METABOLIC AND PROTEOMIC ADAPTATIONS OF METASTATIC PROCESSES IN BREAST CANCER CELLS

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Developments of targeted therapies improved prognoses and outcomes of patients with localized breast tumors. However, treatment options become limited upon occurrence of metastasis, a spread of tumors to distant organs, as surgical removal of tumors are difficult and tumor cells resist to therapies. Tumor cells need to overcome hurdles such as limited access to oxygen, through hypoxia, and matrix detachment during metastasis. To better understand underlying mechanism enabling tumor cells to overcome the two stresses, metabolic and proteomic changes of breast tumor cells when faced with inhibited oxidative phosphorylation (OXPHOS), a model of hypoxia, and detachment are analyzed.

To explore the influence of nutrients on protein expression and that of protein expression on metabolism, Chapter 2 of this dissertation presents a review of previous findings in studies on vitamin intakes affecting tissue proteomes. Metabolic fluxes reflecting cellular utilization of metabolic pathways are quantified with stable isotope tracing, which needs correction for abundances of naturally occurring isotopes if mass isotopologue distributions are directly used to infer fluxes. Chapter 3 introduces a computational tool, PolyMID, which corrects for abundances of naturally occurring isotopes. Among metabolic fluxes, that through pyruvate carboxylase (PC) is reported

to be altered upon breast cancer-derived lung metastasis. However, work presented in Chapter 4 shows that the widely used method to quantify PC flux is inaccurate, and covers the development of a metabolic flux analysis-based approach with improved performance.

Utilizing the above-mentioned tools, the work presented in Chapter 4 continues with analyses of metabolic and proteomic changes in breast cancer cells upon OXPHOS inhibition and culture in suspension. Protein networks regulating metabolic fluxes are identified. PC flux and its positively correlated protein network upon OXPHOS inhibition are found to be negatively correlated in cells resistant to detachment upon culture in forced suspension. Exposure to hypoxia elevates anchorage-independence of the detachment-resistant breast cancer cells. Taken together, hypoxia and matrix detachment induce metabolic and proteomic changes affecting metastatic potential of breast cancer cells.

BIOGRAPHICAL SKETCH

Heesoo completed her Bachelor and Master of Science in Food and Nutrition at Sookmyung Women's University, Seoul, Korea. Her thesis was on antioxidative and inflammatory mechanisms of galactose-induced aging mouse model. Heesoo entered the doctoral program in Molecular Nutrition in the Division of Nutritional Sciences at Cornell University in 2018. She joined Dr.Nathaniel Vacanti's lab where she applied stable isotope tracing and proteomic analyses to study metabolic and proteomic changes of breast cancer cells. She combined experimental and computational analyses and pursued minors in genomics and computational biology. Heesoo received the Genomics Scholars Awards from the Center for Vertebrate Genomics at Cornell in 2021.

This dissertation is dedicated to my parents, Joon Rae Jeong and Min-Seob Yu, and my brother, Jeehun Jeong.

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Chapter 1. Introduction

1.1 Stable Isotope Tracing and Metabolism

1.1.1 Quantifying Cellular Utilization of Metabolic Pathways

Metabolism is defined as biochemical reactions producing or consuming energy. Cells utilize metabolic pathways to chemically process nutrients to produce energy and synthesize macromolecules (1). Tricarboxylic acid (TCA) cycle is the major metabolic pathway cells utilize to produce reducing equivalents to feed into the electron transport chain (ETC) to make energy. TCA cycle intermediates can also exit mitochondria to synthesize fatty acids, amino acids, hemes, and purines (2). As can be seen from TCA cycle, metabolic pathways contribute to production of energy and macromolecules in the cell. Analyzing how cells utilize metabolic pathways provide information on how cells use available nutrients to survive.

Cellular utilization of metabolic pathways can be quantified by tracing stable isotope-labeled nutrients. Isotopes of an element carry the same number of protons but different numbers of neutrons, resulting in having different masses. One of the commonly utilized isotopes to label nutrients are carbon atoms (3, 4). Carbon atoms whose masses are 13 atomic mass units (amu) which are heavier than the ones found in nature (12 amu) can be used to label nutrients such as glucose or glutamine (5-7). By culturing cells with medium containing a stable isotope-labeled nutrient and analyzing the distribution of the isotopes in metabolites, researchers can get information on how cells utilize the nutrient for fuel.

1.1.2 Correcting for Naturally Occurring Isotope Abundances

i. Direct inspection of mass isotopologue distributions for flux analysis

Researchers can perform a computational quantitation using metabolic flux analysis (MFA) to analyze cellular utilization of metabolic pathways. MFA requires researchers to computationally construct a metabolic reaction network. Information on tracers used in experiments and relative abundances of metabolites whose masses are different due to isotope incorporation are used as inputs for MFA. Fluxes of metabolic pathways in a network are computed by minimizing the sum of squared differences between the measured and model-estimated values. Due to complexity in constructing a network, MFA can be time- and data-consuming to researchers not familiar with computational analysis (3, 8).

A more common approach to study cellular utilization of metabolic pathways is direct interpretation of mass isotopologue distributions (MIDs). When cells are cultured with stable isotope-labeled nutrients, metabolites that only differ in isotope compositions called mass isotopologues are present due to incorporation of isotopes. In a ^{13}C tracing analysis, a metabolite with *n* carbon atoms has isotopologues from M0 (all carbons are ^{12}C) to M*n* (all *n* carbons are ^{13}C). Relative abundances of isotopologues from M0 to M*n* of a metabolite is called the MID. MIDs of metabolites can be directly used to infer how cells use specific metabolic pathways when isotope incorporation in metabolites is constant over time (3). For instance, relative abundances of citrate with two ^{13}C atoms (M2 Cit) are frequently used as a measure of pyruvate dehydrogenase (PDH) activities in the cells cultured with glucose carrying six ^{13}C atoms ([U-$^{13}C_6$]glucose). Pyruvate with three labeled

carbon atoms produced from [U-$^{13}C_6$]glucose is converted to acetyl coenzyme A (AcCoA) with two labeled carbon atoms (M2 AcCoA) by the activity of PDH. If M2 AcCoA condenses with oxaloacetate with unlabeled carbon atoms, M2 citrate is produced, which is commonly used as a readout of PDH activities (5, 6, 9). As direct interpretation of MIDs is more intuitive and requires less time, it is widely used to study cellular utilization of metabolic pathways in various disease models such as cancer cell proliferation (10) and metastasis (7, 11), diabetes (12, 13), and neural tube defects (14).

ii. Naturally occurring isotope abundances confounding MID interpretation

However, mass shifts of metabolites can also arise from incorporation of naturally occurring isotopes in stable isotope tracing analyses. Specifically, presence of naturally occurring carbon atoms whose masses are 13 amu are 1.11%. If researchers take MIDs to draw conclusions on cellular utilization of metabolic pathways without correcting for the 1.11%, results can be misleading in ^{13}C tracing analyses (15). In addition to carbon atoms, naturally occurring isotopes exist in other chemical atoms such as ^{2}H (relative abundances of 0.0115%), ^{15}N (relative abundances of 0.368%), ^{17}O (relative abundances of 0.038%), and ^{18}O (relative abundances of 0.205%) (16). Thus MIDs should be corrected for abundances of naturally occurring isotopes to be accurately used as readouts of metabolic pathway utilization.

1.2 Proteomics and Metabolism

Cellular metabolism can be analyzed by inspecting abundances of substrates and

products of metabolic pathways by metabolomics. However, abundances of metabolites do not always correspond to cellular utilization of metabolic pathways. Specifically, accumulation of metabolites does not necessarily imply increased cellular utilization of metabolic pathways producing the metabolites as pathways consuming the metabolites may be inhibited (17). Quantifying activities and abundances of enzymes catalyzing metabolic reactions can aid in better understanding of how cells utilize metabolic pathways. Analyzing cellular proteomes enables researchers to inspect protein abundances of multiple metabolic enzymes simultaneously. Researchers can also examine correlations of protein abundances between metabolic enzymes and how they are correlated with proteins other than metabolic enzymes such as those involved in cell cycle or deoxyribonucleic acid (DNA) repair. Thus proteomics can be a useful tool to study correlations and regulations of metabolic enzymes.

1.2.1 Impact of Vitamin Intakes on Tissue Proteomes

i. Vitamins as cofactors in metabolic reactions

Vitamins are cofactors in majority of metabolic reactions. Vitamin B_3 works as a cofactor in the pentose phosphate pathway, pyruvate oxidation catalyzed by PDH, *de novo* serine synthesis, and TCA cycle (18). Vitamin B_2 is needed for activities of ETC Complex I and II which take reducing equivalents generated from TCA cycle to produce energy (19). In addition to the two vitamins mentioned above, other vitamins participate as cofactors in various metabolic pathways (18, 20). As metabolic enzymes need cofactors to be activated, availability of vitamins in our body may impact abundances of metabolic enzymes, and consequently, cellular proteomes.

ii. Vitamins as regulators of nuclear transcription factors

Vitamins also affect proteomes by regulating transcription of genes which consequently impacts protein abundances of the genes. Vitamin A can activate the retinoic acid receptor (RAR)-retinoid X receptor (RXR) heterodimers which regulate transcription by interacting with retinoic acid-responsive elements in the promoters of genes (21). Vitamin D can bind to vitamin D receptors which regulate transcription together with RXR by interacting with vitamin D receptor elements in the promoter regions of genes (22). Considering their roles in regulation of gene transcription, vitamins can affect abundances of proteins which are the products of transcription and translation and ultimately carry physiological functions in our body.

1.3 Breast Cancer and Metastasis

1.3.1 Breast Cancer

Breast cancer is estimated to have the highest number of new cases among all cancers in 2023 in the United States (US). Approximately 13% of women is estimated to be diagnosed with breast cancer during lifetime (23) and death rate is reported to be 19.6% per 100,000 women in the US (24). Breast tumors are heterogeneous and have been categorized based on immunohistochemical measurements of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in clinical settings (25). In 2000, Perou and colleagues classified breast tumors based on mRNA expressions and identified five molecular subtypes; luminal A and B, HER2, basal, and normal-like subtypes (26).

Among breast cancer-subtypes, patients with triple-negative (TN) breast tumors lack expressions of the ER, PR, and HER2 and account for 15% in the US breast cancer population. Though 70-80% of TN breast tumors are reported to be classified as basal-like molecular subtype, TN breast tumors that are not basal-like subtype exist, indicating the two terms are not synonymous (27, 28). Development of targeted therapies such as ER-targeting endocrine agents and HER2-targeting antibodies improved prognoses and outcomes of patients with hormone receptors-positive breast cancer patients (patients expressing ER, PR, or HER2). However, no targeted therapies are currently available for patients with TN breast tumors which may be associated with higher rates of mortality and earlier relapse compared with patients with other breast cancer-subtypes. Due to lack of targeted therapies, only options available for patients with TN breast tumors are surgery, radiation, and chemotherapy, all of which have severe side effects (25).

1.3.2 Metastasis of Breast Cancer

i. Metastasis as the cause of majority of deaths in breast cancer patients

Metastasis, a spread of tumors into distant organs, occurs through a series of sequential steps. Tumors cells invade and migrate through extracellular matrix (ECM) in primary tumors, move into vasculature or lymphatic system, survive during circulation, exit the circulation, and enter/colonize secondary organs (29). Upon metastasis, removal of tumors with surgery becomes difficult and tumor cells can resist to targeted therapies. Due to these challenges in treatment of metastatic tumors, 90% of cancer deaths are reported to be associated with metastasis (30).

Majority of breast cancer-associated deaths are also due to metastasis rather than

localized tumors themselves (31). According to the US cancer statistics from 2012 to 2018, five-year survival rates of breast cancer patients with metastasis were only one-third of the survival rates of patients with primary breast tumors (23). Gogate and colleagues estimated the number of breast cancer patients with metastasis to increase by 50% from 2015 to 2030 (32), indicating survival of more breast cancer patients may be at risk. Though the development of targeted therapies, early screening, and identification of breast cancer molecular subtypes contributed to increased survival of breast cancer patients with primary tumors, further research is needed to prevent or treat breast cancer metastasis.

ii. Distinct patterns of metastasis among breast cancer-subtypes

Timing and sites of metastasis are reported to differ according to the breast cancer subtypes. Kennecke and colleagues reported patients with TN breast tumors have higher probabilities of developing metastasis within 15 years since diagnosis. Duration of survival after diagnosis of metastasis is reported to be shorter in patients with TN breast tumors compared with those carrying the other breast cancer-subtypes (33).

For patients with hormone receptors-positive breast tumors, bone is reported to be the common site of metastasis. For patients with TN breast tumors, bone and lung are reported to be the two most common sites of metastasis. According to a multivariate analysis comparing preferential metastatic sites across patients with different breast cancer-subtypes, patients with TN breast tumors have higher rates of distant nodal metastasis and lower rates of bone metastasis compared with those with the other breast cancer subtypes. Interestingly, patients with HER2-positive but

ER/PR-negative breast tumors have higher rates of brain metastasis compared with those with the other breast cancer-subtypes (33).

1.3.3 Anchorage-Independent Survival of Tumor Cells During Metastasis

Tumor cells need to overcome multiple hurdles to spread to distant organs and failure to overcome any of the hurdles can halt metastatic cascade. Only those tumor cells that acquired mechanisms to overcome all the hurdles can land at secondary sites and interact with host microenvironments (34). The first barrier tumor cells need to overcome is to maintain survival upon detachment from ECM at primary tumors. Non-malignant or tumor cells without metastatic potential undergo cell death process called anoikis upon detachment from ECM. However, certain tumor cells develop mechanisms to resist anoikis by regulating the tumor necrosis factor receptors superfamily called death receptors or mitochondrial caspases (35), enabling them to survive under anchorage-independent conditions. Cellular mechanism supporting anchorage-independent survival affects viability of tumor cells going through other steps of the metastatic cascade as tumor cells in circulation or at secondary sites (before colonization) are also under anchorage-independent condition (36). Thus, maintaining cellular survival in anchorage-independent condition serves a gatekeeper to successful metastasis.

1.4 Metabolic Changes Promoting Anchorage-Independent Survival of Breast Tumor Cells

Tumor cells are reported to change phenotypes to maintain viability during metastatic cascade. For instance, tumor cells change from epithelial to mesenchymal morphology to detach from ECM and move to circulatory system. Throughout the metastatic cascade, anchorage-independent tumor cells are exposed to different types and amounts of metabolites upon losing contacts with ECM (37). Thus, tumor cells alter utilization of nutrients and metabolic pathways to meet metabolic requirements during metastasis.

A previous study reported decreased utilization of glucose in glycolysis, pentose phosphate pathway, and TCA cycle upon detachment from ECM in a mammary epithelial cell line (38). In an anchorage-independent mammary epithelial cell line, HER2-overexpression increased glucose utilization in glycolysis, pentose phosphate pathway, PDH activities, and TCA cycle , indicating metabolic changes upon ECM detachment may be different according to distinct molecular signatures in mammary epithelial cells (39).

In a TN breast cancer cell line, anchorage-independent cells are reported to depend more on glutamine than glucose to maintain viability (40). Fendt and colleagues reported increased dependence on pyruvate upon detachment from ECM in a breast cancer cell line (41). These previous findings suggest breast cancer cells may rewire metabolism to survive in anchorage-independent condition.

1.5 Proteomic Changes Promoting Anchorage-Independent Survival of Breast Tumor Cells

i. Proteomes reflecting survival and metastasis occurrence of breast cancer patients

Proteomic signatures are reported to stratify breast tumors into survival outcomes according to a quantitative proteomics on breast tumors of 178 patients (42), indicating analyzing proteomes may provide information directly related to patient survival. As proteins carry physiological functions in our body, proteomes may contain mechanisms crucial to survival of breast cancer patients. As metastasis is the cause of majority of cancer-associated deaths (30), analyzing proteomes of patients with metastasis may enable researchers to identify molecular mechanisms associated with patient survival. A previous study on plasma proteomes of 48 TN breast cancer patients reported cell adhesion and migration as pathways proteins whose abundances were differentially expressed in patients with metastasis compared with those without metastasis (43). The primary purpose of tumor cell adhesion and migration is to support anchorage-independent survival in metastasis (44). Thus analyzing proteomic changes associated with anchorage-independent survival of breast tumor cells can provide mechanisms critical to metastasis occurrence.

ii. Reported proteomic changes upon detachment from ECM in breast cancer cells

A previous study on the proteome of a hormone receptor-positive breast cancer cell line reported increased abundances of proteins regulating cell-cell or cell-ECM interactions upon detachment from ECM (45). In two hormone receptor-positive breast cancer cell lines, detachment from ECM increased abundances of proteins involved in degradations of fatty acids and ketone bodies, an oxidative branch of pentose phosphate pathway, and pyruvate carboxylase-catalyzed glucose anaplerosis (46). In addition to the abundance changes in the metabolic enzymes,

decreased abundances of proteins in phosphoinositide 3-kinases (PI3K)-AKT serine/threonine kinases (AKT) signaling and an integrin signaling pathways, all of which regulate cell cycle, are reported in the two anchorage-independent hormone receptor-positive breast cancer cell lines (47).

iii. Associations of metabolomes and proteomes in breast tumors

A proteomic analysis on breast tumors of 45 patients reported proteomic signatures are associated with glycolytic properties of breast tumors (48), suggesting a close association between metabolomes and proteomes in breast tumors. As metabolic rewiring and proteome alterations are both critical to development of metastasis, analyzing metabolic and proteomic changes simultaneously may enable identification of more efficient molecular targets that affect both.

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