

Metabolic Analyses of Crosstalk between Pancreatic Stromal and Cancer Cells

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Cite This: *Anal. Chem.* 2026, 98, 27340–27353



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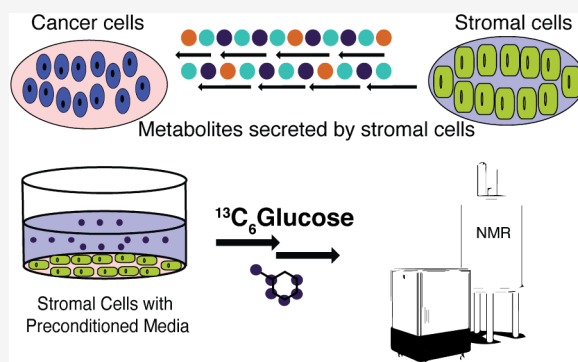


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ABSTRACT: Metabolic crosstalk between cell types is critical to our mechanistic understanding of pancreatic cancer. The lack of methods to investigate metabolic crosstalk has impeded the comprehensive OMIC characterization of these complex cellular systems. To address this need, an NMR metabolomics method employing a selective isotope-labeling strategy is presented to study metabolic crosstalk in routine cell cultures. 2D ^1H – ^{13}C HSQC NMR experiments were used to simultaneously monitor pancreatic cancer cells and stromal cells, which revealed a cancer-cell-induced metabolic remodeling of stromal cells. Cancer-cell-conditioned medium was used to stimulate stromal cells, which identified a unique and divergent metabolism in stromal cells in contrast to the classical Warburg metabolism in cancer cells. The two phenotypically different stromal cells exhibited distinct stimulated metabolic profiles; however, the exo-metabolome from both stimulated stromal cells revealed that $^{13}\text{C}_6$ -glucose-derived carbon is excreted as alanine and pyruvate. Furthermore, intracellular $^{13}\text{C}_6$ -glucose was converted to a variety of tricarboxylic acid cycle-linked and redox metabolites. Stromal cells, unlike cancer cells, mainly convert $^{13}\text{C}_3$ -pyruvate to alanine and lactate at an early growth time point, followed by the incorporation of $^{13}\text{C}_3$ -pyruvate-derived carbon into multiple amino acids. Interestingly, unstimulated stromal cells depend on pyruvate for survival, whereas cancer cells do not. Simply put, efficient metabolic crosstalk between cancer and stromal cells enables the cells to utilize different metabolic pathways and nutrient sources. Although presented to study cancer-stromal crosstalk, the method can be easily adapted to a variety of cellular crosstalk systems, such as neuron-astrocyte, host–pathogen, or host-microbiota systems.



INTRODUCTION

Biological processes in living organisms are complex and interconnected, wherein different cell types are grouped to form tissues, organs, or even cancerous tumors.¹ Dynamic and intertwined cellular relationships are therefore required to maintain homeostasis and perform core functions. Nevertheless, single-cell lines are routinely the focus of scientific inquiry despite the inherent complexity of multicellular organisms.² The availability of individual cell lines has significantly contributed to the understanding of cell biology and human disease, enabling larger pursuits such as drug discovery and personalized medicine.^{3,4} Unfortunately, a reductionist approach to understanding cellular function does eliminate important contributions from the complex cellular organization found in tissues or tumors. The routine failure to translate laboratory successes to a clinical setting may be attributed to the fact that a single cell is an insufficient surrogate for the complex cellular environment of a tissue or tumor.⁵ Accordingly, there is a need to design protocols that facilitate the investigation and characterization of multicellular systems.

Genomics, proteomics, and metabolomics may provide a means to decipher the complexity of cell-to-cell communication in a complex system.^{6,7} Metabolomics is an obvious choice for characterizing cellular communication involving an exchange of metabolites between cell types. Metabolites, in addition to their role in providing energy, are building blocks for cellular structure and complex macromolecules (e.g., DNA, RNA, proteins), regulate enzyme function, gene transcription, and signaling, and dictate the cellular phenotype.⁸ Metabolites that perform these aforementioned functions also participate in cellular communication, such as chemotaxis,⁹ quorum sensing,^{10,11} autocrine, paracrine signaling,^{12,13} and synaptic signaling.¹⁴ This metabolite-driven cellular communication,

Received: April 1, 2026

Revised: August 18, 2026

Accepted: August 31, 2026

Published: September 9, 2026



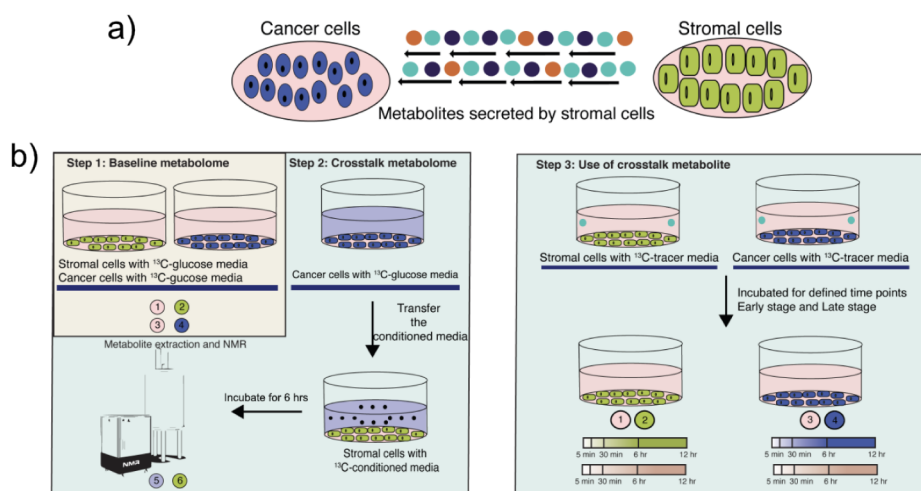


Figure 1. Overview of the SIRM workflow to measure metabolic crosstalk. (a) Simplified diagram of the overall goal of the study to measure the uptake by cancer cells (blue) of the metabolites excreted (colored circles) by stromal cells (green). (b) A pictorial diagram of the three primary steps that comprise the SIRM workflow. The first step (**Step 1**) consists of measuring the individual baseline metabolomes for both the stromal (green) and cancer (blue) cells. In both cases, the cell culture media (pink) is supplemented with $^{13}\text{C}_6$ -glucose for 6 h. ^{13}C -labeled metabolites from stromal cells that were excreted into the culture medium (exo-metabolome, pink 1) and from the cell lysate (cellular metabolome, green 2) were analyzed using 2D ^1H - ^{13}C HSQC NMR experiments. Similarly, ^{13}C -labeled metabolites from cancer cells that were excreted into the culture medium (pink 3) and from the cell lysate (blue 4) were analyzed using 2D ^1H - ^{13}C HSQC NMR experiments. The next step (**Step 2**) measures the metabolic crosstalk between cancer and stromal cells. The cancer cells were cultured in media supplemented with $^{13}\text{C}_6$ -glucose (light blue) for 6 h, while the stromal cells were cultured in unlabeled media. The stromal cell culture medium was then replaced by the cancer cell-conditioned medium (light blue). The stromal cells were incubated for an additional 6 h. ^{13}C -labeled metabolites from the stimulated stromal cells that were excreted into the conditioned medium (light blue 5) and cell lysate (green 6) were analyzed using 2D ^1H - ^{13}C HSQC NMR experiments. The final step (**Step 3**) characterized how cancer and stromal cells metabolized crosstalk metabolites. Stromal cells and cancer cells were incubated in culture media (pink) supplemented with a ^{13}C -labeled crosstalk metabolite (e.g., $^{13}\text{C}_3$ -pyruvate or $^{13}\text{C}_3$ -alanine) identified from **Step 2**. Cells were harvested and media were collected at four different time points (5 min, 30 min, 6 h, 12 h). ^{13}C -labeled metabolites from stromal cells that were excreted into the culture medium (pink 1) and from the cell lysate (green 2) at each time point were analyzed using 2D ^1H - ^{13}C HSQC NMR experiments. Similarly, ^{13}C -labeled metabolites from cancer cells that were excreted into the culture medium (pink 3) and from the cell lysate (blue 4) at each time point were analyzed using 2D ^1H - ^{13}C HSQC NMR experiments.

i.e., metabolic crosstalk, can promote cell survival or proliferation and has garnered significant interest.^{15–17}

Alterations in cellular metabolic crosstalk can provide cues to disease states and distinguish cellular responses to environmental stress and more.^{18,19} Different cell types are likely to incur signature metabolic crosstalk that may simultaneously reduce cellular stress while exchanging needed nutrients and creating a dependent, symbiotic relationship between different cell types.

Pancreatic ductal adenocarcinoma (PDAC) has a dismal 5-year survival rate of 13% in the United States²⁰ and 10% in Canada.²¹ PDAC is the third leading cause of cancer-related deaths in the United States and the fourth in Canada, and its incidence continues to rise in both countries.^{20,21} PDAC tumors are characteristically aggressive, exhibit a heterogeneous microenvironment, and have a fibrotic stromal response to the invasive cancer, where the stroma forms the bulk of the primary tumor.²² The resulting desmoplastic reaction is a hallmark of PDAC that facilitates interactions between different cell types and influences cell survival, proliferation, and migration.^{22,23} The stroma and its associated extracellular matrix (ECM) assist in tumor invasion and metastasis by providing metabolites needed for tumor growth and by creating a barrier that reduces the effectiveness of anticancer agents.²⁴ Overall, the aggressiveness of PDAC is often attributed to a crosstalk between the cancer cells and stroma.^{25,26} Understanding the role of cancer-stromal cells is critical for enhancing our repertoire of therapeutic strategies against aggressive PDAC cancers.

NMR is an invaluable analytical tool for deciphering and tracking small-molecule-based cellular communication in PDAC. Accordingly, NMR metabolomics methods are used to investigate PDAC and other cancers.^{18,27,28} NMR metabolomics has enhanced our fundamental understanding of cancer pathogenesis,^{27,29} assisted the development of new drug treatments,¹⁹ improved disease diagnosis,³⁰ and calibrated patient-specific efficacy of treatment.³¹ One-dimensional (1D) ^1H NMR experiments, in combination with univariate and multivariate statistical techniques, provide a means to characterize the global metabolic profile from cells in cultured environments, tumors, or patient-derived biofluids.³² Metabolites and metabolic pathways responsible for phenotypical variations in pancreatic cancer cell models are readily identified by NMR methods and are now routine.^{18,27,29,33} Isotopically labeled metabolites (e.g., ^{13}C -glucose, ^{15}N -alanine, etc.) combined with two-dimensional (2D) NMR (e.g., 2D ^1H - ^{13}C HSQC) and statistical methods are commonplace to improve metabolite identification and to define specific use of metabolites across pathways. Although these approaches have provided important insights into PDAC metabolism, most studies have focused on defining metabolic alterations in isolated cell populations.¹⁹ In PDAC, however, cellular metabolism is strongly influenced by interactions between cancer cells and the surrounding stromal compartment. In fact, up to 80% of the total tumor mass is not represented by the cancer cells but by the desmoplastic stroma.³⁴ As a result, there is increasing interest and need for methods to study metabolite crosstalk between cancer and stromal components of

PDAC.^{15,16} To address these limitations, we present a methodology to study metabolic crosstalk that combines an isotopic labeling strategy with an optimized cell culture protocol to characterize metabolite exchange between two cell types, pancreatic cancer and stromal cells. This methodology can tease out the mechanistic role of divergent metabolism between pancreatic cancer cells and the surrounding stromal cells that aid in supporting the cancer cells overall survival. We hypothesized that pancreatic cancer cells actively remodel stromal cell metabolism to generate metabolites that compensate for cancer-specific metabolic deficiencies, thereby establishing mutually dependent metabolic crosstalk. To test this hypothesis, our study pursued three specific aims: (1) define the baseline metabolic phenotypes of pancreatic cancer and stromal cells using $^{13}\text{C}_6$ -glucose tracing; (2) determine how cancer-derived conditioned media alter stromal cell metabolism and survival; and (3) characterize the temporal utilization of key crosstalk metabolites, specifically $^{13}\text{C}_3$ -alanine and $^{13}\text{C}_3$ -pyruvate, by each cell type. Our experiments establish a generalizable platform for dissecting metabolic communication within multicellular systems and provide new insights into the metabolic interdependence of pancreatic tumors.

METHODS

Study Design

An overview of the series of experiments used to characterize the metabolic crosstalk between pancreatic cancer cells and stromal cells is diagrammed in Figure 1. Pancreatic cancer cells (S2-013), pancreatic stromal cells, human pancreatic stellate cells (HPS), and cancer-associated fibroblasts (CAF-0911) were used for all experiments. S2-013 cells are male pancreatic ductal adenocarcinoma cells derived from a metastatic site in the liver. HPS and CAF-0911 are the main fibroblastic cells of the pancreas. The study design consisted of three major steps: (1) profiling the baseline metabolome for the stromal and cancer cells, (2) identifying metabolites that have net extracellular accumulation by the stromal cells when triggered by cancer cells, and (3) characterizing how the crosstalk metabolites were utilized by the recipient cancer or stromal cell.

We establish the baseline metabolome of cancer and stromal cells (Step 1). To initiate all cell culture experiments, S2-013, HPS, and CAF-0911 cells were separately cultured overnight until the cells attached, in triplicate, at 37 °C and 5% CO_2 , supplemented with Dulbecco's modified Eagle medium (^{12}C DMEM). After overnight attachment of cells, ^{12}C DMEM was removed, the cells were washed three times with 1X PBS, and the cells were supplied with DMEM with 25 mM $^{13}\text{C}_6$ -glucose as the stable isotope tracer (^{13}C DMEM), at 37 °C with 5% CO_2 . A 2 mL aliquot of the medium was reserved to serve as a control. Cells were then harvested at the 6 h time point, lysed, and the metabolome was extracted using our standard protocols using cryogenically cold 80% methanol (see Supporting Information Methods). A 2D ^1H – ^{13}C HSQC spectrum was acquired for each cell lysate and medium sample.

We then characterized the metabolic crosstalk (Step 2). The stromal cells (HPS and CAF-0911) were cultured in ^{12}C DMEM, and the cancer cells (S2-013) were cultured in ^{13}C DMEM to obtain cancer-conditioned media for the stromal cells. The stromal cell culture medium was swapped with the cancer-conditioned medium from the cancer cell culture. The stromal cells were incubated for another 6 h. A 2 mL aliquot of the medium was collected, and the cells were then harvested and lysed, and the metabolome was extracted (see Supporting Information Methods). A 2D ^1H – ^{13}C HSQC spectrum was collected for each cell lysate and medium aliquot.

To characterize how the crosstalk metabolites are utilized (Step 3), after overnight culture of the cells with regular complete DMEM, the media were aspirated, and the cells were washed with ice-cold 1X

PBS; then the cells were cultured with fresh DMEM supplemented with 1 mM of $^{13}\text{C}_3$ -pyruvate or $^{13}\text{C}_3$ -alanine to investigate the fate of selected crosstalk metabolites (see Supporting Information Methods). Cells were then harvested at 5 min, 30 min, 6 h, or 12 h after the ^{12}C DMEM was replaced with the ^{13}C -tracer-supplemented DMEM. A 2 mL aliquot of the medium was collected, and the cells were then harvested and lysed, and the metabolome was extracted (see Supporting Information Methods). A 2D ^1H – ^{13}C HSQC spectrum was acquired for each cell lysate and media sample.

Each resulting 2D ^1H – ^{13}C HSQC NMR spectrum was analyzed to identify and quantify all observed metabolites using our standard protocols (see Supporting Information Methods). For each assigned metabolite, an average relative metabolite concentration (i.e., peak intensity), a standard deviation, a fold change, and a false discovery rate (FDR)-corrected p -value from pairwise comparisons were calculated. All metabolite changes were used for network analysis to create the metabolic pathways.

RESULTS

Stable Isotope-Resolved Metabolomics (SIRM) Protocol to Monitor Metabolic Crosstalk

Our optimized SIRM workflow (Figure 1) combines a cell culture protocol using conditioned media, a selective isotope-labeling strategy, and a 2D ^1H – ^{13}C HSQC NMR experiment to trace the metabolic flow in a two-cell system. Here, we were able to monitor the impact of the cancer cell (S2-013) stimulation on stromal cell (HPS and CAF-0911) metabolism. Our SIRM protocol for characterizing cell-to-cell communication consists of three primary steps. The first step was to establish the baseline central carbon metabolism for each cell type. $^{13}\text{C}_6$ -glucose was selected as a stable isotope tracer because it is the primary carbon source for cellular metabolism.³⁵ As a result, our baseline SIRM study utilized media supplemented with $^{13}\text{C}_6$ -glucose. This approach enabled the identification of alterations in glucose metabolism and informed the experimental conditions used to investigate the metabolic crosstalk. An overnight culture in ^{12}C DMEM (DMEM without $^{13}\text{C}_6$ glucose supplementation) followed by a 6 h culture in ^{13}C DMEM, which corresponds to a total cell culture time of 18 h, was determined to be the most practical approach that also maximizes the incorporation of ^{13}C into the metabolome (Figure 1b).

For the second step, the transfer of cancer-conditioned medium from the cancer cells to the stromal cell culture required complete replacement of the spent stromal medium. To ensure no spent stromal medium remained, the cells were washed three times with 1X PBS. A series of 2D ^1H – ^{13}C NMR HSQC spectra of the aspirated 1X PBS (Figure S1a,b) confirmed the complete elimination of detectable ^{13}C -labeled metabolites from the stromal culture before the addition of ^{13}C -labeled cancer-conditioned medium. To avoid inadvertent transfer of cancer cells with the conditioned media, the ^{13}C -labeled conditioned medium was centrifuged (e.g., 20,800 g for 5 min at 4 °C) before adding the medium to the stromal cells. Overall, these two steps generated three extracellular metabolite pools: cancer-conditioned medium, stromal cell-conditioned medium, and double-conditioned medium (cancer-conditioned medium subsequently conditioned by stromal cells). These media were used to investigate metabolites involved in tumor–stromal metabolic crosstalk. Lastly, to expand upon how stromal cells utilize crosstalk metabolites, we studied the time-dependent metabolism of selected crosstalk metabolites (Figure 1b).

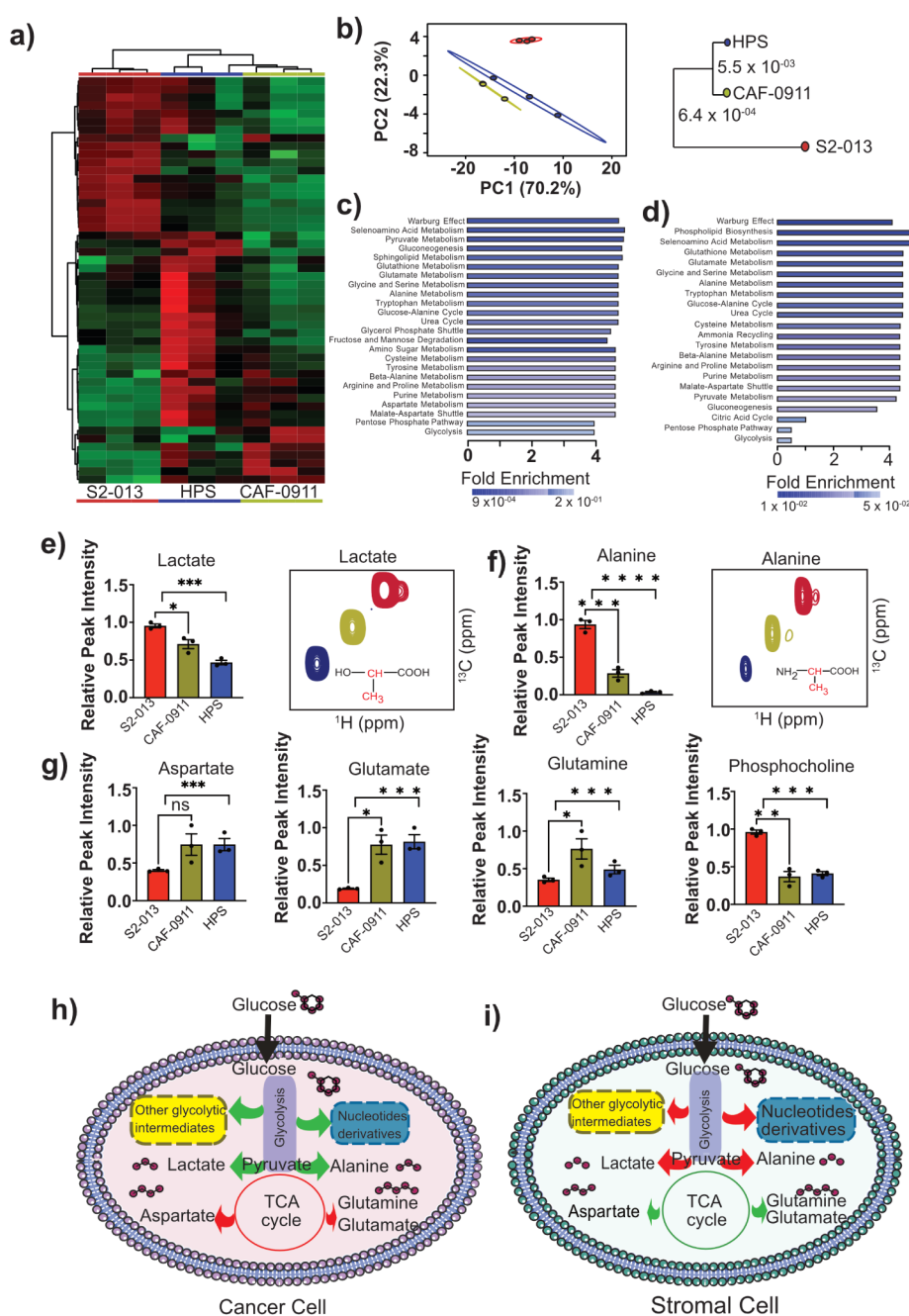


Figure 2. Baseline metabolome differences between cancer and stromal cells. (a) Heatmap summarizing the metabolome differences between cancer (S2-013) and stromal (HPS and CAF-0911) cell lysates. Cells were cultured for 6 h in DMEM supplemented with $^{13}\text{C}_6$ -glucose. For simplicity, only the top 50 NMR peaks (p -value ≤ 0.05) are represented in the heatmap. Red corresponds to a relative metabolite increase and green corresponds to a relative metabolite decrease in cancer cells compared to stromal cells. Both the NMR peaks and the cell-type triplicates were hierarchically clustered in the heatmap. NMR peaks were grouped based on their relative increase or decrease between the two cell types. The two sets of stromal cell triplicates were grouped together and separately from the cancer cell triplicates. The S2-013 triplicates are colored red, the HPS triplicates are colored blue, and the CAF-0911 triplicates are colored yellow. (b) PCA scores plot generated from the 2D ^1H – ^{13}C HSQC spectral data set obtained from the cancer (S2-013) and stromal (HPS and CAF-0911) cell lysates. The PCA model (R^2 0.84, Q^2 0.28) was created using all of the 2D ^1H – ^{13}C HSQC NMR peaks from each of the three replicates per cell type. The associated metabolomics tree diagram was generated from the PCA model, where each node contains a Mahalanobis distance-defined p -value. The S2-013 replicates are colored red, the HPS replicates are colored blue, and the CAF-0911 replicates are colored yellow. MSEA pathway enrichment map (<https://www.metaboanalyst.ca/>) showing the top-most differentially used metabolic pathways when comparing the (c) S2-013 and HPS cellular metabolomes and the (d) S2-013 and CAF-0911 cellular metabolomes. Metabolic pathways are listed in decreasing order of corrected p -value. Variations in (e) lactate and (f) alanine levels between cancer and stromal cells calculated using the shown 2D ^1H – ^{13}C HSQC peak intensities. The NMR peak assignments are indicated by the red-colored atoms in the chemical structures. S2-013 peaks are colored red, HPS peaks are colored blue, and the CAF-0911 peaks are colored yellow. (g) Dot scatter plots showing variations in metabolite levels between cancer and stromal cells based on selected 2D ^1H – ^{13}C HSQC peak intensities. S2-013 dots are colored red, HPS dots are colored blue, and the CAF-0911 dots are colored yellow. Each solid line represents the mean value of the replicates. Schematic summary of the baseline metabolic variations between (h) cancer and (i) stromal cells. A red arrow indicates a

Figure 2. continued

decrease, and green arrows indicate an increase in the total metabolite content in comparing cancer cells to stromal cells. Significance was assessed using Student's *t* test (ns > 0.05, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

Differential Glucose Metabolism and a Distinct Metabolic Phenotype for Pancreatic Cancer and Stromal Cells

Baseline metabolites from pancreatic cancer (S2-013) and stromal (HPS and CAF-0911) cells derived from $^{13}\text{C}_6$ -glucose were identified from triplicate 2D ^1H – ^{13}C HSQC spectra. ^{13}C -labeled metabolites from both the cell lysates and the culture media were identified, and a heatmap summarizing the top 50 NMR peaks for the three different cell lysates (S2-013, HPS, and CAF-0911) is shown in Figure 2a. Hierarchical clustering analyses of the mean-centered 2D ^1H – ^{13}C HSQC peaks using the Ward clustering algorithm³⁶ resulted in two distinct branches. Not surprisingly, the two stromal cell types (HPS and CAF-0911) formed a distinct branch from the cancer cells (S2-013). Hierarchical clustering of the NMR peaks identified several distinct branches of similarly altered NMR peaks, which were used to simplify the assignments of highly variable metabolites, such as glucose, lactate, and alanine. Further, a principal component analysis (PCA) model was generated from the 2D ^1H – ^{13}C HSQC data set. The resulting PCA score plot (Figure 2b) demonstrated a similar group separation between the cancer and the two stromal cell lines. The magnitude and statistical significance of the group separation are readily apparent from the tree diagram (Figure 2b) using our PCA/PLS-DA utilities (<http://bionmr.unl.edu/pca-utils.php>).^{37,38} The *p*-value at each node is based on the group separation in the PCA score plot using a Mahalanobis distance.³⁷ In summary, these analyses demonstrate that the pancreatic cancer cells have a clearly distinct metabolic phenotype from pancreatic stromal cells.³⁷

A metabolite set enrichment analysis (MSEA) pathway enrichment plot (Figure 2c,d) was generated from the complete list of 14 metabolites identified from the cancer and stromal cell lysates (Table S1). Importantly, the pathway enrichment analysis software emphasizes human metabolic processes (<https://www.metaboanalyst.ca>).^{39,40} The metabolite pathway enrichment plot further demonstrates the distinct metabolic phenotypes of the pancreatic cancer and stromal cells. A univariate analysis of NMR peak intensities (Figure 2e–g) clearly indicated a significant increase (*p* < 0.01) in cellular lactate for the cancer cells when compared to the stromal cells. Higher levels of alanine (*p* < 0.001) and phosphocholine (*p* < 0.05) were also observed in the cancer cells. In contrast, a relative decrease in amino acid levels was observed in the cancer cells. HPS cells exhibited higher levels of glutamate (*p* < 0.05), glutamine (*p* < 0.05), and aspartate (*p* < 0.05). The cancer and stromal cells have a fundamentally distinct metabolic profile as summarized in the cell diagram (Figure 2h,i). As illustrated, the six glucose ^{13}C carbons are traced through the cellular metabolism after uptake from the extracellular environment.

A similar NMR analysis of the spent media showed the cancer and stromal cells excreted a diverse set of metabolites (Figure S2 and Table S1). The ^{13}C -labeled metabolites excreted into the media mainly included amino acids, amino acid-derived metabolites, and glycolysis intermediates (Table S1). All these excreted metabolites were derived from the $^{13}\text{C}_6$ -glucose supplemented in the media, which was taken up by the

cells and metabolized. Like the cell lysates, metabolites from the spent media show a systematic difference between the cell types. The hierarchically clustered heat map indicates that the exo-metabolome from cancer and stromal cells is distinctly different. The dendrogram shows CAF-0911 clustering more closely with the S2-013 cancer cells than with HPS, with HPS forming the most distinct branch overall (Figure S2a). This pattern differs from the PCA-based group separation (Figure S2b), where HPS and CAF-0911 are statistically indistinguishable from one another (*p* = 0.27) and both differ significantly from S2-013 (*p* = 4.3×10^{-4}), indicating that while individual peak-level clustering shows some overlap between CAF-0911 and cancer cell profiles, the overall multivariate metabolic separation remains consistent with a stromal-versus-cancer distinction. Surprisingly, a univariate analysis of the media NMR data set indicated no difference in glucose uptake between cancer and stromal cells (Figure S2f). The systematic alteration in the exo-metabolome and a comparable consumption of glucose show that the stromal cells are just as metabolically prolific as cancer cells. A comparative analysis of the pathway enrichment maps (Figure S2c,d) identified pyruvate metabolism as the largest metabolic difference between cancer and stromal cells. Pyruvate media concentrations were significantly increased (*p* < 0.01) in cancer cell-conditioned media. A direct metabolic derivatives of pyruvate, lactate was also increased (*p* < 0.01) in conditioned media of cancer cells compared to stromal cells (Figure S2f and Table S1). A MetaboAnalyst (<https://www.metaboanalyst.ca/>) pathway impact map (Figure S2e)⁴⁰ was consistent with the pathway enrichment maps and shows major metabolic pathways related to pyruvate metabolism, glucose metabolism, and amino acid metabolism to be differentially activated. It is important to note that the pathway impact software emphasizes metabolic pathways identified from KEGG,⁴¹ while the MSEA pathway enrichment software emphasizes human metabolic pathways.^{39,40}

Altered Stromal Cell Metabolism May Replenish Cancer Cell Deficiencies

Stromal cell metabolites were compared before and after stimulation with conditioned media from cancer cells (Figure 3). Stromal metabolomes exhibited a dramatic shift after stimulation by the exo-metabolome of cancer cells, as seen from the hierarchically clustered heat map (Figure 3a), the PCA score plot, and its accompanying tree diagram (Figure 3b,c). The stimulated stromal cells form a distinct branch in the hierarchical cluster and a separate cluster in the PCA score plot from the untreated cells. Stimulated stromal cells exhibited an increase in the cellular levels of alanine, lactate, citrate, serine, and glutathione (Figure 3d). Of note is the surge in the alanine level in the stromal cell lysate following treatment with conditioned media. To distinguish metabolites already present in the cancer-conditioned medium from additional changes occurring during stromal cell incubation, we directly compared the cancer-conditioned medium (S2-013) with the corresponding double-conditioned medium (CAF-0911-C). Alanine and lactate were significantly higher in the double-conditioned medium than in the original cancer-conditioned medium,

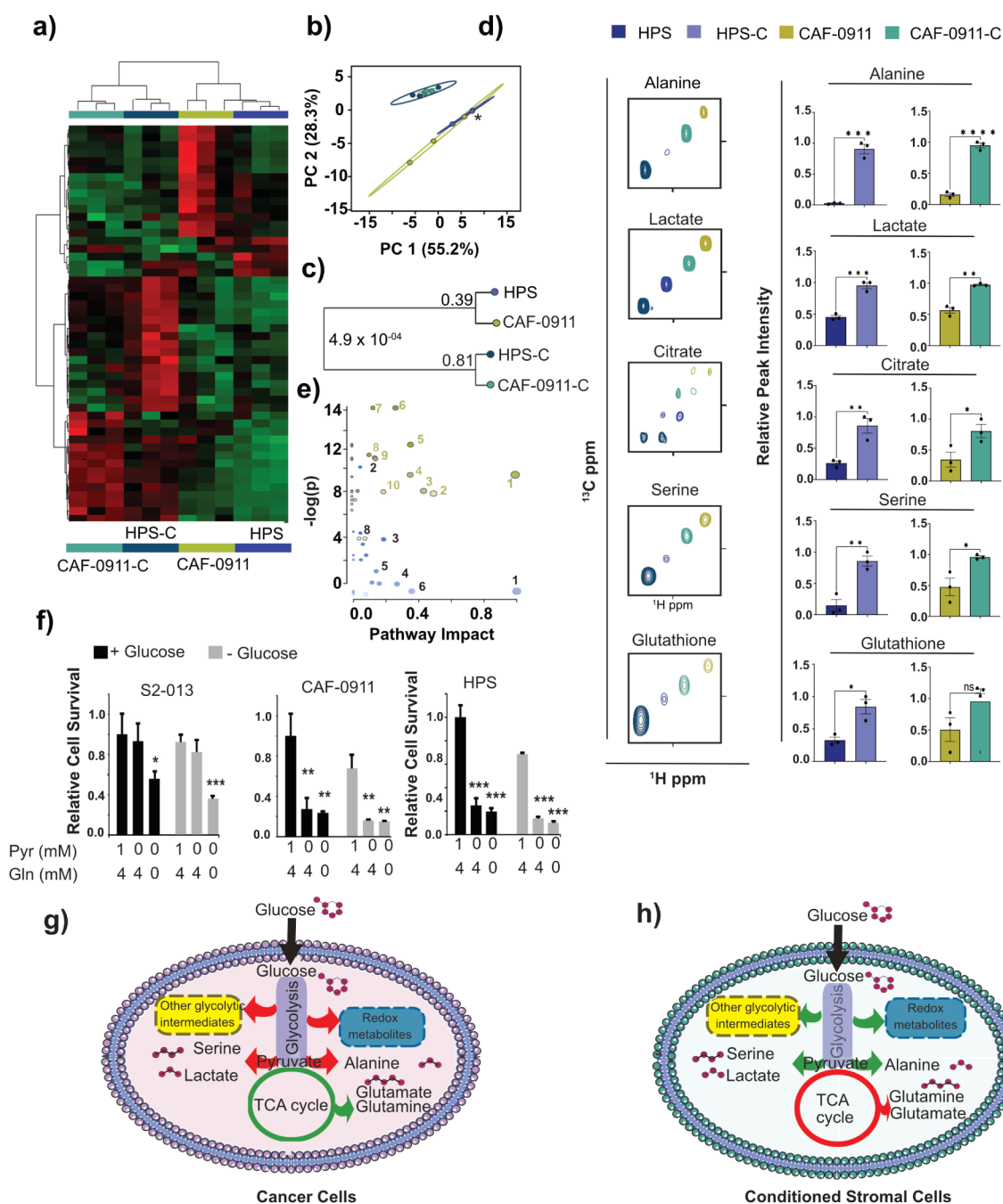


Figure 3. Alterations in the stromal cellular metabolome following cancer cell stimulation. (a) Heatmap summarizing the cellular metabolome differences between stromal cells with (HPS-C and CAF-0911-C) and without (HPS and CAF-0911) stimulation from cancer cell-conditioned media. Cells were cultured for 6 h after replacing the ^{12}C -DMEM (regular DMEM with naturally occurring isotopic abundance) with ^{13}C -labeled cancer cell-conditioned media. For simplicity, only the top 30 NMR peaks (p -value ≤ 0.05) are represented in the heat map. Red corresponds to a relative metabolite increase and green corresponds to a relative metabolite decrease in stimulated stromal cell lysates compared to unstimulated stromal cell lysates. Both the NMR peaks and the cell-type triplicates were hierarchically clustered in the heatmap. Accordingly, NMR peaks were grouped based on their relative increase or decrease between the two cell types. The two sets of stimulated stromal cell triplicates are grouped together and separately from the stromal cell triplicates without stimulation. The HPS triplicates are colored blue, HPS-C triplicates are colored teal, the CAF-0911 triplicates are colored yellow, and the CAF-0911-C triplicates are colored green. (b) PCA scores plot generated from the 2D ^1H - ^{13}C HSQC spectral data set obtained from the stimulated (HPS-C and CAF-0911-C) and unstimulated stromal (HPS and CAF-0911) cell lysates. The PCA model (R^2 0.95, Q^2 0.77) was created using all of the 2D ^1H - ^{13}C HSQC NMR peaks from each of the three replicates per cell type. The HPS triplicates are colored blue, HPS-C triplicates are colored teal, the CAF-0911 triplicates are colored yellow, and the CAF-0911-C triplicates are colored green. (c) The associated metabolomics tree diagram generated from the PCA model, where each node contains a Mahalanobis distance defined p -value. The HPS triplicates are colored blue, HPS-C triplicates are colored teal, the CAF-0911 triplicates are colored yellow, and the CAF-0911-C triplicates are colored green. (d) Bar charts showing variations in alanine, lactate, citrate, serine, and glutathione metabolite levels between the stimulated (HPS-C and CAF-0911-C) and unstimulated stromal (HPS and CAF-0911) cell lysates based on the shown 2D ^1H - ^{13}C HSQC peak intensities. HPS dots and peaks are colored blue, HPS-C dots and peaks are colored teal, CAF-0911 dots and peaks are colored yellow, and CAF-0911-C dots and peaks are colored green, where peaks are shown with an offset to avoid overlap. (e) MetaboAnalyst

Figure 3. continued

(<https://www.metaboanalyst.ca/>)⁴³ pathway impact map showing the top-most differentially used metabolic pathways between the stimulated (HPS-C and CAF-0911-C) and unstimulated stromal (HPS and CAF-0911) cellular metabolomes. Metabolic pathways are listed in decreasing order of impact. Yellow circles are metabolic pathways from CAF-0911 and CAF-0911-C comparison and are labeled as 1: alanine, aspartate, and glutamate, 2: starch and sucrose, 3: glutamine and glutamate, 4: glycine, serine, and threonine, 5: glutathione, 6: amino and nucleotide sugar, 7: nicotinamide, 8: glyoxylate. Blue circles are metabolic pathways from HPS and HPS-C comparison and are labeled as 1: alanine, aspartate, and glutamate, 2: glutamine and glutamate, 3: starch and sucrose, 4: glutathione, 5: glycine, serine, and threonine, 6: arginine and proline metabolism, 7: TCA, 8: glyoxylate metabolism. (f) Relative cell survival of cancer (S2-013) and stromal cells (HPS and CAF-0911) as a function of cell culture media supplemented with glutamine (4 mM) and/or pyruvate (1 mM) with (black bars) or without (gray bars) glucose. Schematic summary of the metabolic variations between (g) unstimulated and (h) stimulated stromal cells from cancer cell-conditioned medium. A red arrow indicates a decrease, and a green arrow indicates an increase in the total metabolite content in comparing stimulated to unstimulated stromal cells. Significance was assessed using Student's *t* test (ns > 0.05, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

demonstrating additional net metabolite accumulation during the stromal cell conditioning step (Figure S3a,b).

The canonical use of glucose through the glycolytic process forms two major end products: lactate and alanine. Both metabolic end points are linked through pyruvate. The significant increase in the level of glucose-derived alanine by the stimulated stromal cells raised some obvious questions: how are the stromal cells utilizing the excess alanine and pyruvate? Are the stimulated stromal cells dependent on the availability of pyruvate for their survival? Because pyruvate occupies a central position linking glucose, alanine, and lactate metabolism, we next examined stromal and cancer cell dependence on pyruvate and compared this with their dependence on glucose and glutamine, a major alternative carbon and nitrogen source (Figure 3f).

Stromal cell survival was observed to be dependent on the availability of pyruvate (*p* < 0.01). The absence of glucose further diminished the cell survival. This response was consistent across both stromal cell lines (CAF-0911 and HPS). Notably, stromal cell survival was not further diminished in the absence of both glutamine and pyruvate. Glutamine may serve as an alternate carbon and nitrogen source for cancer cells.⁴² Thus, a lack of dependence on glutamine may suggest that the stromal cells have a reduced reliance on glutaminolysis. Cancer cells exhibited no measurable shift in cell survival in the absence of pyruvate; however, survival was negatively impacted by the absence of glutamine (*p* < 0.05). This was exacerbated by the absence of glucose (*p* < 0.001). Accompanying this phenotypic observation was the revelation that the most impacted pathways were linked to amino acid metabolism and redox glutathione metabolism, which were dysregulated in the stimulated stromal cells (Figure 3e).

The stimulated stromal cells have an altered metabolic profile summarized in the cell diagram (Figure 3g,h). Following conditioned-medium stimulation, the stromal cells exhibited an increase in the end products of aerobic glycolysis and a reduction in the tricarboxylic acid cycle (TCA cycle) intermediates. The switch in central carbon metabolism is coupled with an increase in the levels of the redox metabolite glutathione.

The stromal cell exo-metabolome was similarly analyzed to characterize the impact of stimulation by conditioned media from cancer cells. The exo-metabolome, like the cellular metabolome, exhibited a dramatic metabolic shift following treatment with conditioned media. The hierarchically clustered heat map and PCA model (Figure 4a,b and Figure S4) clearly illustrate the overall global changes to the excreted metabolome. Pathway enrichment and impact analysis indicate

that pyruvate and amino acid metabolism were the most impacted metabolic processes following treatment with conditioned media (Figure 4d,e and Figure S4). Interestingly, a surge in the number of metabolites with net extracellular accumulation from the stimulated stromal cells was observed. These metabolites include alanine, leucine, isoleucine, glutathione, glutamine, lysine, phenylalanine, and lactate (Figure 4c and Figure S4). Altogether, our data demonstrate that the conditioning of stromal cells by cancer cells-conditioned media remodels stromal cell metabolism and promotes the net accumulation of metabolites that could potentially replenish cancer cell metabolic needs.

Temporal Metabolism of Alanine and Pyruvate by Cancer and Stromal Cells

To further investigate the differential utilization of alanine by cancer and stromal cells, cancer (S2-013) and stromal cells (CAF-0911) were cultured in media supplemented with 1 mM alanine to measure the intracellular and media alanine levels at 5 min, 30 min, 6 h, and 12 h. Our results show that the intracellular alanine levels in both cancer and stromal cells did not change at early time points (Figure 5a,c). However, at later time points (at 6 and 12 h), the intracellular alanine levels in cancer cells show a reduction in alanine levels by 45% at 6 h and 65% at 12 h, while stromal cells showed no significant change. Measurement of residual ¹³C₃-alanine in the media revealed that cancer cells consumed most of the alanine by 12 h (Figure 5b), whereas the stromal cells had only taken up a little over 40% of the available alanine (Figure 5d). The preferential consumption of exogenous alanine by cancer cells demonstrates that alanine is not only secreted into the tumor microenvironment but also serves as an important metabolic substrate. In contrast, stromal cells maintained relatively stable intracellular alanine levels, indicating fundamentally different utilizations between the two cell types.

Accordingly, we next evaluated metabolites generated from ¹³C₃-alanine. We observed an increased accumulation of ¹³C₃-alanine-derived lactate in the cancer cell lysate (Figure 5f); in contrast, stromal cells maintained a steady lactate level over 12 h (Figure 5h). The level of lactate in the medium was then measured to determine the efflux of lactate from the cells. The results show that both cancer and stromal cells increase the efflux of lactate over time (Figure 5g and 5i).

Upon evaluating ¹³C₃-alanine-derived metabolite incorporation into the TCA cycle, a sharp increase in succinate was observed in cancer cells at 6 h, followed by a decrease at 12 h (*p* < 0.05). This indicates that during a 6- to 12 h time period, cancer cells redirect alanine-derived carbon to the TCA cycle and reduce this flux (Figures S5 and S6a). In stromal cells, succinate increased steadily over the full 12 h (Figures S5 and

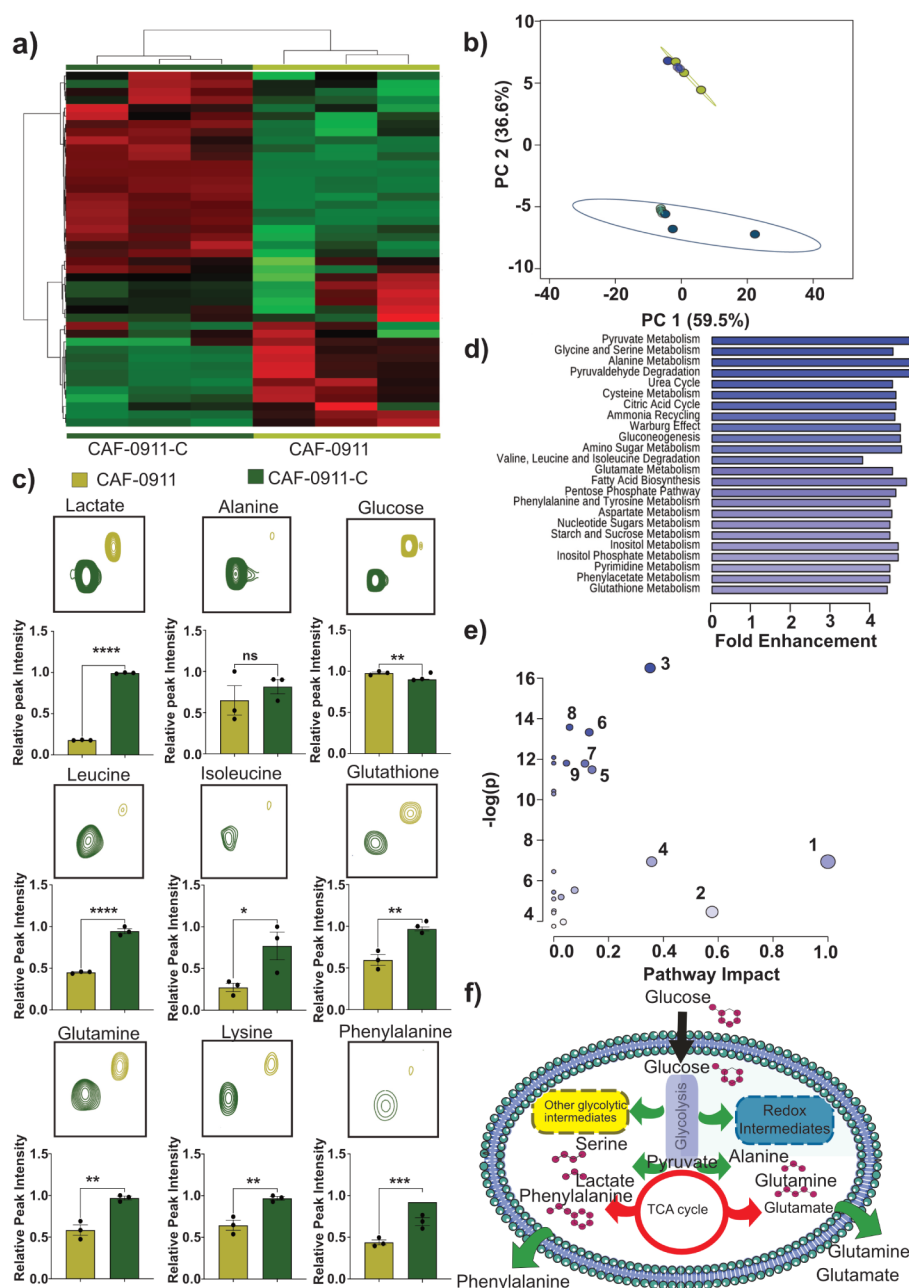


Figure 4. Alterations in the stromal exo-metabolome following cancer cell stimulation. (a) Heatmap summarizing the exo-metabolome differences between stromal cells with (CAF-0911-C) and without (CAF-0911) stimulation from cancer cell-conditioned medium. Cells were cultured for 6 h after replacing the ^{12}C -DMEM medium with ^{13}C -labeled cancer cell-conditioned medium. For simplicity, only the top 30 NMR peaks (p -value ≤ 0.05) are represented in the heatmap. Red corresponds to a relative increase in excreted metabolites, and green corresponds to a relative decrease in excreted metabolites comparing stimulated to unstimulated stromal cell media. Both the NMR peaks and the cell-type replicates were hierarchically clustered in the heatmap. NMR peaks were grouped based on their relative increase or decrease between the stimulated and unstimulated stromal cells. The stimulated stromal cell triplicates are grouped together and separately from the stromal cell triplicates without stimulation. The CAF-0911 triplicates are colored yellow, and the CAF-0911-C triplicates are colored green. (b) PCA score plot generated from the 2D ^1H - ^{13}C HSQC spectral data set obtained from the stimulated (CAF-0911-C) and unstimulated stromal (CAF-0911) exo-metabolomes. The PCA model (R^2 0.96, Q^2 0.37) was created using all the 2D ^1H - ^{13}C HSQC NMR peaks from each of the three replicates per cell type. The CAF-0911 triplicates are colored yellow, and the CAF-0911-C triplicates are colored green. (c) Bar charts showing variations in lactate, alanine, glucose, leucine, isoleucine, glutathione, lysine, glutamine, and phenylalanine metabolite levels between the stimulated (CAF-0911-C) and unstimulated stromal (CAF-0911) cell exo-metabolomes based on the shown 2D ^1H - ^{13}C HSQC peak intensities. CAF-0911 dots and peaks are colored yellow, and CAF-0911-C dots and peaks are colored green, where peaks are shown with an offset to avoid overlap. (d) MSEA pathway enrichment map (<https://www.metaboanalyst.ca/>)^{42,43} showing the top-most differentially used metabolic pathways when comparing the exo-metabolomes from the stimulated (CAF-0911-C) and unstimulated stromal (CAF-0911) cells. Metabolic pathways are listed in decreasing order of corrected p -value. (e) MetaboAnalyst (<https://www.metaboanalyst.ca/>)⁴³ pathway impact map showing the top-most differentially used metabolic pathways between the exo-metabolomes from the stimulated (CAF-0911-C) and unstimulated stromal (CAF-0911) cells. Metabolic pathways are listed in decreasing order of impact and correspond to 1: phenylalanine and tyrosine metabolism, 2: starch and sucrose metabolism, 3: phenylalanine metabolism, 4: pyruvate metabolism, 5: tyrosine metabolism, 6: glycolysis and gluconeogenesis, 7: alanine, aspartate, and glutamate metabolism, 8: arginine

Figure 4. continued

synthesis, 9: TCA. (f) Schematic summary of the exo-metabolic variations between unstimulated and stimulated stromal cells. A red arrow indicates a decrease, and a green arrow indicates an increase in the total metabolite content in comparing stimulated to unstimulated stromal cells. Significance was assessed using Student's *t* test (ns > 0.05, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

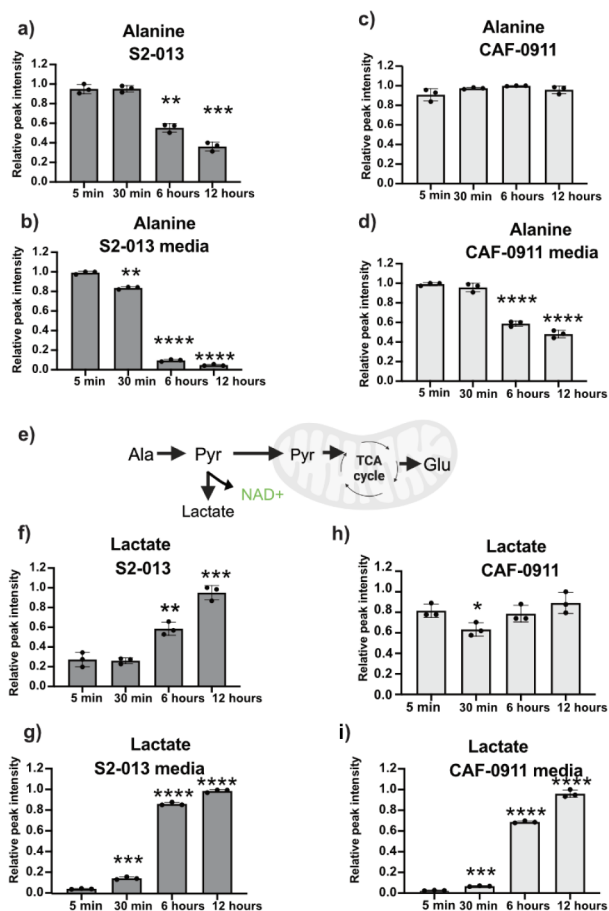


Figure 5. Consumption of alanine and production of alanine-derived lactate in cancer (S2-013) and stromal (CAF-0911) cells over time. (a–d) Relative peak intensities of intracellular and extracellular alanine following supplementation with ^{13}C -alanine. (a) Intracellular alanine in S2-013 cells decreases steadily over 12 h. (b) Alanine abundance in S2-013 media decreases correspondingly, indicating rapid uptake. (c) Intracellular alanine in CAF-0911 stromal cells remains relatively constant across time points. (d) Alanine levels in CAF-0911 media decrease gradually, reflecting slower uptake than in S2-013 cells. (e) Schematic representation of alanine metabolism. Alanine is converted to pyruvate, which contributes carbon to the TCA cycle or is reduced to lactate, generating NAD^+ . Pyruvate-derived carbon also contributes to glutamate synthesis through α -ketoglutarate. (f–i) Relative peak intensities of intracellular and extracellular lactate. (f) S2-013 cells exhibit a time-dependent increase in ^{13}C -lactate, with the highest levels at 12 h. (h) In contrast, CAF-0911 cells maintain more stable lactate levels over the time course. (g, i) Lactate levels in the media demonstrate increasing lactate efflux from both cell types, though S2-013 cells show a more pronounced accumulation. Bars represent mean relative peak intensities; individual points denote biological replicates, and error bars represent the standard deviation. For time points showing a statistical difference compared to the 5 min time point, significance was assessed using Student's *t* test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). Only significant comparisons are shown.

S6b). Like succinate, glutamate levels increased at 6 h (*p* < 0.05) and decreased by 12 h (*p* < 0.05) in cancer cells (Figure S6c), while stromal cells' glutamate levels remained unchanged (Figure S6d). We also observed multiple essential amino acids, including isoleucine, lysine, and valine, in the extracellular media of both cancer and stromal cells (Figure S7).

We next examined the temporal fate of pyruvate in the cancer and stromal cells. S2-013 (cancer) and CAF-0911 (stromal) cells were cultured in medium supplemented with 1 mM pyruvate. Intracellular (Figure S8) and extracellular (Figure S9) ^{13}C -labeled metabolites were quantified after 5 min, 30 min, 6 h, and 12 h of incubation.

In stromal cells, a rapid accumulation of intracellular ^{13}C -alanine and lactate was observed within 5 min of ^{13}C -pyruvate supplementation (Figure S8a and b). Labeling of TCA cycle-linked metabolites was also evident at this early time point as ^{13}C -enriched citrate and glutamate were observed (Figure S8c and d). Alanine and lactate levels then declined, followed by a gradual increase that reached a second maximum at 12 h. Citrate followed a similar biphasic pattern, whereas glutamate peaked at 30 min and subsequently decreased over the remaining time points (Figure S8a–c). Glutamine labeling lagged glutamate, reaching a maximum at 6 h before declining, consistent with its derivation from glutamate (Figure S8e). Cancer cells exhibited a temporal pattern broadly similar to that of stromal cells, but with an apparent delay of ~ 30 min. The early rise in ^{13}C -alanine, ^{13}C -lactate, and TCA cycle intermediates occurred at later time points relative to stromal cells. One notable difference was the relative abundance of glutamate, glutamine, and serine: cancer cells accumulated higher levels of labeled glutamate and glutamine at early time points (5–30 min), whereas stromal cells showed a greater accumulation at the later time points (6–12 h) (Figure S8). These observations suggest that the two cell types differ in the timing and extent to which pyruvate-derived carbon is routed into amino acid biosynthesis.

DISCUSSION

Although NMR metabolomics is a powerful tool widely used for biomarker discovery and mechanistic understanding of disease, only a small percentage of metabolomics studies have utilized isotope labeling in their investigations.⁴³ The method we present here further demonstrates the potential of the scarcely utilized SIRM-based NMR metabolomics approach. Specifically, our method offers a unique approach to investigating metabolite-based cellular communication. Prior studies have also used conditioned media to characterize the protein secretome or the presence of signaling peptides and metabolites in an extracellular environment.^{44,45} For example, cancer-conditioned media treated with a fluorescent dye were used to elucidate the role of gap-junction intracellular communication.⁴⁶ Serum-free conditioned media were used to identify protein biomarkers for colon cancer and to quantify the secretome in pancreatic cancer.^{47,48} Conversely, detecting low-concentration biomarkers or extracellular proteins in traditional culture media containing high amounts of serum

would be prohibitive.^{47,48} Various *in vitro* coculture systems have been designed and used to study cell communication, cell migration, and extracellular matrix composition changes.⁴⁹ Cocultures consist of either direct cell-to-cell contact or cell separation via a permeable membrane.⁵⁰ Two-dimensional or three-dimensional culture systems entail porous membranes, micropatterning, and permeable or degradable hydrogels.⁴⁹ Compared with our approach, all of these systems are technically challenging, difficult to implement, and still do not represent a true, heterogeneous cancer environment.⁵¹ Thus, segregated cell cultures employing conditioned media provide a simple and elegant means to study cell communication.

To understand how metabolic communication between pancreatic cancer cells and stromal cells is established, it is first necessary to characterize the baseline metabolic phenotypes of each cell type. Differences in central carbon metabolism provide the foundation for identifying how stromal cells respond to tumor-derived signals and ultimately support the metabolic demands of cancer cells. The PDAC stroma, which comprises most of the tumor mass, contains fibroblastic cell populations that play key roles in tumor progression and therapeutic responses.^{34,52–56} Multiple lines of evidence have revealed that cancer cells cue and reprogram stromal cells' metabolism to exploit the stromal cell machinery to promote tumorigenesis.^{54,57} The cancer and stromal cells have fundamentally distinct metabolic phenotypes (Figure 2h,i). Our ¹³C₆-glucose-tracing experiments revealed a clear partitioning of central carbon metabolism between the two cell types. Cancer cells preferentially directed glucose-derived carbon toward aerobic glycolysis, whereas stromal cells exhibited greater incorporation into TCA cycle-associated metabolites and amino acid biosynthesis. The analysis confirms the Warburg effect, a hallmark of cancer and the most pronounced metabolic difference between cancer and stromal cells. The Warburg effect is characterized by a high rate of glycolysis and the corresponding accumulation of lactate.⁵⁸ Importantly, the systematic alteration in the exo-metabolome, despite comparable consumption of glucose, shows that stromal cells are just as metabolically prolific as cancer cells.

The differences between baseline metabolic states suggest that cancer and stromal cells fulfill distinct metabolic roles within the tumor microenvironment. This raises the question of whether these intrinsic states remain fixed or can be remodeled through communication between the two cell types. Cancer cell-conditioned media induced a marked increase in the glucose-derived alanine level by stromal cells, consistent with a substantial remodeling of central carbon metabolism. The contrasting nutrient dependencies of the two cell types, with stromal cells showing greater reliance on pyruvate and cancer cells remaining more dependent on glutamine, further support this metabolic divergence. This shift, together with the alteration of amino acid and glutathione metabolism (Figure 3e), suggests that cancer-induced stromal remodeling extends beyond central carbon metabolism to pathways involved in biosynthesis and redox homeostasis.

The observed increase in intracellular serine is particularly important, because serine contributes to glycine and one-carbon metabolism and can support glutathione synthesis.⁵⁹ Although serine is a non-essential amino acid, cancer cells utilize extracellular serine to support one-carbon metabolism and cancer cell proliferation.⁶⁰ This increase in stromal serine levels may also support stromal cell redox homeostasis through

serine-dependent glutathione synthesis.⁶¹ In addition to altering central carbon metabolism and redox balance, conditioned media also influenced amino acid availability within the stromal exo-metabolome, suggesting that stromal cells actively modify the extracellular environment. Essential amino acids, such as leucine, isoleucine, and phenylalanine, are not directly derived from glucose. Thus, the increase of leucine, isoleucine, and phenylalanine in the exo-metabolome is likely a result of a decreased consumption. Collectively, these metabolic adaptations extend beyond individual metabolite changes and point to a coordinated remodeling of stromal metabolism.

These adaptations suggest that stromal cells adopt a metabolic phenotype capable of supporting the biosynthetic and energetic requirements of the neighboring cancer cells. Our analysis indicates that the baseline metabolism in cancer cells is dominated by glucose flux through Warburg metabolism with a lower TCA-cycle-linked labeling. Cancer cells are depleted in intermediates for amino acid synthesis and one-carbon transfer metabolism and experience a sharp reduction in their redox capabilities. Amino acids such as lysine, leucine, and isoleucine are important protein building blocks that stimulate growth and survival in cells.^{28,62} All of these amino acids were significantly increased in the exo-metabolome of stromal cells treated with the cancer cell-conditioned media. Aromatic amino acids, phenylalanine and tyrosine, are abundant in the albumin present in fetal bovine serum routinely used to supplement growth media,⁶² but the ability of cancer cells to scavenge these amino acids is limited. Thus, the excretion of phenylalanine and tyrosine by the stimulated stromal cells may remedy this cancer-cell nutrient deficiency. Aspartate and glutamate are key TCA cycle intermediates that are downregulated in cancer cells. Cytosolic aspartate is essential for purine and pyrimidine synthesis,⁵⁹ and aspartate metabolism serves as a source of NADPH to neutralize reactive oxygen species, making the availability of aspartate crucial for cancer cell survival and proliferation even under hypoxic conditions.^{63,64} Similarly, the increased usage and dependency on glutamine for growth and survival are hallmarks of cancer cells.⁶⁵ Of course, both aspartate and glutamate can be derived from glutamine metabolism, and cancer cells may also rely on glutamine-derived glutamate.⁶⁶ Clearly, enrichment of the exo-metabolome with these metabolites highlights the various ways cancer cells may benefit from the altered metabolism of stromal cells. A summary of the metabolic changes incurred by stromal cells following stimulation by cancer cell-conditioned media is shown in Figure 4f.

Among the numerous metabolic alterations induced by conditioned medium, alanine showed one of the largest changes. We therefore investigated its temporal metabolic fate using direct ¹³C₃-alanine tracing (Figure 5). A prior study using mass-spectrometry-based metabolomics also observed an increase in alanine by pancreatic stromal cells. This previous study demonstrates that stromal-derived alanine is generated in an autophagy-dependent manner to alleviate the cancer cells from depending on glucose and glutamine, as those nutrients are limited in the pancreatic tumor microenvironment. Inhibition of the alanine transporter in pancreatic cancer cells also impairs tumor growth.^{16,67} The dynamic reduction of intracellular alanine in cancer cells suggests its incorporation into other metabolic pathways. These findings highlight a distinct metabolic adaptation in cancer cells characterized by

the increased consumption and redirection of alanine carbons into other pathways.

The preferential consumption of alanine by cancer cells prompted an investigation of how this carbon source was subsequently metabolized after its uptake. Alanine can be converted to pyruvate by alanine transaminases in both the cytoplasm (ALT1) and the mitochondria (ALT2). The subsequent conversion of pyruvate to lactate, catalyzed by lactate dehydrogenase (LDH), occurs only in the cytoplasm. The time-resolved data suggest that cancer cells (S2-013) actively metabolize alanine to lactate. The conversion regenerates NAD^+ , thereby maintaining the intracellular NAD^+ pool required to sustain the glycolytic flux.⁶⁸ This NAD^+ -supporting role of alanine in S2-013 cells contrasts with prior work showing predominant mitochondrial use of alanine in other PDAC models,¹⁶ underscoring metabolic diversity within PDAC (Figure 5e).

Although conversion of alanine to lactate represents one major metabolic fate, pyruvate generated from alanine can also enter mitochondrial metabolism. We therefore examined whether alanine-derived carbons also contribute to TCA cycle activity. Using succinate as a proxy for alanine-derived TCA cycle flux, our results indicate that both cancer and stromal cells channel alanine into the TCA cycle, although cancer cells display a more dynamic pattern of flux. Glutamate can be derived from the TCA cycle intermediate, α -ketoglutarate, through transamination (Figure S5). These temporal fluctuations in the levels of TCA cycle-linked metabolites in cancer cells could be consistent with cell-cycle-dependent changes in carbon flux, as suggested by prior studies, although our data did not directly test this hypothesis.⁶⁹ Although our cells were not synchronized at the start of the experiment, the oscillating fold changes of TCA cycle intermediates highlight the proliferative phenotype of cancer cells compared to stromal cells. The observation of essential amino acids in both cancer and stromal cells is perhaps indicative of a higher uptake and scavenging of these amino acids that were readily recorded in a 2D NMR spectrum (Figure S7).

Since alanine and pyruvate are interconnected through alanine transaminase activity, we further investigated the fate of pyruvate in cancer and stromal cells. Cancer cells rapidly excreted a majority of pyruvate-derived lactate into the medium, consistent with a Warburg-like glycolytic phenotype and relatively lower reliance on oxidative TCA metabolism (Figure S9a). In this regard, stromal cells utilized lactate (i.e., pyruvate ^{13}C -carbons) to produce other metabolites. Specifically, a spike in the excretion of choline, myo-inositol, isoleucine, serine, and threonine was observed at the 30 min time point. These secreted metabolites, including choline and serine (linked to one-carbon metabolism), threonine (which can feed into TCA-related pathways), and essential amino acids such as isoleucine, could, in principle, help fulfill cancer cells' demand for biosynthetic and redox-supporting metabolites (Figure S9b-f). These patterns are consistent with stromal cells channeling pyruvate/lactate-derived carbon into biosynthetic and redox-related pathways, potentially influencing the availability of reducing equivalents (e.g., NADH/NADPH). This raises the possibility that stromal cells could act as a metabolic source of "redox currency" for neighboring cancer cells.^{63,64}

CONCLUSIONS

An SIRM workflow was developed to understand cancer-stromal metabolic crosstalk in a pancreatic cancer micro-environment. Our SIRM protocol combines a selective isotopic labeling strategy with 2D NMR experiments to characterize individual cellular metabolomes to monitor the exchange of ^{13}C -labeled metabolites between stromal and cancer cells. In this regard, characterizing the cellular communication between pancreatic cancer and stromal cells may provide a new perspective on advancing PDAC drug development. Pancreatic cancer cells were observed to have distinct cellular and exo-metabolomes from pancreatic stromal cells. In our system, the pancreatic cancer cells showed relatively higher glycolytic flux and a lower TCA-cycle-linked labeling compared to the stromal cells. The stromal cells increased their utilization of pyruvate, but interestingly, glucose uptake was the same for both cancer and stromal cells. Stromal cells' (HPS and CAF-0911) survival was dependent on pyruvate but not on glutamine. In contrast, cancer cells' (S2-013) survival was dependent on glutamine but not on pyruvate. Cancer cells were observed to dramatically remodel the stromal cell metabolism. Specifically, stromal cells stimulated with cancer cell-conditioned media exhibited metabolite changes consistent with an increase in metabolites associated with redox homeostasis, a switch in central carbon metabolism, and a surge in the number of excreted metabolites. Stimulated stromal cells have higher intracellular levels and a net extracellular accumulation of alanine. The cancer cells are dominated by Warburg metabolism, which resulted in a decrease in intermediates for amino acid synthesis and one-carbon transfer metabolism and a reduction in metabolites associated with redox capabilities. Accordingly, stimulated stromal cell metabolism was directed in a manner consistent with replenishing these metabolic deficiencies through the efflux of essential metabolites. Metabolic flux analysis further demonstrated the temporal differences in the metabolomes of the cancer and stromal cells. The two cells exhibited markedly distinct and often complementary metabolic profiles, which were characterized using our simplified SIRM workflow. The workflow presented here is adaptable to studying other metabolic crosstalks, illuminating unique metabolic features in different systems.^{70,71}

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.6c02205>.

Materials and methods related to chemicals, cells and culture media, metabolite extraction for NMR analysis, NMR experiments and data analyses, and the cell survival assay; Figures showing the effectiveness of our washing procedure to remove ^{13}C -labeled metabolites from cell cultures, and the summary of changes in the cancer and stromal cell exo-metabolome, stimulated stromal cell (HPS) exo-metabolome, cancer and stromal cell exo-metabolome with media supplemented with $^{13}\text{C}_3$ -pyruvate, and cancer and stromal cells' cellular and exo-metabolome with media supplemented with $^{13}\text{C}_3$ -alanine (PDF)

A table summarizing the detected metabolites and the FDR-corrected p -values determined from relative

concentration changes between cancer and stromal cells (XLSX)

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F.B., T.G., J.S., and N.V.C. performed the experiments; F.B., T.G., P.K.S., and R.P. designed the experiments; F.B., T.G., F.A., and R.P. analyzed the data and wrote the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

National Institutes of Health (Grant No. R01 CA163649, NCI) to P.K.S. and R.P. and (Grant No. R01 CA270234, NCI) to P.K.S.; the Redox Biology Center (Grant No. P30 GM103335, NIGMS) to R.P.; the Nebraska Center for

Integrated Biomolecular Communication (Grant No. P20 GM113126, NIGMS) to R.P.; the American Association for Cancer Research (AACR)-Pancreatic Cancer Action Network (PanCAN) Career Development Award (Grant No. 30-20-25-SING) to P.K.S.; the Specialized Programs for Research Excellence (Grant No. SPORE, 2P50 CA127297, NCI) to P.K.S.; the Pancreatic Tumor Microenvironment Research Network (Grant No. U54, CA163120, NCI) to P.K.S.; and the Fred & Pamela Buffett Cancer Center Support Grant (Grant No. P30 CA036727) to P.K.S. and R.P. The research was performed in facilities renovated with support from the National Institutes of Health (Grant No. RR015468-01). T.G. is supported by funding from the Natural Sciences and Engineering Research Council of Canada NSERC-Discovery Grant (RGPIN-2025-06578 and DGEGR-2025-00277) and the Verspeeten Family Cancer Centre Catalyst Grant. F.B. is supported by funding from the National Cancer Institute Career Transition Award (Grant No. K99CA300901). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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