**Metabolic reprogramming and dependencies associated with epithelial cancer stem cells uncoupled from epithelial-mesenchymal transition**

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**Cell proliferation and viability assay.**  Proliferation and viability were assessed by Hoechst staining (HO33342; Sigma-Aldrich). Briefly, cells were seeded in 96-well plates and media was replaced after 24 h with complete fresh media containing the drug under study or nutrient-deprived media. At the end of the experiment, media was removed, cells were washed with PBS, the supernatant was aspirated and 100 µL of 0.01% SDS were added to each well. Plates were frozen at -20 °C until analyzed. For analysis, plates were thawed at 37 °C and HO33342 stain added (1 µg/mL in 1 M NaCl, 0.1 M EDTA, 1 M Tris, pH 7.4). Plates, protected from light, were placed on a shaker and incubated at 37 °C for 1 h and fluorescence quantified in a fluorescence plate reader at 355 nm excitation and 460 nm emission.

**Cell cycle analysis.** Cell cycle was analyzed by flow cytometry. Briefly, 1·105 cells were seeded on 6-well plates and after 24 h media was replaced with complete fresh media containing the drug under study or nutrient-deprived media. After 48 h, cells were collected and fixed with 70% cold ethanol at -20 °C for at least 1 h. For analysis, cells were centrifuged, washed with phosphate-buffered saline (PBS), resuspended in PBS containing 0.2 mg/mL RNAse A (REAL Laboratories) and incubated for 1 h at 37 °C. Prior to analysis, 0.05 µg/mL propidium iodide was added to the cell suspension. DNA content was determined on an Epics-XL flow cytometer (Coulter Corporation, Hialeah, FL) and cell cycle phase distribution analyzed with the FlowJo software.

**Apoptosis assay.** Apoptosis was assessed by evaluating the binding of Annexin-V to phosphatidylserine. Briefly, 1·105 cells were seeded on 6-well plates. After treatments, cells were collected, centrifuged and were resuspended in binding buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM CaCl2. Then, Annexin V-FITC conjugate (1 µg/mL; eBioscience) was added and the suspension was incubated in the darkness for 30 min at room temperature. After incubation, binding buffer was added to the cell suspension and just before FACS analysis cells were stained with 20 µL propidium iodide (1 mg/mL). Flow cytometry was performed on an Epics-XL flow cytometer (Coulter Corporation, Hialeah, FL). Data from 1·104 cells was collected and analyzed.

**Concentration of media metabolites.** Glucose, lactate, glutamate and glutamine were determined by spectrophotometry (COBAS Mira Plus, Horiba ABX) from cell culture media by monitoring at 340 nm wavelength the production of NAD(P)H in specific reactions for each metabolites[1]. Glucose concentration was measured using a commercial kit based in hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) coupled enzymatic reactions. Lactate concentration was determined by lactate dehydrogenase (LDH) reaction, which was carried out at 37 °C by adding media sample to a cuvette containing 1.55 mg/mL NAD+ and 87.7 U/mL LDH in 0.2 M hydrazine, 12 mM EDTA buffer, pH 9.0. Determination of glutamate concentration was done by its conversion to α-ketoglutarate through glutamate dehydrogenase reaction in the presence of ADP. This reaction was performed at 37 °C by adding media sample to a cuvette containing 2.41 mM ADP, 3.9 mM NAD+ and 39 U/mL GLDH in 0.5 M glycine, 0.5 M hydrazine, pH 9.0. Glutamine was determined by first its conversion to glutamate through glutaminase reaction and subsequently quantification of glutamate concentration as described above. GLS reaction was performed by adding media sample to a cuvette containing a mixture consisted of 125 mU/mL GLS in 125 mM acetate, pH 5.0. Reaction was carried out for 30 min at 37 °C in agitation.

Amino acids concentrations in cell media were determined by ion-exchange chromatography with a Biochrom 30 amino acid analyzer (Pharmacia Biochrom Ltd, Cambridge, UK). 150 µM norleucine was used as internal standard and added to media in a relationship 7:50. Solvent was evaporated to complete dryness using a SpeedVac concentrator (Thermo Scientific, Waltham, MA). Samples were resuspended in lithium citrate, pH 2.2 and filtered through a 0.22 µm filter. 30 µL of sample were injected onto the Biochrom 30 lithium system according to the manufacturer’s protocol. A set of lithium citrate buffers were used as mobile phase for separation during 115 minutes and post column derivatization with ninhydrin allowed amino acid detection at 570 and 440 nm. The retention time of the peak on the chart allowed the identification of the amino acid and the area under the peak indicated the quantity of amino acid present.

The metabolite consumption/production normalized rates were derived from the measured metabolites concentrations and corrected according to the measured cell proliferation (under exponential growth conditions). Cell numbers were scored in the same plates from which sample media were obtained. All values are expressed in micromol or nanomol of metabolite consumed or produced per hour per 106 cells (µmol h-1 per 106 cells or nmol h-1 per 106 cells)

**Enzyme activities.** Freshly plated cells were rinsed with PBS and scraped with lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 0.2% Triton X-100, 0.02% sodium deoxycholate) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were disrupted by sonication using a titanium probe (VibraCell, Sonics & Materials Inc., Tune: 50, Output: 30) and immediately centrifuged at 12,000 x g for 20 min at 4°C. Supernatants were collected and used for the determination of specific enzyme activities by spectrophotometric monitoring of NAD(P)H production or disappearance at 340 nm wavelength[1]. All enzymatic activities were normalized by protein content in the supernatant, determined by the BCA assay.

Lactate dehydrogenase (LDH, EC 1.1.1.27).LDH specific activity was measured by adding diluted sample to a cuvette containing 0.2 mM NADH in 100 mM KH2PO4/K2HPO4, pH 7.4, at 37 °C. Reaction was initiated by the addition of pyruvate at a final concentration of 0.2 mM.

Transketolase (TKT, EC 2.2.1.1). TKT specific activity was determined by adding samples to a cuvette containing 5 mM MgCl2, 0.2 U/mL triose phosphate isomerase, 0.2 mM NADH, 0.1 mM thiamine pyrophosphate in 50 mM Tris-HCl, pH 7.6, at 37 °C. The reaction was initiated by the addition of a substrate mixture containing ribose-5-phosphate (R5P) and xylulose-5-phosphate. This substrate mixture was prepared by dissolving 50 mM R5P in 50 mM Tris-HCl, pH 7.6, in the presence of 0.1 U/mL ribulose-5-phosphate-3-epimerase and 1.7 mU/mL phosphoriboisomerase and incubated with agitation at 37 °C for 1 h and then stored at -20 °C until use.

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). G6PDH specific activity was measured by adding samples to a cuvette containing 0.5 mM NADP+ in 50 mM Tris-HCl, pH 7.6, at 37 °C. Reaction was initiated by the addition of glucose-6-phosphate at a final concentration of 2 mM.

Glutamic-pyruvate transaminase (GPT, EC 2.6.1.2). ALT specific activity was measured using a two-reagent commercial kit (ABX Pentra). Reagent 1 consisted of 140 mM Tris pH 7.5, 709 mM L-alanine, ≥ 1700 U/L LDH and reagent 2 consisted of 85 mM α-KG and 1.09 mM NADH. Reaction was initiated by the addition of samples to a cuvette containing reagents 1 and 2 mixed in a 4:1 proportion.

**Western Blotting**. Cell extracts were obtained from either fresh or frozen cells using RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentration from the supernatant was determined by the BCA assay. Aliquots (30-40 μg of protein) were loaded and separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked by incubation with PBS-Tween (0.1% (v/v)) containing 5% non-fat dried milk for 1-2 h at room temperature. Then, membranes were incubated with primary antibodies, rinsed with PBS-Tween (0.1% (v/v)), incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and washed with PBS-Tween (0.1% (v/v)). Blots were treated with the Immobilon ECL Western Blotting Detection Kit Reagent (Millipore) and developed after exposure to Fujifilm X-ray film. The following primary antibodies were used: ACLY (ref. 4332, Cell Signaling, 1/1000, o/n incubation at 4 °C), actin (ref. 69100, MP Biomedicals, 1/120000, 30 min at room temperature), CPT1 (ref. SAB1410234, Sigma, 1/200, o/n incubation at 4 °C), GAC (ref. 19958-1-AP, Proteintech, 1/200, o/n incubation at 4°C), GLS1 (ref. ab93434, Abcam, 1/1000, o/n incubation at 4 °C), KGA (ref. 20170-1-AP, Proteintech, o/n 1/1000, incubation at 4 °C), PDH (ref. ab110330, Abcam, 1/1000, o/n incubation at 4 °C), PDHK1 (ref. 3820S, Cell Signaling, o/n 1/1000, at 4 °C ) and PDH-P (ref. ABS204, Millipore, 1/10000, o/n incubation at 4 °C).The following secondary antibodies were used: anti-mouse (ref. PO260, Dako, 1/3000, 1 h at room temperature) and anti-rabbit (ref. NA934V, Amersham Biosciences, 1/3000, 1 h at room temperature).

**ATP measurement.** Cellular ATP was quantified with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) and luminescence measured with a FLUOstar Optima plate reader (BMG Labtech, Offenberg, Germany). The resulting ATP values were normalized to cell number determined by Hoechst staining.

**RNA isolation, reverse transcription and gene expression analysis.** RNA isolation was performed from fresh or frozen pellets or cultured plates using Trizol reagent (Invitrogen). Complementary DNA was synthesized from 1 µg of RNA using random hexamers (Roche) and M-MLV reverse transcriptase (Invitrogen). Gene expression analysis was performed by quantitative real-time RT-PCR (ABI Prism 7700 Sequence Detector System, Applied Biosystems), using either gene-specific TaqMan assays (Applied Biosystems) or the Universal Probe Library system (UPL; Roche). For the TaqMan assays, cDNA was mixed with PCR Master MixM containing the specific TaqMan probes for each gene (TKT: Hs00169074\_m1; G6PD: Hs00166169\_m1; SOX2: Hs01053049\_s1; PPIA: Hs99999904\_m1). Subsequently, the ΔΔCt method was used for calculating the fold changes in gene expression using PPIA as reference (housekeeping) gene. In the case of the transcripts quantified with the UPL system, RT-PCR assays were performed on a LightCycler 480 instrument (Roche) and analyzed with the LightCycler 480 Software release 1.5.0. The amplification levels of RN18S1 and HMBSE were used as an internal reference to estimate the relative levels of specific transcripts, and relative quantification was determined by the ΔΔCt method. Probes and sequences from the UPL system were:

|  |  |  |  |
| --- | --- | --- | --- |
| **GENE** | **UPL PROBE** |  | **OLIGONUCLEOTIDES 5’🡪3’** |
| **PDHK1** | #21 | FW | aaatgccacgtaaccaaagc |
| REV | agagcggagaccctgtcttga |
| **PDP2** | #49 | FW | agctgggtcctgactaggg |
| REV | tccgtccggtcagttcag |
| **SNAI1** | #11 | FW | gctgcaggactctaatccaga |
| REV | atctccggaggtgggatg |
| **KLF4** | #82 | FW | gccgctccattaccaaga |
| REV | tcttcccctctttggcttg |
| **GAC** | #85 | FW | ctgcagagggtcatgttgaa |
| REV | atccatgggagtgttattcca |
| **KGA** | #11 | FW | gcaaaataatgaaccccaaatta |
| REV | tggcataaatgtaaacacaagctaa |
| **RN18S1** | #40 | FW | ggagagggagcctgagaaac |
| REV | tcgggagtgggtaatttgc |
| **HMBS** | #26 | FW | tgtggtgggaaccagctc |
| REV | tgttgaggtttccccgaat |

**Gene Set Enrichment Analysis (GSEA)**. Transcriptomic expression data for PC-3M and PC-3S cells (GSE24868; [2]) were used to select for metabolic genes included in Recon2 [3]. A gene set of metabolic genes with significant differential expression between PC-3M and PC-3S cells was generated by applying a rank product [4] with the following criteria: q-value ≤ 0.01 for false discovery rate (FDR) with Benjamini-Hochberg correction [5]; p-value ≤ 0.001; fold change ≥ 1.4. The resulting PC-3M-enriched metabolic gene set was used in GSEA analysis of expression datasets for prostate cancer (GEO accession GSE21034; [6]) and tumor types shown in Supplemental Table 7 retrieved from The Cancer Genome Atlas (<https://tcga-data.nci.nih.gov/tcga/>). For analysis, datasets were selected for those containing clinical stage information, with samples grouped as T1-2, T3, or T4 stages and metastatic. GSEA [7-8] was performed on these datasets by applying a weighted scoring scheme and a Pearson metric with 1,000 phenotype permutations. Enrichment along tumor progression of the PC-3M metabolic gene set in these datasets was considered significant for FDR values ≤ 0.250.

**Mitochondrial staining.** 105 cells were seeded onto sterile glass coverslips in 24-well plates 16-24 h before treatment. For immunofluorescence analysis, PC-3M and PC-3S cells seeded on coverslips were incubated with 300 nM MitoTracker CMXRos (Invitrogen, CA, USA) 30 min at 37˚C. After incubation, cells were washed in PBS, fixed in 4% formaldehyde and mounted in ProLong Gold Antifade Mountant with DAPI. Optical sections were acquired using a Leica TCS SP5 confocal system (Leica Microsystems, Manheim).

**Reactive oxygen species (ROS) levels.** Total intracellular ROS levels were determined by means of flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) as a cell-permeant probe (Invitrogen). Cells were incubated with incubation buffer (5.5 mM glucose in PBS containing 5 µM H2DCFDA) for 30 min at 37 ˚C and 5% CO2. Subsequently, this solution was replaced by warm culture media and cells incubated for 45 min at 37 ˚C and 5% CO2. Next, cells were trypsinized and resuspended in a PBS containing 50 µM H2DCFDA and 20 µg/mL propidium iodide. Internalized and activated probe reacts with ROS and emits fluorescence when excited at 492 nm. Emitted fluorescence was quantified by flow cytometry at 520 nm. For ROS analysis, only negative PI cells were considered.

**Intracellular glutathione content.** Total glutathione content was determined by the glutathione reductase enzymatic method. Freshly plated cells were lysed with 5% 5-sulfosalicylic acid (Sigma-Aldrich) solution, vortexed and disrupted by two freezing/thawing cycles in liquid N2 and 37˚C water bath. 50 µL of this solution was separated for subsequent protein quantification. Cell extracts were kept at 4˚C for 10 min and centrifuged at 10,000 x g for 10 min. For glutathione quantification, a working solution consisted of 15 U/mL of glutathione reductase and 40 µg/mL of 5,5’-Dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich) dissolved in assay buffer (100 mM K2HPO4/KH2PO4, pH 7.0, 1 mM EDTA) was prepared. Glutathione standards were prepared from a 50 mM oxidized glutathione (GSSG) stock solution. Reaction was initiated by mixing 150 µL of working solution with 10 µL of cell extract (diluted 1:5 or 1:10) or 10 µL of GSSG standard (final concentrations from 0 to 12.5 µM). Next, 50 µL of 0.16 mg/mL NADPH solution were added and the increase in absorbance was recorded at 340 nm wavelength. Total glutathione concentration was normalized by protein content as determined by the BCA assay.

**Mapping of metabolic fluxes (Isodyn).** Metabolic flux distribution was evaluated through computer simulations of the measured distribution of 13C isotopologues of metabolites. The measured isotopologue dynamics was simulated with a corresponding kinetic model implemented in Isodyn [9-13], adapted to the experimental data. The kinetic model is represented by a system of ordinary differential equations (ODEs) considering total metabolites and their isotopomer concentrations as variables. The adaptation includes introduction in the model of more variables corresponding to the experimentally determined concentrations and isotopologue distributions (mainly the metabolites of the TCA cycle and aminoacids), and considering two different pools (cytosolic or mitochondrial) for some of the TCA cycle metabolites (pyruvate, citrate, α-ketoglutarate, malate, oxaloacetate). Isodyn optimizes parameters of the kinetic model by fitting the model to the measured dynamics of metabolite concentrations and isotopologue distributions using the Simulated Annealing algorithm [14] for minimization of the sum of squared deviations from the experimental data normalized per respective standard deviations (χ2) in the global space of parameters, combined with the Powell method of coordinate descent in local area [15]. After finding a best fit, and, thus, optimal sets of parameters and corresponding metabolic fluxes, Isodyn calculates the confidence intervals of model parameters and corresponding fluxes using as criteria the fixed threshold values of χ2 [16].

Biochemical data presented in Supplemental Table 4. These data were integrated with the data measured from the 13C-based experiments (Supplemental Tables 2 and 3) and the whole set was simulated with Isodyn. To make the experiments comparable, the concentrations of amino acids were recalculated by using a consistency factor determined as a ratio of glutamine consumptions in these experiments, and these recalculated data were used for the Isodyn analysis. This was an important supplement to the measurements of 13C-labeling in these amino acids. The other amino acids, not detected by GC/MS analysis or whose labeling was insignificant, were grouped by metabolic pathways (highlighted in yellow), considering their relation to the synthesis of Pyr, α-KG, OAA, Fum and AcCoA. Their total flux was simulated by Isodyn as a constant rate of uptake of the corresponding unlabeled metabolites.

**GC/MS analysis.** Culture media was replaced by fresh media (10 mM glucose and 2 mM glutamine) containing either 100% or 50% [1,2-13C2]-glucose (Sigma-Aldrich), 100% [U-13C6]-glucose (Sigma-Aldrich) or 100% [U-13C5]-glutamine (Sigma-Aldrich) and media, pellets and plates collected and stored at -80 ºC until analysis. Metabolites were isolated and derivatized previous to the analysis of 13C-isotopologue distributions, which were done by mass spectrometry-coupled gas chromatography (GC/MS) on an Agilent 7890A GC instrument equipped with a HP5 capillary column connected to an Agilent 5975C MS. Fatty acids were analyzed with a GCMS-QP 2012 Shimadzu instrument equipped with a bpx70 (SGE) column. Samples (1 μL) were injected at 250 °C using helium as a carrier gas at 1 mL/min flow rate.

Glucose.Glucose was collected from cell culture media using a tandem set of Dowex-1X8/Dowex-50WX8 ion-exchange columns, through which glucose was eluted with water. Subsequently, water was evaporated to dryness under airflow and the collected glucose was heated to 100 °C first with 2% (v/v) hydroxylamine hydrochloride in pyridine for 30 min and then acetic anhydride for 60 min more for derivatization. Excess reagent and solvent were removed by evaporation with N2 flow, and glucose derivatives were dissolved in ethyl acetate for GC/MS analysis under chemical ionization mode. Sample injection was done at 250 °C and oven temperature was held at 230 °C for 2 min after injection and increased to 260 °C at 10 °C/min. Detector was run in SIM, recording ion abundance of C1-C6 molecule in the range of 327-336 m/z. RT was 3.8 min.

Lactate.Culture media were acidified by addition of HCl and the lactic acid produced was extracted with ethyl acetate and evaporated to dryness under N2 flow. Dried lactate was incubated at 75 °C for 1 h in presence of 2,2-dimethoxypropane and methanolic HCl. Next, n-propilamine was added to the reaction mixture, kept at 100 °C for 1 h and evaporated to dryness under N2 flow. To remove the n-propylamine-HCl salt, the precipitate obtained was resuspended in ethyl acetate and filtrated through a glass wool packed Pasteur pipette. After drying the filtered solution under N2 flow, dichloromethane and heptafluorobutyric anhydride were added and incubated at room temperature for 10 min. Subsequently, sample was evaporated to dryness under N2 and resuspended in dichloromethane for GC/MS analysis under chemical ionization mode. Sample injection was done at 200 °C and oven temperature was held at 100 °C for 3 min after injection and increased to 160 °C at 20 °C/min. Detector was run in SIM recording ion abundance of C1-C3 molecule in the range of 327-332 m/z. RT was 5.4 min.

Amino acids. Media were passed through a Dowex-50WX8 (H+) column, amino acids eluted with 2 N NH4OH and the amino acids-containing solution evaporated to dryness under airflow. To separate glutamate from glutamine, the amino acid mixture was passed through a Dowex-1X8 (C2H3O2-) column. Glutamine was removed with water, and glutamate was collected by elution with 0.5 N acetic acid. The acid fraction containing glutamate was evaporated to dryness. Dried amino acids or glutamate samples were incubated in butanolic HCl at 100 °C for 1 h. The excess reagents was removed under N2, the precipitate dissolved in dichloromethane and trifluoroacetic anhydride, and left at room temperature for 20 min. Samples were dried under N2 and the derivative was dissolved in dichloromethane for GC/MS analysis. The amino acids derivatives were analyzed under chemical ionization mode, and the analysis of glutamate derivatives was performed under electron impact mode, which yields C2-C4 and C2-C5 glutamate fragments.

For amino acids, sample injection was done at 250 °C and oven temperature was held at 110 °C for 1 min, increased to 125 °C at 10 °C/min, then to 153 °C at 5 °C/min, to 200 °C at 50 °C/min, to 216 °C at 5 °C/min and finally to 250 °C at 25 °C/min. The following table shows the metabolites and m/z ranges monitored by SIM.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **m/z range** | **RT (min)** |
| Alanine | 241-246 | 5.28 |
| Glycine | 227-231 | 5.56 |
| Serine | 353-358 | 6.52 |
| Proline | 295-302 | 9.58 |

For glutamate, sample injection was done at 250 °C and oven temperature was held at 215 °C for 2 min after injection and increased to 225 °C at 9 °C/min and then to 233 °C at 3 °C/min. Detector was run in SIM mode recording ion abundance in the range of 151-157 m/z for C2-C4 and 197-203 m/z for C2-C5. RT was 3.9 min.

Ribose.Ribose from RNA was isolated from the aqueous phase after addition of Trizol (Invitrogen) to the cell pellets. Purified RNA was hydrolyzed in 2 N HCl at 100 °C for 2 h and the solvent was evaporated to dryness under airflow. RNA ribose was derivatized as previously described above for glucose and GC/MS analysis was performed under chemical ionization mode. Sample injection was done at 250 °C and oven temperature was held at 150 °C for 1 min after injection, increased to 275 °C at 15 °C/min and finally to 300 °C at 40 °C/min. Detection was run in SIM recording ion abundance of C1-C5 molecule in the range of 256-261 m/z. RT was 5.3 min.

Palmitate and stearate.Fatty acids from cultured cells were hydrolized from the interphase and lower phase after addition of Trizol to the cell pellets, as described above, by adding 100% ethanol and 30% potassium hydroxide. Samples were then incubated at 70 °C overnight, after which free fatty acids were extracted with petroleum ether, followed by evaporation to dryness under N2 flow. Fatty acids were derivatized adding methanolic HCl, incubated at 70 °C for 1 h and evaporated under N2. Fatty acids derivatives were dissolved in hexane and GC/MS analysis was performed under chemical ionization mode. Sample injection was done at 250 °C and oven temperature was held for 1 min at 120 °C after injection and increased to 220 °C at 5 °C/min. Detector was run in SIM recording ion abundance in the range of 269-278 m/z for palmitate (RT 9.2 min) and 297-307 m/z for stearate (RT 11.85 min).

TCA cycle intermediates (citrate, glutamate, fumarate, malate, aspartate), pyruvate, lactate, alanine.These intracellular metabolites from cultured cells were extracted from liquid nitrogen-frozen cultured plates with the addition of methanol:H2O (1:1) and scraping on ice. Then, cell extracts were sonicated using a titanium probe (VibraCell, Sonics & Materials Inc., Tune: 50, Output: 30). Chloroform was added to the cell lysate and tubes were placed in a shaker for vigorous agitation at 4 °C for 30 min. Subsequently, samples were centrifuged and the upper aqueous phase was separated and evaporated to dryness under airflow at room temperature. TCA cycle intermediates were derivatized by adding 2% (v/v) methoxyamine hidrochloride in pyridine and shaken vigorously at 37 °C for 90 min. Next, N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MBTSTFA) + 1% tert-butyldimetheylchlorosilane (TBDMCS) was added and samples were incubated for 1 h at 55 °C and finally GC/MS analysis performed under electron impact ionization mode. Sample injection was done at 270 °C and oven temperature was held at 100 °C for 3 min, increased to 165 °C at 10 °C/min, then to 225 °C at 2.5 °C/min, to 265 °C at 25 °C/min and finally to 300 °C at 7.5 °C/min. The following table shows the metabolites and m/z ranges monitored by SIM.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **m/z range** | **RT (min)** |
| Pyruvate | 173-181 | 8.2 |
| Lactate | 260-269 | 11.78 |
| Alanine | 259-268 | 12.62 |
| Fumarate | 286-295 | 18.17 |
| Malate | 418-428 | 27.58 |
| Aspartate | 417-428 | 28.90 |
| Glutamate | 431-442 | 32.59 |
| Citrate | 458-469 | 37.67 |

**GC/MS data reduction.** Spectral data obtained from a mass spectrometer represent the distribution of ions of a certain compound with different molecular weights. The value for each observed m/z is given by the combination of 13C and 12C isotopes presents in the background and the precursor used as a tracer. The derivatizing reagents often contain isotopes (e.g. Si isotopes) which contribute to the isotopologue distribution of the derivatized compound. Therefore, correction for all such contributions is necessary before the amount of isotope incorporation and its distribution in the compound of interest can be determined. This correction was performed by using regression analysis employing in-house developed software. The algorithm used corrects all the previous detailed contributions over the observed spectral intensities of each ion cluster, and provides the isotopologue distribution in the analyzed metabolite due to incorporation of 13C atoms from the tracer used as precursor. Results of the isotopologues in any of the ion clusters were reported as fractional enrichments of molecule isotopomers, defined as the fraction of molecules having a certain number of isotope substitutions. Thus, they are designated as m0, m1, m2, etc. where the number indicates the number of labeled carbons (13C) in the molecule as a consequence of the 13C incorporation from the tracer. Note that the sum of all isotopologues of the ion clusters is equal to 1 (or 100%).

**Contribution of glycolysis, PPP and other pathways to the synthesis of lactate.** The amount of lactate produced from glucose via glycolysis, pentose phosphate pathway (PPP) or other substrates can be estimated by combining lactate concentrations and the isotopologue distribution of lactate in cell culture media, using 10 mM 50% [1,2-13C2]-glucose as a tracer. These calculations are made assuming that cells only produce lactate (without lactate consumption). Thus, the amount of unlabeled lactate present in the media at the beginning of the incubation will contribute to the m0 lactate pool at the end of the experiment. Isotopologue distribution of the lactate present in cell culture media at the end of incubation (*LacTotal(m0,m1,m2,m3)tf*) was multiplied by final lactate concentration to obtain the absolute isotopologue distribution of total lactate in mM (*[LacTotal(m0,m1,m2,m3)tf)]* (mM)). Next, initial lactate concentration was subtracted from the concentration of total lactate m0 (mM) to obtain the produced lactate in mM (*LacProd(m0,m1,m2,m3)tf)]* (mM)). Next, relative isotopologue distribution of produced lactate (*LacProd(m0,m1,m2,m3)tf)* was recalculated by dividing the (*[LacProd(m0,m1,m2,m3)tf)]* (mM)) values by total produced lactate ([LacProd]), obtained by subtracting initial lactate concentration from final lactate concentration.

From the recalculated isotopologue distribution of produced lactate, the percentage of lactate that comes from glucose through direct glycolysis(glycolytic tax, GT) was calculated as follows:

*GT = LactProd(m2) \* 2/ Glc(m2)ti* (%)

where Glc(m2)ti is the percentage of [1,2-13C2]-glucose in the cell culture media at the beginning of the incubation. Next, maximum feasible amount of lactate coming from glycolysis (*[LacGlycolysis]*) was obtained by multiplying GT by produced lactate:

*[LacGlycolysis]* = GT \* *[LacProd]* (mM)

In order to calculate the amount of lactate from glucose coming through the PPP, that is, the Pentose Cycle (Pc) parameter, we used the next expression, which includes values of isotopologue distribution of total lactate:

*Pc = {LacTot(m1)tf/LacTot(m2)tf}/{3+LacTot(m1)tf/LacTot(m2)tf}*

The Pc parameter is defined as the relative amount of glucose metabolized through glycolysis related to the glucose metabolized through PPP. A detailed description of this parameter and the deduction of the equation can be found in *Lee et al*. [17].

Lactate from glucose coming through PPP [*LacPPP*] was obtained by multiplying Pc value per the maximum feasible amount of lactate coming from glycolysis, determined before:

*[LacPPP]* = Pc \* *[LacGlycolysis]* (mM)

In order to calculate the amount of lactate produced from other sources different from glucose [Lac*OS*], the maximum lactate produced from glycolysis and lactate coming from glucose through PPP was subtracted to produced lactate:

*[LacOS] = [LacProd] – [LacGlycolysis] – [LacPPP]* (mM)

**Tests performed using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience).** A XF24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to measure the rate of change in dissolved oxygen and protons in media immediately surrounding adherent cells cultured in a XF24-well microplate (Seahorse Bioscience). Cells were seeded in 24-well plates and incubated overnight at 37 °C. One hour before assays, growth media was replaced by basal media (unbuffered DMEM; Sigma-Aldrich) with or without carbon supplements. The sensor cartridge was loaded with the test agents and calibrated prior to the assays. Responses to the different treatments are expressed as Log2 of the fold change comparing the measured point immediately before and after agent injection.

Glucose test.The OCR decrease after glucose addition (Crabtree effect) was analyzed at a final glucose concentration of 18.75 mM. Basal media contained 2 mM glutamine.

Etomoxir test.Analysis of the contribution of fatty acid β-oxidation to OCR was analyzed by injecting etomoxir, a CPT1 inhibitor, which impairs the transport of fatty acids into the mitochondria. Etomoxir was used at a final concentration of 30 µM. Basal media contained 3 mM glucose and 5 mM carnitine.

Mito Stress test.Mitochondrial function was analyzed by injection of oligomycin (ATP synthase inhibitor) to a final concentration of 5 µM, carbonilcyanide p-trifluoromethoxyphenylhydrazone (FCCP) (mitochondrial uncoupler) to a final concentration of 600 nM and rotenone + antimycin (mitochondrial complex I and III inhibitors, respectively) at 2 µM each. FCCP response was further enchanced by 2 mM pyruvate. For the different experiments using this test, four types of basal media were used: full media (17 mM glucose and 2 mM glutamine), minimal media (without glucose and glutamine), media without glucose (with 2 mM glutamine) and media without glutamine (with 17 mM glucose).

Oxamate and DCA titrations.Oxamate is a LDH inhibitor whereas dichloroacetate (DCA) inhibits pyruvate dehydrogenase kinase (PDHK) and consequently allows the activation of the pyruvate dehydrogenase (PDH) complex. This experiment was performed to analyze the effect of promoting the entry of pyruvate into the mitochondria and elucidate the degree of PDH activity in PC-3M and PC-3S cells. Basal media contained 10 mM glucose and 2 mM glutamine. Final oxamate concentrations were 10, 30, 50 and 70 mM and final DCA concentrations 10, 20, 30 and 40 mM. In experiments where the effect of the coinjection of oxamate and DCA was analyzed, oxamate reached a final concentration of 30 mM and DCA of 10 mM.

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