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Original Research Article

Exo-MFA – A 13C metabolic flux analysis framework to dissect tumor microenvironment-secreted exosome contributions towards cancer cell metabolism

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ABSTRACT

Dissecting the pleiotropic roles of tumor micro-environment (TME) on cancer progression has been brought to the foreground of research on cancer pathology. Extracellular vesicles such as exosomes, transport proteins, lipids, and nucleic acids, to mediate intercellular communication between TME components and have emerged as candidates for anti-cancer therapy. We previously reported that cancer-associated fibroblast (CAF) derived exosomes (CDEs) contain metabolites in their cargo that are utilized by cancer cells for central carbon metabolism and promote cancer growth. However, the metabolic fluxes involved in donor cells towards packaging of metabolites in extracellular vesicles and exosome-mediated metabolite flux upregulation in recipient cells are still not known. Here, we have developed a novel empirical and computational technique, exosome-mediated metabolic flux analysis (Exo-MFA) to quantify flow of cargo from source cells to recipient cells via vesicular transport. Our algorithm, which is based on ^{13}C metabolic flux analysis, successfully predicts packaging fluxes to metabolite cargo in CAFs, dynamic changes in rate of exosome internalization by cancer cells, and flux of cargo release over time. We find that cancer cells internalize exosomes rapidly leading to depletion of extracellular exosomes within 24 h. However, metabolite cargo significantly alters intracellular metabolism over the course of 24 h by regulating glycolysis pathway fluxes via lactate supply. Furthermore, it can supply up to 35% of the TCA cycle fluxes by providing TCA intermediates and glutamine. Our algorithm will help gain insight into (i) metabolic interactions in multicellular systems (ii) biogenesis of extracellular vesicles and their differential packaging of cargo under changing environments, and (iii) regulation of cancer cell metabolism by its microenvironment.

1. Introduction

The tumor microenvironment (TME) is a complex milieu of several types of cells, blood vessels and extracellular matrix proteins in which cancerous cells thrive. Fibroblasts, immune cells, endothelial cells and pericytes are collectively known as stroma and constitute the TME. These cells become reactive and develop characteristics that support and even enhance tumor progression and metastasis due to proximity and constant interaction with cancer cells (Chu et al., 2007; Valencia et al., 2014). Tumor development is thus being looked at in a new light, one in which TME plays a pleiotropic role in tumor progression and metastasis. Cancer-associated fibroblasts (CAFs), the most abundant constituent of TME, have been implicated in promoting tumor growth, conferring resistance to immune response by secreting soluble factors (Feig et al., 2013; Rupp et al., 2015), aiding tumor cells in acquiring resistance to therapeutic agents (Chen et al., 2015). Stromal cells also provide carbon sources like free fatty acids and amino acids to meet bioenergetic demands of cancer cells (Yang et al., 2016; Salimian et al., 2014; Sanità et al., 2014). Given the multiple modes of support provided by stromal cells, the TME needs to be investigated with the

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same vigor and focus as afforded to cancer cells. More importantly, knowledge of seemingly indispensable intercellular interactions within the TME has presented new avenues for therapy in cancers that form solid tumors (Ghesquière et al., 2014; Hansen et al., 2016). However, to aid such therapeutic discoveries, we should learn to assess complex multicellular systems with a level of granularity that is currently overlooked.

Researchers have revealed that microvesicles secreted by cells are an important apparatus of transporting vital biological cargo between stromal and tumor cells that can support tumor progression (D'Souza-Schorey and Clancy, 2012; Penfornis et al., 2016). Exosomes are microvesicles that are 30–100 nm in diameter and transport proteins, nucleic acids including miRNA intercellularly. Exosomes secreted by cancer cells condition the extracellular environment and stromal cells in close proximity to aid proliferation and metastasis (Costa-Silva et al., 2015). Recently, our group showed that CAFs secrete exosomes to regulate the metabolism of recipient cancer cells. This metabolic regulation takes place in two ways; (i) miRNA-mediated inhibition of mitochondrial oxidative metabolism and (ii) nutritional supplementation via *de novo* "off-the-shelf" metabolites (Zhao et al., 2016). ^{13}C tracer experiments provided compelling evidence that exosomes transport free metabolites that are directly incorporated into cancer cells' central carbon metabolite pools. Quantitatively assessing the contribution of metabolite cargo to cancer cell metabolism will be the first step for dissecting the pleiotropic effects of CAF-secreted exosomes (CDEs) on tumor growth. This will help achieve a mechanistic understanding of exosome-mediated rescue of nutrient-deprived cancer cells to identify suitable therapeutic targets.

In the current study, we have designed a novel paradigm, exosome-mediated metabolic flux analysis (Exo-MFA), to predict fluxes involved in metabolite trafficking from CAFs to cancer cells. Exo-MFA integrates a novel experimental protocol using ^{13}C-labeled substrates with an enhanced metabolic flux analysis to provide insight into metabolic crosstalk within the TME. Stable-isotope tracing techniques have emerged as a powerful empirical tool to provide insight into nutrient utilization and metabolic pathway activities by measuring isotopic enrichment of intracellular metabolites (Yang et al., 2014; Bennett et al., 2008; Hosios et al., 2016; Jiang et al., 2016; Lewis et al., 2014; Zamboni et al., 2009; Caneba et al., 2012; Bellance et al., 2012). A range of ^{13}C, ^{15}N and ^{2}H labeled substrates have been employed to study altered metabolism affecting cancer pathology. However, due to the presence of branches and cycles in metabolic networks, manually analyzing isotope labeling data can only provide relative changes in limited number of metabolic pathways. Fortunately, computational flux analysis techniques such as 13-C metabolic flux analysis, (^{13}C-MFA) have been developed that can estimate intracellular fluxes based on the reaction stoichiometry of metabolic networks, carbon atom transitions and extracellular fluxes within complex mammalian systems (Bordbar et al., 2014; Huang et al., 2014; Quek et al., 2009; Weitzel et al., 2013; Young, 2014). Exo-MFA utilizes the fundamentals of the ^{13}C-MFA algorithm and tracer experiments, traditionally used for single cell systems that only exchange metabolites with their media, to provide an enhanced platform to analyze metabolite fluxes in multicellular systems.

Herein, we study the effects of metabolite cargo of CDEs on pancreatic ductal adenocarcinoma (PDAC) cells under nutrient-deprived conditions. We successfully employ Exo-MFA to estimate the packaging fluxes in CAFs that constitute the metabolite cargo of exosomes. Furthermore, Exo-MFA predicts the rate of exosome internalization by PDAC cells within physically reasonable ranges. Finally, Exo-MFA estimates contribution of exosomal cargo to intracellular metabolites in PDAC cells, proving that metabolite cargo significantly alters their intracellular fluxes and supports TCA cycle fluxes. We observe that the effect of metabolite cargo diminishes within 24 h due to depletion of exosomes in the media, but our results strongly indicate that the metabolites supplied by CDEs can support PDAC metabolism during the initial stages of nutrient deprivation.

2. Methods

2.1. Cell culture

MiaPaCa-2 cells were cultured in DMEM (high glucose) medium with 10% FBS, 1% penicillin-streptomycin. CAF19 cells were cultured in DMEM (high glucose) medium with 15% FBS, 1% penicillin-streptomycin. For labeled exosomes collection, CAF19 cells were cultured in RPMI medium with uniformly ^{13}C labeled glucose, glutamine, phenylalanine (Cambridge isotope laboratories). RPMI deprived media were constructed from medium of cat no. R9010 (USBiologicals). For all experiments with nutrient-deprivation condition, MiaPaCa-2 were cultured in RPMI reconstructed from R9010 medium. MiaPaCa-2 cells in deprivation conditions were cultured in 6-well plates with seeding density of 200,000 cells per well.

2.2. Protein assays

Protein amount for CAF19, MiaPaCa-2 and exosomes are measured per the Bicinchoninic Acid (BCA) Protein Assay protocol. In brief, protein reagent (200 µl) was added to a 96-well assay plate and mixed with samples or standards (10 µl), and then incubated at 37 °C for 30 min. The absorbance was read on a spectrophotometer at 562 nm.

2.3. Metabolic assays

2.3.1. Glucose assay

Glucose assays were performed according to the instructions of the Wako Glucose kit. In brief, a 250 µl of reconstituted Wako glucose reagent was added to a 96-well assay plate followed by adding 2 µl sample in each well. The plate was incubated at 37 °C for 5 min with vortex. The change in absorbance, which indicates the amount of glucose present, was measured at 505 nm and 600 nm by using a spectrophotometer (SpectraMax M5; Molecular Devices).

2.3.2. Lactate assay

Lactate secretion was measured using the Trinity Lactate Kit. Media samples were diluted 1:10 in PBS, and lactate reagent was reconstructed and added to the diluted samples in a 96-well assay plate. The plate was incubated for 1 h at 37 °C, protecting from light. Afterwards the change in absorbance was read on a spectrophotometer at 540 nm.

2.3.3. Amino acid measurements

Ultra-high-performance liquid chromatography was used to assess amino acid uptake and secretion using Waters Acquity UPLC device. Briefly, media samples were deproteinized, and MassTrak Reagent was added to the samples, along with Borate Buffer/NaOH. Samples were then heated and analyzed using the Waters ACQUITY UPLC system. Eluents were prepared according to Waters' protocol. MassTrak AAA eluent A concentrate was diluted 1:10 in milliQwater, and MassTrak AAA eluent B was inputted in undiluted form. Flow rate of eluents was 0.4 ml/min, and UV detection was at 260 nm.

2.3.4. Viability assay

Cells viability was measured by Cell counting kit-8. Cells were cultured on 96-well plate in the indicated conditions. Viability assay solution was added to the plate for incubation period of 2 h and absorbance was measured at 450 nm.

2.4. Isotope labeling experiments and GC-MS analysis

2.4.1. Intra-exosomal metabolites extraction

The exosomes pellet was extracted by adding 75 µl of cold methanol; 150 µl of cold water with norvaline was added, which