft Rejection	-2.41	0
Interferon Alpha Response	-2.33	0
IL6/JAK/STAT3 Signaling	-2.17	0
Inflammatory Response	-1.97	0
TNFα Signaling Via NFKB	-1.82	0.001
IL2/STAT5 Signaling	-1.80	0.001
Complement	-1.70	0.002

Table 1. Hallmark gene sets related to immune processes with statistically significant normalized enrichment scores (FDR q-value < 0.05). 8 out of 12 pathways significantly downregulated in the Dox group were involved in the host immune response.

To further investigate the specific pathways contributing to the transcriptomic differences caused by PC knockdown, gene ontology (GO) Bioprocesses pathways were also analyzed via GSEA. GO bioprocesses is a more granular database than Hallmarks, with 3,992 gene sets. This increased breadth of different biological mechanisms may lend further insight into phenotypic differences between samples, as well as indicate pathways poorly represented in Hallmark gene sets. However, the size, complexity, and partial redundancy of the results makes interpretation difficult. Network-based visualization tools are one method to address this redundancy and allow for useful interpretation of the data. Cytoscape's Enrichment Map software was utilized for visualization and cluster formation of the GO Bioprocesses results in order to identify the major pathways present within related gene sets (Figure 3). Circles (nodes) represent gene sets significantly enriched in either condition (FDR q-value < 0.05) and are sized proportionally to

the number of genes within the gene set. Lines (edges) connecting two nodes represent at least 50% overlap of genes between those two sets, with increasing line weight corresponding to overlap over 50%.

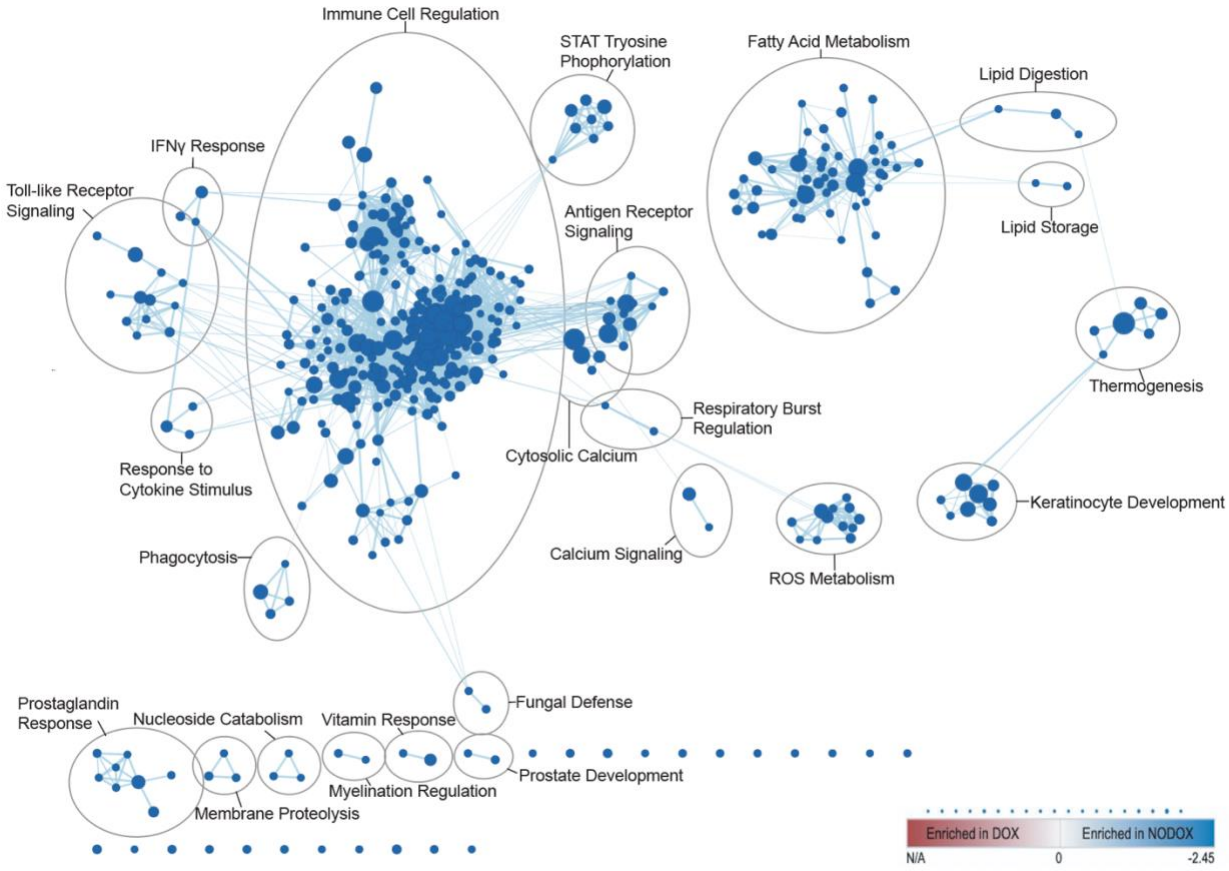

Figure 3. Pathway enrichment analysis of GO Bioprocesses gene sets to visualize pathway clusters with redundant genes. Circles (nodes) represent individual gene sets and are sized relative to the number of genes they contain. Lines (edges) connecting nodes represent at least 50% overlap of genes between the two gene sets, with increased line weight indicating amount of overlap above 50%. All gene sets shown are enriched in control (indicated in blue), as enrichment analysis with GO Bioprocesses did not yield any gene sets significantly enriched in the Dox condition.

The analysis of GO Bioprocesses resulted in no gene sets that were significantly enriched in the Dox condition, with no FDR q-values at or below 0.05. The control condition had 461 significantly enriched gene sets, resulting in only blue (enriched in control) nodes on the enrichment map. Similar to the findings of the Hallmarks analysis, immune-related gene sets were the most numerous of the Bioprocesses gene sets enriched in the control group, indicating downregulation of these processes in the PC knockdown condition. The effectiveness of this network-based approach to identify pathways associated with PC not identified in Hallmarks was supported by a vitamin response cluster, which represents well-established regulation of PC by vitamin D[82]. Cytoscape's clustering algorithms group similar pathways into biological themes. Multiple clusters with similar characteristics to the significantly enriched Hallmarks gene sets such as regulation of innate and adaptive leukocytes, interferon-γ response, and STAT signaling were present, as well as newly identified pathways such as antigen and toll-like receptor signaling. Together these results indicate suppression of PC promotes a transcriptomic profile indicative of immunosuppression and tumor immune evasion.

To determine the subset of genes most commonly contributing to the enrichment of gene sets, leading edge analysis was conducted using 131 gene sets from the GO Bioprocesses analysis that were enriched in the control condition and were significant at a highly conservative statistical cutoff (FDR q-value ≤ 0.001). The leading-edge subset is the genes within a gene set that appear in the ranked list before the running sum for NES reaches its peak. These genes are thus the main drivers of the normalized enrichment scores for the selected gene sets. The top ten most common genes from the 131 leading edge subsets analyzed are shown in table 2. The majority of the genes encode cytokines or cytokine receptors (IL12B, TNFSF4, IL6, XCL1, IL23R, IL1B, and IL10), suggesting that the Dox group had impaired immune signaling within

the tumor microenvironment, an observation consistent with downregulation of gene sets related to immune response seen in both Hallmarks and Go Bioprocesses analysis. These findings from GSEA suggest that critical pathways involved in the host immune response were suppressed in PC knockdown.

Genes	Number of Gene Sets	Classification
IL12B	74	Cytokine (T-cell differentiation)
TNFSF4	68	Cytokine (OX40 ligand)
IL6	62	Cytokine (Inflammatory Response)
XCL1	62	Cytokine (Leukocyte migration/activation)
IL23R	57	Cytokine Receptor (JAK2/STAT3 signaling)
HLA-E	55	MHC Class I Antigen (NK cell recognition)
PTPRC	53	CD45 Antigen (T-cell activation)
IL1B	53	Cytokine (Inflammatory Response)
HAVCR2	51	Immune Checkpoint Receptor ($CD8^+$ T-cell exhaustion)
IL10	50	Cytokine (Anti-inflammatory)

Table 2. Most common leading edge subset genes from 131 gene sets with the greatest enrichment in the control group (FDR value ≤ 0.001).

III. Cell fractions of critical immune populations are altered in response to PC knockdown

Having discovered the downregulation of critical pathways involved in immune signaling and activation in response to loss of PC, gene expression data was then utilized to determine immune cell composition in the TME. Common methods for deriving cell fractions from bulk

tissue, such as immunohistochemistry and flow cytometry, are often limited in their study of cell heterogeneity by the availability of phenotypic markers for cell recognition. An alternative approach is to use computational methods and previously described expression signatures for deconvolution of cell types from bulk expression data. CIBORSORTx is the leading such tool, with a regression coefficient of 0.97 in comparison to flow cytometry of healthy lung tissue[83]. This form of digital cytometry was utilized to discover if the downregulation of immunological pathways in response to loss of PC results in alterations to the abundance of key tumor infiltrating leukocytes (TILs).

Indeed, the cell fractions of 3 immune cell populations involved in the immunological response to tumors were significantly different between groups at a p-value of 0.05 (Figure 4). M0 macrophages were found at a higher cell fraction, while Th1 and resting natural killer cells (NK cells) were found at lower cell fractions in the Dox group. Differences in cell fraction of M1 macrophages and Naïve $CD8^+$ T-cells between groups were not statistically significant. Taken together, the differences in cell fractions between groups for M0 macrophages, Th1 cells, and Resting NK cells support previous findings that immunological pathways in tumors with PC knockdown were altered relative to control. Cause and effect are difficult to separate as lower abundance of immune cells in the TME could cause the downregulation of immunological pathways and vice versa. Nevertheless, gene expression data has revealed that PC knockdown results in a TME with diminished activation of inflammatory and anti-tumor immune cell regulatory pathways.

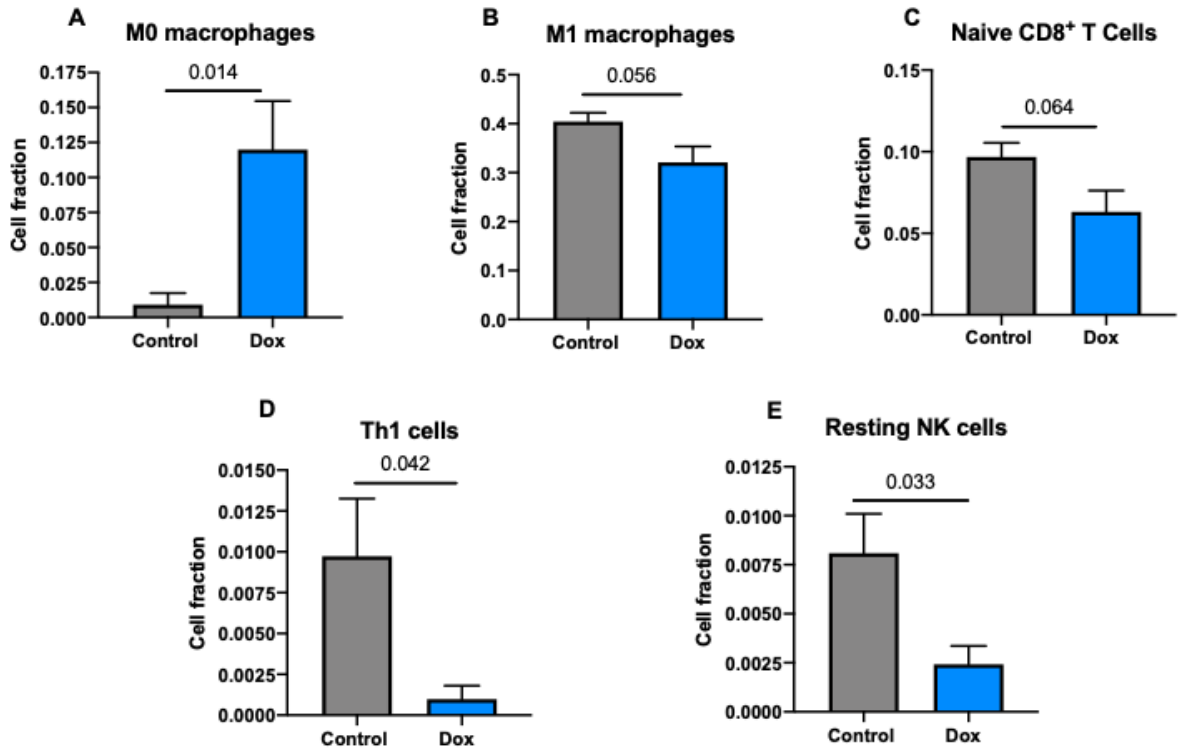

Figure 4. Digital cytometry using CIBERSORTx estimated immune cell fractions of tumor infiltrating leukocytes. Cell fractions were determined for M0 macrophages (**A**), M1 macrophages (**B**), naïve $CD8^+$ T cells (**C**), Th1 cells (**D**), and resting natural killer (NK) cells (**E**) and compared between control and dox groups (n = 5). Statistical analysis conducted using unpaired t tests was used to determine statistical significance (p-value < 0.05).

IV. PC knockdown results in metabolic reprogramming marked by increased lactate efflux and impaired mitochondrial metabolism

To investigate the mechanisms by which loss of PC may lead to an immunosuppressive TME, *in vitro* experiments with the doxycycline-inducible ShPC M-Wnt cell line were conducted. As PC serves as a critical enzyme in both gluconeogenic and anaplerotic pathways, its knockdown was expected to result in considerable metabolic reprogramming that could alter the TME nutrient pool, impacting not only malignant cells but tumor-infiltrating leukocytes.

Changes to glucose consumption and lactate production were first assessed to determine if PC suppression altered flux through glycolysis and lactate dehydrogenase (LDH). Indeed,

colorimetric assays measuring concentrations of L-lactate and D-glucose in cell culture media found a statistically significant increase in lactate concentration in the media (Figure 5A), as well as a significant decrease in glucose consumption in the Dox condition after 24 hours (Figure 5B). While both results were statistically significant, the effect size of the changes in lactate concentration were greater than the relative changes in glucose consumption, with an approximately 25% increase and 6% decrease, respectively. These results indicate that loss of PC results in more carbon being shunted through lactate dehydrogenase (LDH) and exported as lactate rather than into the TCA cycle or other biosynthetic pathways that branch from glycolysis. This effect comes without a marked increase in glucose consumption, implying the increased lactate export is a function of increased flux through LDH, rather than simply a greater intake of glucose with a concomitant increase in lactate production.

To investigate the effect of reduced pyruvate derived TCA cycle intermediates, extracellular flux analysis was used to assess perturbations to mitochondrial metabolism in response to PC suppression. As PC is an important anaplerotic enzyme, its knockdown was expected to alter mitochondrial metabolism and subsequently, the relative oxygen consumption rate (OCR) of the doxycycline treated ShPC cells. To gain more insight into the effects of extracellular carbon sources on mitochondrial metabolism, the cells were transferred from control media $\pm$ doxycycline to Seahorse media supplemented with either 2mM glutamine and 10mM D-glucose (Figure 5C) or 2mM glutamine, 10mM D-glucose, and 10mM L-lactate (Figure 5D) prior to experimentation. The addition of L-lactate to the media in one of the conditions was informed by the increased lactate efflux seen in the previous lactate assays, and used to better emulate nutrient availability PC knockdown tumors would experience *in vivo*. As expected, the Dox group had a significant decrease in relative OCR relative to control in both conditions. The condition which

included L-lactate saw a greater drop in relative OCR between the Dox group and control, indicating that lactate availability does not rescue mitochondrial metabolism perturbations. These *in vitro* results suggest that to compensate for loss of PC, M-Wnt cells shunt more carbons from glucose into lactate production, resulting in less pyruvate-derived TCA cycle intermediates and a subsequent decrease in OCR.

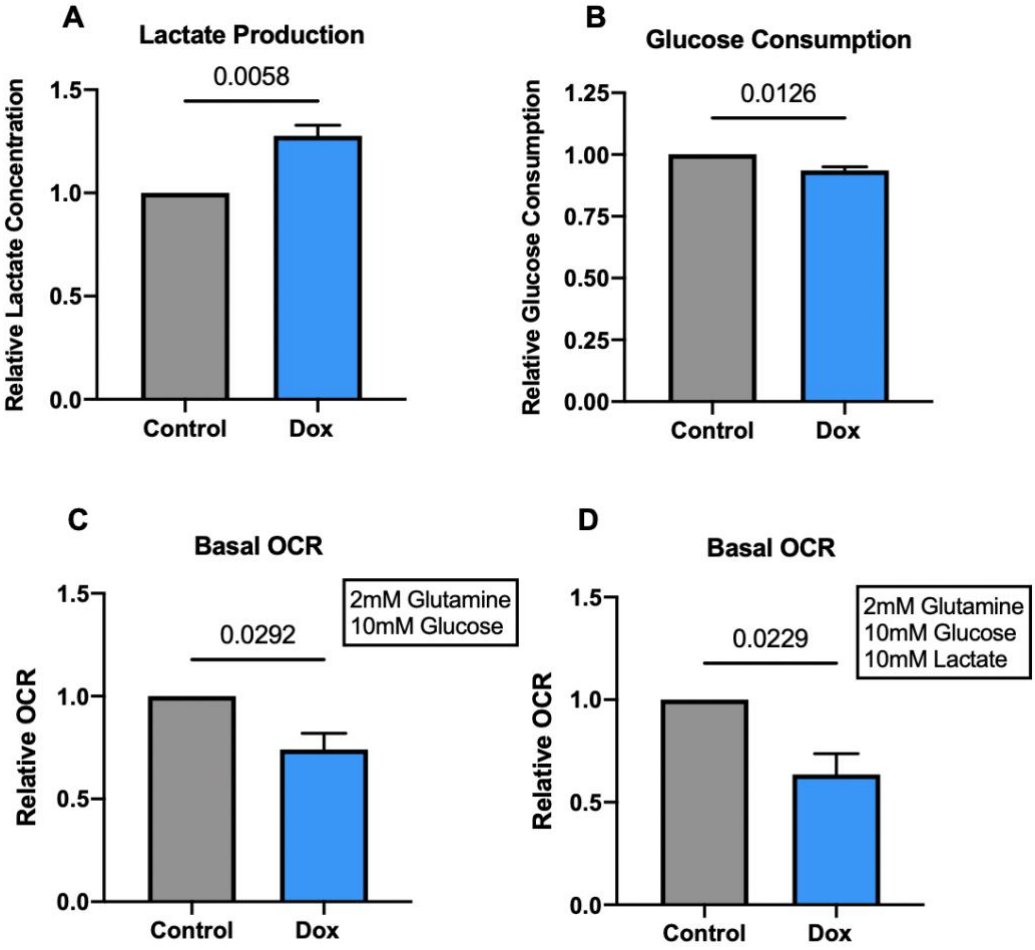

Figure 5. *In vitro* metabolic effects of PC knockdown. Lactate concentration (**A**) and glucose consumption (**B**) were measured in cell culture media of ShPC M-Wnt cells following 24 hours of treatment with control media ± doxycycline (n = 5). To determine relative oxygen consumption rate (OCR), ShPC M-Wnt cells were cultured in control media ± doxycycline for 24 hours then transferred to Seahorse media supplemented with 2mM glutamine and 10mM D-glucose (**C**) or 2mM glutamine, 10mM D-glucose, and 10mM L-lactate (**D**). Statistical analysis conducted using unpaired t tests was used to determine statistical significance (p-value < 0.05).

Discussion

PC expression in breast cancer is correlated with increased tumor size, stage, and metastatic potential, as well as survival of breast-cancer derived pulmonary tumors[72, 74]. Less understood is the impact of PC activity on metabolic reprogramming and the TME within primary tumors. Thus, we utilized orthotopic injection of ShPC M-Wnt cells to investigate how PC expression impacts primary tumors in a mouse model of TNBC. PC knockdown resulted in increased tumor mass and volume relative to control. Previous work modulating PC expression in models of breast cancer have either detected no primary tumor growth effect or a reduction in primary tumor growth following PC suppression[73, 75, 76]. Thus, our results represent a novel relationship between PC and primary tumor growth in models of TNBC. This study sought to investigate the transcriptomic and metabolic effects of PC suppression to delineate potential mediators of the observed pro-growth phenotype and add to the growing body of literature surrounding PC and tumor growth in models of breast cancer.

We discovered that the transcriptomes of PC knockdown tumors were altered relative to control, with strong intergroup clustering revealing that the transcriptomic changes were more similar between PC knockdown samples than to the control samples. This indicates that the metabolic reprogramming resulting from loss of PC activity led to a distinct gene expression profile that was consistent between PC knockdown tumors. Alterations to tumoral gene expression in response to loss of PC activity was expected, as gene expression and metabolism reciprocally regulate one another in order to maintain cellular homeostasis[84]. Metabolic reprogramming and the subsequent changes to metabolic enzyme levels not only affects flux through metabolic pathways but can also direct the subcellular localization of these enzymes to the nucleus, where they can then regulate gene expression through multiple mechanisms,

including chromatin-remodeling, histone-modification, and serving as transcription factors[85]. Further, these metabolically driven gene expression changes can result in a favorable transcriptome for cancer progression, as multiple metabolic enzymes are known to be essential components of oncogene driven transcriptional programs[86]. For example, the PDH complex is one of the primary enzymes which generates acetyl-CoA for use in the TCA cycle or fatty acid synthesis. While typically thought of as a mitochondrial enzyme, PDH has been shown to localize to the nucleus in response to mitochondrial stress where it produces a nuclear pool of acetyl-CoA that increases the acetylation of histones important for S-phase entry[87]. As PC is an anaplerotic enzyme influencing the levels of TCA cycle intermediates, its suppression-mediated changes to acetyl-CoA/PDH levels may be one of numerous mechanisms by which its knockdown is altering tumoral gene expression. Another potential mechanism that may be driving the transcriptomic changes is an altered immune profile in the TME, as changes in nutrient and metabolite concentrations in response to metabolic changes are potent mediators of immune infiltration and activation[88]. As immune cells make up a substantial portion of the cells within the TME[89], changes to their activity could be driving the overall transcriptomic profile of the tumors.

GSEA has emerged as a powerful analytical tool capable of identifying biological themes from genome wide expression data and is now widely used to gain insight into metabolic, immunological, and other oncogenic processes in various cancers. In our GSEA of transcriptomic data from control and Dox-treated tumors, the majority of Hallmarks gene sets downregulated following PC suppression were related to immunological pathways. Indeed, all immunologic gene sets described in Hallmarks were suppressed by PC knockdown. These downregulated pathways involve regulation of innate and adaptive immune processes, both of

which are critical for tumoral immunosurveillance and can be reprogrammed to support tumor progression[90]. Hierarchal clustering using these immunological gene sets again shows strong clustering within conditions relative to between, indicating that the immunological changes are consistent between PC knockdown samples. This effect of PC expression on immune regulation has not been described in the literature and may contribute to enhanced tumor growth, as immune evasion is a hallmark of cancer[38].

Two of the downregulated Hallmarks pathways in the PC knockdown group were the interferon gamma (IFN-γ) and interferon alpha (IFN-α) responses. Impaired signaling of Type-I (IFN- α) and Type-II (IFN- γ) interferons is a key mechanism underlying immune dysfunction in breast cancer[91]. Effective interferon signaling is vital to the anti-tumor immune response by both innate and adaptive immune cell populations, stimulating clonal expansion and differentiation of $CD8^+$ T-cells as well as positively regulating natural killer cell-mediated cytotoxicity[92, 93]. However, the net effect of IFNs on the TME is complex, as IFN signaling has also been identified as a key driver of immunosuppression and resistance to immunotherapies[94, 95]. The conflicting effects of IFNs in the TME can in part be explained by the differential responses to IFN signaling in cancer cells versus immune cells. Inactivation of the type I IFN receptor IFNAR1 in $CD8^+$ T-cells results in inhibition of CTL viability and decreased efficacy of immune checkpoint therapies[96]. However, loss of IFNAR1 in cancer cells rendered them more susceptible to $CD8^+$ T-cell mediated killing[97], indicating that while IFN signaling may promote an activated immune cell profile, type 1 IFN signaling may also be a mechanism of CTL-independent immune evasion in cancer cells.

Inflammatory response, TNFα signaling, and IL6/STAT3 signaling Hallmark gene sets were also reduced following suppression of PC, indicating diminished pro-inflammatory

signaling in the TME. Inflammatory signaling in cancer is complex, with roles in both immunosuppression and immune-mediated cancer elimination[98]. Ultimately, the relative expression of various signaling molecules (cytokines, metabolites) as well as the makeup of the immune cell population in the TME are what determine if inflammatory signaling leads to an anti-tumor or pro-tumor immune response[99]. IL-6 and TNFα are markers of an inflammatory TME and are produced by a multitude of immune cells, namely activated macrophages, dendritic cells, and T cells[100]. Thus, downregulation of IL-6/ TNFα signaling and the inflammatory response in the PC knockdown tumors may be indicative of an immune profile marked by diminished presence or function of these cytokine secreting immune cells, a characteristic of immune "cold" tumors[101]. Cold tumors are classified as having low levels of T-cell infiltration and activation, caused in part by defective sensing from innate immune cells[102]. Taken together, the downregulation of interferon and inflammatory signals is emblematic of an immunosuppressed microenvironment, with diminished infiltration and activation of TILs.

Enrichment mapping of significant GO Bioprocesses gene sets following GSEA revealed similar findings to Hallmarks, with the majority of identified clusters relating to immune processes that regulate innate and adaptive responses. Multiple clusters were consistent with identified Hallmark gene lists, such as IFN- γ response, STAT signaling, and response to cytokine stimulus, further supporting those findings. Clusters identified such as toll-like receptor signaling and antigen receptor signaling are not covered in Hallmarks analysis, however, are consistent with the proposed immune profile of diminished signaling and activation of TILs.

To further characterize the immune profile of the TME, leading edge analysis was used to find genes that were commonly contributing to differences in gene expression between groups. The majority of the ten most common genes identified via leading edge encoded cytokines,

cytokine receptors, or other immune activating antigens, again supporting the idea of an immune cold TME. Several genes directly related to T-cell differentiation, activation, and survival were common to the leading edges, including IL-12, PTPRC (CD45), and TNFSF4 (OX40-ligand). IL-12 is an interleukin produced by macrophages, dendritic cells, and neutrophils in response to antigenic stimulation, and mediates differentiation of naïve T-cells into Th1 cells[103]. As Th1 cells are major producers of IFN-γ, this finding provides a possible mechanism for the downregulation of the IFN-γ signaling found in Hallmarks analysis. PTPRC is present on all immune- differentiated hematopoietic cells and is an important regulator of T-cell activation[104], while TNFSF4 supports T-cell survival as a costimulatory signal[105]. Decreased expression of these genes would indicate impaired signaling between antigen-presenting cells and T-cells, further supporting an immunosuppressive TME profile.

Given the downregulation of immunological gene sets found in the PC knockdown condition, we next determined if these changes resulted in alterations to the abundance of immune cells in the TME. Traditionally, identification and quantification of immune cell populations is conducted using immunohistochemistry or flow cytometry. Alternatively, digital cytometry is a computational method of calculating cell fractions using deconvolution of gene expression profiles from bulk tissues to infer and quantify specific cell types. We utilized CIBORSORTx in our digital cytometry analysis, as it has been proven effective in distinguishing closely related cell populations as well as determining activation states of the same cell type, all while maintaining concordance with traditional methods[80, 83]. CIBORSORTx revealed that PC suppression resulted in decreased cell fractions of Th1 and resting NK cells, as well as an increased cell fraction of M0 macrophages. Tumor associated macrophages (TAMs) make up the majority of the immune cells in the TME of breast cancer and their infiltration has been

correlated with increased metastatic risk and poor prognosis[106]. M0 are the resting, nonactivated subgroup of macrophages, distinct from the polarized M1 and M2 states. Polarization of TAMs from M0 to M1 or M2 is orchestrated by the cytokine milieu of the TME[107]. M1 polarization occurs in response to microbial products or IFN$-\gamma$ and is associated with proinflammatory cytokine release, nitric oxide production, and protection against bacteria, viruses, and cancer. M2 polarization occurs in response to various signals including Il-4, IL-13, or glucocorticoids, and is associated with wound healing and tissue repair[108]. TAMs in the TME predominantly resemble M2-like macrophages and contribute to immunosuppression and tumor development[109]. A higher cell fraction of M0 macrophages in PC knockdown tumors indicates a lack of polarizing stimuli in the TME. This result further supports the previous findings of impaired innate signaling following PC suppression.

Th1 cells, reduced in the PC knockdown tumors, are a subset of $CD4^+$ T-cells that are involved in proinflammatory responses. IFN-γ, IL-2, and TNFα are important Th1-cytokines[110]; thus, data from GSEA of Hallmarks and GO Bioprocesses which found downregulation of these pathways in the PC knockdown condition reveal a potential pathway of Th1 cell-mediated signaling dysfunction in the TME. As stated previously, leading edge analysis from GO Bioprocesses found decreased expression of the gene encoding IL-12, which promotes differentiation of naïve T-cells into Th1 cells. As IL-12 has potent signaling effects in the TME, its downregulation may have contributed to the decreased cell fraction of Th1 cells, although further investigation is needed to control for other factors affecting naïve T-cell differentiation, and to delineate the role of IL-12 signaling in the TME following PC suppression.

Resting NK cells were also found in lower proportion in response to PC suppression. NK cells are the predominant innate lymphocyte that mediates anti-tumor immunity[111]. The

transition from resting to activated NK cells depends on the integration of signals from both activating and inhibitory receptors[112]. Decreased cell fractions of resting NK cells could imply either impaired infiltration of NK cells into the TME or a higher proportion of activated NK cells, although no significant difference was found in the cell fractions of activated NK cells. Taken together, the increased abundance of nonpolarized, M0 macrophages and the decreased abundance of Th1 cells and resting NK cells indicate that the transcriptomic changes caused by PC suppression result in an altered immune cell population indicative of diminished innate signaling and polarizing stimuli.

Following transcriptomic analysis, *in vitro* metabolic assays were conducted in order to determine the metabolic consequences of PC suppression that may direct TME remodeling. As PC is a critical enzyme for both gluconeogenesis and anaplerotic refilling of TCA cycle intermediates[113], we anticipated its knockdown to result in reprogramming of tumor cell metabolism. This reprogramming not only alters metabolic pathways within cancer cells, but affects metabolite concentrations in the TME, which can have profound effects on the metabolism and function of TILs and APCs[88]. Without normal function of PC, pyruvates other primary metabolic fates in the cell are reduction into lactate via lactate dehydrogenase, conversion to acetyl-CoA for entry into the TCA cycle via PDH, or transamination to form alanine[114]. As PDH is heavily regulated by acetyl-CoA and cellular redox status, it is limited in its capability to enhance pyruvate to acetyl-CoA flux in the face of increase pyruvate concentrations. Further, acetyl-CoA requires oxaloacetate to condense with and form citrate for flux through the TCA cycle; however, PC suppression reduces pyruvate-derived oxaloacetate. Instead of entry into the TCA cycle, increased flux through LDHA and a subsequent increase in lactate production was expected, as infants born with a genetic PC deficiency often present with

severe lactic acidosis in the blood[65]. Further, PC suppression in pancreatic beta cells results in decreased malate and citrate, increased concentrations of pyruvate and lactate, and no changes to alanine concentrations[115]. Although PC suppression in pancreatic beta cells lends insight into the metabolic reprogramming that results from loss of PC activity in the pancreas, these metabolite alterations may not be concordant with changes seen in cancer cells. Thus, metabolomic analysis of the tumor interstitial fluid in PC knockdown tumors is necessary to confirm the relative levels of these metabolites in response to PC suppression in cancer.

We investigated the effect of PC suppression on lactate production and glucose consumption by measuring the concentration of these metabolites in the cell culture media following 24H treatment of control media $\pm$ doxycycline. As expected, PC suppression led to a ~25% increase in lactate production relative to control. Importantly, increased glucose consumption was not the cause of this increase in lactate production, as PC suppression led to a small but significant decrease in glucose consumption. Lactate has profound signaling properties in the TME, and promotes immunosuppression via interactions with both innate and adaptive immune cells[116, 117]. In cytotoxic T lymphocytes, lactic acid was shown to suppress both proliferation and cytokine production, with up to 50% decreases in IFN- γ and Il-2 production[50], both of which were involved in pathways shown to be downregulated in our GSEA. Lactate exposure has also been shown to promote immunosuppressive phenotypes in innate immune cells, including polarization of M2-like macrophages, myeloid-derived suppressor cells (MDSC), and immature DCs[116, 118]. Thus, increased lactate production and export into the TME may play an important role in promoting the immunosuppression observed in tumors following PC suppression. It is important to note that these assays were conducted in 2D culture, as 3D tumors may have more complex interplay between tumors cells. Oxidative

cancer cells are known to uptake lactate derived from hypoxic cells located farther from the tumor vasculature to fuel their TCA cycle, a phenomenon known as metabolic symbiosis[119]. This effect could be an immunosuppression-independent mechanism fueling the pro-growth effect of PC suppression as a result of increased lactate production; hence, further investigation in the *in vivo* environment is needed.

Given its role as a key anaplerotic enzyme supplying oxaloacetate to the mitochondria, PC suppression was also expected to reduce the concentration of TCA cycle intermediates. Radiotracing experiments in PC knockdown breast cancer cell lines have shown lowered glucose incorporation into downstream metabolites of oxaloacetate including malate, citrate, and aspartate[120]. Our finding of increased production of lactate without a concomitant increase in glucose consumption agree with this model, as increased flux through LDHA rather than PC and PDH would leave less glucose-derived carbons for entry into the TCA cycle. To test if loss of PC affected mitochondrial metabolism, extracellular flux analysis was used to measure relative oxygen consumption rate (OCR) as a proxy for electron transport chain (ETC) activity. As extracellular carbon sources other than glucose could also affect TCA filling and consequently ETC function, OCR was measured in the presence of either 2mM glutamine and 10mM D-glucose or 2mM glutamine, 10mM D-glucose, and 10mM L-lactate. Exported lactate in the TME can serve as a potential nutrient for tumors and is a primary source of carbon for the TCA cycle[32]; thus, it is possible that adding lactate to the media prior to OCR measurements would rescue the mitochondrial perturbations expected from PC suppression. However, both conditions saw a significant decrease in OCR following suppression of PC. This finding supports the hypothesis that PC suppression results in alterations to mitochondrial metabolism, with three additional carbon sources unable to rescue this effect.

Taken together, these in vitro findings explain important alterations to central carbon metabolism following PC suppression and offer a potential mechanism underlying the immunosuppression of the TME. The fate of pyruvate is a key regulatory point governing the metabolic reprogramming of cancer cells. We have found that loss of pyruvate-derived oxaloacetate from PC results in increased production and export of lactate without an increase in glucose consumption, resulting in perturbations to mitochondrial metabolism due to the diminished flux of carbons for the TCA cycle. As lactate is a potent immunosuppressive signaling molecule, it may be a driver of the immunosuppression in the TME following PC suppression. Given this increase in lactate production and its known roles in the TME, targeting of LDHA with inhibitors such as FX11 may hold promise in reversing the immunosuppression and pro-growth phenotype caused by increased lactate export in conditions such as those caused by PC suppression. However, future studies controlling for lactate concentrations and other TME modulators (hypoxia, vascularization, metabolites) are needed to better discern the most consequential effects of PC suppression and reveal effective therapeutic targets. Future experimentation involving radiolabeling of carbon sources would also further elucidate the mechanisms by which PC suppression alters incorporation of carbons into TCA cycle intermediates and elucidate other important metabolic pathways under the regulation of pyruvate metabolism, including alanine formation via transamination, 3-phosphoglycerate derived serine production, and flux through the pentose phosphate pathway (PPP) for production of NADPH and nucleotide precursors. These pathways were not investigated in this study, however, may be contributing to the metabolic reprogramming and transcriptomic changes caused by PC suppression. These studies would yield further insight into the remodeling of the TME and the mechanisms underlying the observed immunosuppression

Conclusion

Pyruvate carboxylase's role in breast cancer is a growing area of interest, with important connections to tumor growth and metastasis. This study investigated the mechanisms underlying a pro-growth tumor phenotype observed in response to PC knockdown. Transcriptomic analysis revealed that PC suppression induced changes to the gene expression of bulk tumors, with pathways involved in immune signaling discovered to be downregulated. This gene expression profile is indicative of an immunosuppressed TME, which may be driving the observed pro-growth effect. Metabolic reprogramming caused by loss of PC activity was also observed, with increased production of lactate and diminished OCR. Lactate is a potent signaling molecule and may play an important role in the observed immunosuppression of the TME. Immunosuppressive TMEs are currently being targeted to enhance the efficacy of immunotherapies, such as ICIs. Our results suggest that targeting of lactate dehydrogenase or lactate's transporters MCT1 and MCT4 may hold promise in reversing the immunosuppressive effects of increased lactate export and make ICI more effective in the face of PC suppression. Taken together, this study revealed a novel relationship between PC expression and primary tumor growth and TME composition in a model of TNBC. The metabolic alterations seen in our model and the subsequent remodeling of the immunological network in the TME should be considered in future studies to better understand the effects of PC modulation in breast cancer models.

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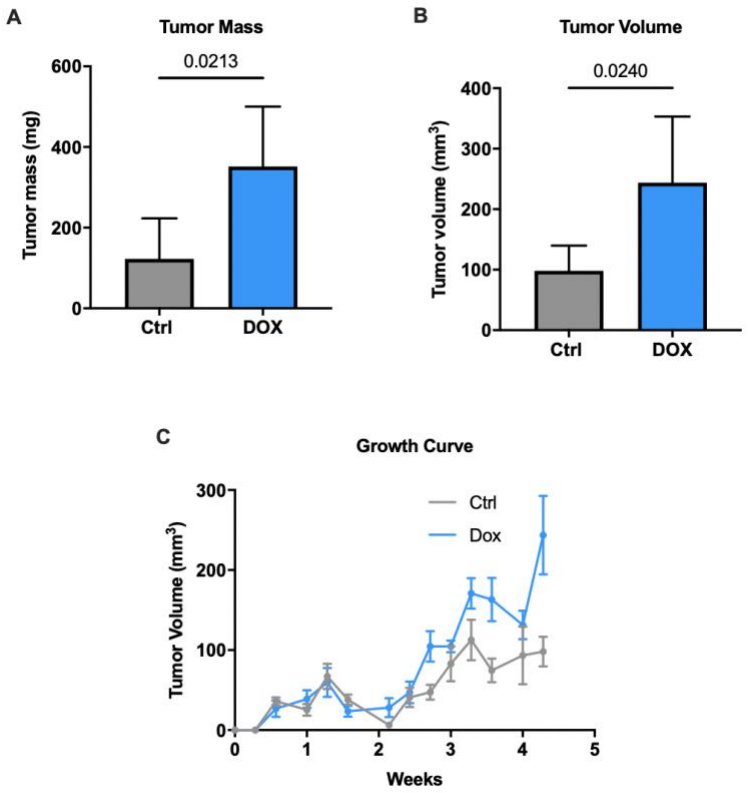
Supplemental Information



Supplemental Figure 1A-C. Tumor data from mouse study (n = 5). C57Bl/6 mice were injected with M-Wnt cells transduced with doxycycline-inducible ShRNA targeting PC. Doxycycline treatment began once tumors were palpable. Tumors were harvested 4 weeks following injection.