SUPPLEMENTAL METHODS and DATA

LDH-A knockdown and control cell lines. This manuscript focuses on one component of the metabolic phenotype, LDH-A, because it is a bridge between several metabolic pathways, and because the product of LDH-A activity (lactate) can be assessed non-invasively and quantitatively using magnetic resonance spectroscopic (MRS) imaging (MRSI). Lactate dehydrogenase (LDH) is a tetrameric enzyme comprising two major subunits (A and B) that are coded by two different genes (*LDH-A* and *LDH-B*), resulting in five isozymes (1). All five isozymes catalyze the forward and backward conversion (pyruvate<=>lactate). LDH-A (LDH-5, M-LDH, or A4) kinetically favors the conversion of pyruvate to lactate and is predominantly expressed in anaerobic skeletal muscle. LDH-B (LDH-1, H-LDH, or B4) predominantly converts lactate to pyruvate, is predominantly expressed in aerobic cardiac muscle (1, 2) and is oxidized through the tricarboxylic acid (TCA) cycle.

SUPPLEMENTAL METHODS

Analysis of Breast Cancer Microarray Datasets.

A compendium of four breast cancer microarray datasets was analyzed using the Bioconductor set of tools (www.bioconductor.org) in R statistical language (www.r-project.org). Data was downloaded from GEO. Four breast cancer datasets were analyzed: A) MSKCC-82 GSE-2603 (3), B) EMC-286 GSE-2034 (4), C) ECM 192

GSE12276: 204 samples (5), and D) EMC-344 (EMC 286 AND 58 cases of ER- tumors, GSE 5327)(6). All four datasets were profiled on the HG_U133 Affymetrix platform. Three datasets are on the HG-133A platform (one group) and one dataset is on the HG133 Plus2 platform (another group). Both groups of samples were normalized using the standard gcrma procedure (7), and then the data for common probes were combined into one dataset. Values for multiple probes that correspond to the same genes were averaged. Survival analysis was performed using the R package survival. Survival data were separated into low-medium and high groups according to the expression level of LDHA: 2/3 of data-points are in the low group (dashed line) and 1/3 are in the high group (solid line). The two Kaplan-Meir estimators are significantly different ($p<10^{-16}$), indicating longer survival for patients with lower and medium expression of LDH-A compared to the high expression group.

Generation of LDH-A knockdown and control cell lines.

We have transfected 4T1 murine breast tumor cells with four different Sure Silencing shRNA bearing plasmids targeting mouse LDH-A mRNA (KD-knockdown). Two of the shRNAs were most effective in suppressing LDH-A (plasmid #2, and plasmid #3). A sub-cloning strategy of cells transduced with plasmid #2 and plasmid #3 was used to obtain cells that displayed the lowest-levels of LDH-A protein expression. In brief, transfected cells were dissociated with trypsin and were then seeded at a concentration of approximately 1 cell per well into 96-well micro-plates. Approximately 20 different sub-clonal colonies from each plasmid were expanded and analyzed as separate subclones of the original parental cell line.

***In vitro* Assays.**

qRT-PCR. For RNA purification, cells were grown for 48 hours (exponential growth phase). RNA was isolated using the RNeasy total RNA isolation kit (Qiagen), following the manufacturer's protocol. Quantitative RT-PCR for LDH-A and β-actin were performed by the Genomics Core Lab at MSKCC.

Western blotting. Cell pellets were lysed with RIPA Buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Thermo Scientific) and protease inhibitors cocktail (1:100, Thermo Scientific Halt Protease Inhibitor Cocktail). Protein concentrations were determined with bicinchoninic acid assay (BCA Protein Assay Kit, Pierce). A total of 10-20 microgram of proteins were separated by electrophoresis using a NuPAGE 4-12% Bis-Tris Gradient Gel (Invitrogen) and transferred to an Immun-Blot PVDF Membrane (BioRad). Membranes were blocked in 5% milk in Tris-buffered saline - Tween 20 buffer. Membranes were then immunoblotted with anti-LDH-A antibody (Cell Signaling Technology, #2012) at a 1:1,000 dilution, anti-LDH-B antibody (Epitomics) at 1:5,000 dilution and anti-ß-actin antibody (Abcam) at a 1:5,000 dilution. Bound primary antibodies were visualized with appropriate horseradish peroxidase–conjugated secondary antibodies (1:2,000) using enhanced chemiluminescence reagent (Western Lightning-ECL).

LDH-activity. Total LDH activity was assessed using the Cytotoxicity Detection Kit PLUS (LDH) (Roche Diagnostics). Different numbers of cells were plated in 96-well

plates and incubated (37^{o}C, 5%CO_2, humidified incubator) for 2 hours for their attachment. LDH activity from lysed cells was measured as described (8).

Assessment of *in vitro* L-lactate production. Lactate production by KD9 and NC cells during growth was assessed by assaying the culture medium using a L-Lactate Assay Kit (Eton Bioscience). In brief, a series of cell culture supernatant dilutions (1/2, 1/4, 1/8) were made and were added in the wells of 96 well plate. Then L-Lactate assay solution was added and the absorbance spectra were measured at 490 nM, as described by the manufacturer. The data were normalized to the number of viable cells and to background lactate levels of the DMEM medium and quantified by comparing known lactate solution (8).

Glucose utilization. Glucose utilization of NC and KD9 cells was assessed using the Glucose Assay Kit (Eton Bioscience) where glucose is measured by adding cell culture supernatant into 96-well plates. The glucose assay solution was then added and the plates were read for absorbance at 570 nm using a micro-plate reader, as described by the manufacturer. The data were normalized to the number of viable cells (8).

Oxygen consumption rate. Oxygen consumption was measured using the Oxylite system (Oxford Optronics, Oxford, UK). Cells were suspended in 5 ml medium, and incubated in sealed Reacti-vials (Pierce Scientific) at 37^{o}C, with continuous stirring. The Oxylite probe was introduced into the cell mix using a 19 G needle to pierce the rubber septum. Measurements were recorded over 30-60 minutes (8).

Concurrent measurements of glycolysis and oxygen consumption. Glycolytic activity and maximal respiratory capacity of both cell lines were measured using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Cells were seeded at 10,000 cells per well in Seahorse XF96 96-well plates and allowed to attach overnight in a 37°C incubator under 95% air/5% CO_2 in standard growth media containing 10% FCS supplemented with either 25 or 5 mM glucose and 6 mM L-glutamine, penicillin/streptomycin and 4 mg/L of puromycin.

A non-buffered DME medium was used as standard medium in the XF assays and is referred to as "assay medium." In the assays to measure ECAR, glucose was omitted and 2 mM glutamine was used as a substrate. In the assays to measure OCAR, glucose was added to 5 or 25 mM and media also contained 6 mM glutamine. Assays were initiated by removing the growth medium from each well and replacing it with 160 µl of the Seahorse assay medium prewarmed to 37°C. The cells were incubated at 37°C for 60 min to allow media temperature and pH to reach equilibrium before the measurement. The extracellular acidification rate (ECAR, largely reflecting glycolysis) and the oxygen consumption rate (OCR) were measured simultaneously for 4 min to establish a baseline rate. The assay medium was then gently mixed again for 2 min between each measurement to restore normal oxygen tension and pH in the microenvironment surrounding the cells (9). After the baseline measurement, 20 µl of a test agent (FCCP, Glucose, Glutamine, 2-DG) was then injected into each well to reach the desired final working concentration (FCCP, 300 nM; Glucose 25 mM; Glutamine 6 mM; 2-DG 50 mM). Experiments were performed by simultaneously measuring multiple replicates of each cell line. Relative effects were expressed using calculated areas

under the curve (AUC) that were generated by the manufacturer's software and used to compare the various cell lines and conditions.

The area under the curve (AUC) is calculated as follows. For each rate, the group's average is multiplied by the time interval for that rate. This represents the area under the defined part of the curve. The results are then summed to provide the total area. We used the Baseline rate (first rate) for the further calculations and subtracted the Baseline rate each from each subsequent rate. These totals are then summed to provide the total baseline-subtracted AUC.

Assessment of *in vitro* Intracellular Reactive Oxygen Species (ROS) production. The intracellular ROS level was detected by using carboxy-H2DCFDA (C400, Invitrogen) (10). In brief, cells were grown on 96 well black plates. After attachment, cells were washed with warmed PBS and were incubated for 30 min with 20 μM carboxy-H2DCFDA. The dye was removed and the cells washed twice with warm PBS. The cells were returned to prewarmed growth medium and incubated. A 96 well plate reader (TECAN) with an excitation wavelength of 495 nm and an emission wavelength of 529 nm measured fluorescence. The data were normalized to the number of viable cells and background as described by the manufacturer.

Assessment of cellular mitochondria. Cellular mitochondria were assessed using MitoTracker® Red CMXRos (Invitrogen) kit (11). To label mitochondria, cells are incubated with MitoTracker® probes at different concentrations (100 μm, 200 μm, and 500 μm). The probe passively diffuses across the plasma membrane and accumulates in active mitochondria. A 96 well plate reader (TECAN) with an excitation wavelength of

579 nm and an emission wavelength of 599 nm measured fluorescence intensity as described by the manufacturer.

Cell migration and invasion using Transwell (Boyden Chamber) and Wound healing scratch migration assay. In the transwell cell migration and invasion assays, $1x10^5$ cells were allowed to migrate (without a matrigel coating) and invade (with a matrigel coating) across the transwell inserts (BD science, cell culture Inserts for 24-well plates, transparent PET (Polyethylene Terephthalate) membrane 8 μm pore size). 10% serum was used in the lower chamber as a chemoattractant. For the invasion assay, matrigel was diluted using serum free cold DMEM at a concentration of 200 μg/ml. 100 μl of the diluted matrigel was plated into the transwell and was incubated for 30 minutes at 37^{o}C for gelling. The total growth area in the transwell is about 33 mm^2. Addition of matrigel creates a 3 mm thick invasion barrier. The experiments were run over 24 hours at 37°C, and repeated three times. Membranes were processed according to the manufacturer's instructions for migration assessment. In brief, un-migrated cells from the upper side of the membrane were washed and removed. The migrated cells in the lower surface of the membrane were fixed, stained and dried. The average number of pixels that were positive for cells, in randomly chosen fields of view, were counted to quantify the extent of migration and invasion using Matlab software. Statistical analysis of the data was performed using the paired Student's t-test.

For the wound healing scratch assay, cells were cultured as confluent monolayers for 24 h in a 6 well plate, and a ~ 500-μm wide strip was cut across the well with a standard 200-μl pipette tip (12). The cut monolayers were washed with PBS twice to remove any

cell debris. Wound healing was monitored at 6 hours and 24 hours. Wound healing was imaged using a Nikon inverted microscope and an Olympus digital camera. Wound healing was quantified using Matlab software as 100 minus the percent mean cell-free area compared with the area of the initial wound.

For all of the cellular assays, n=3 unless otherwise stated.

In vivo Assay Methods

MRSI Lactate measurements. Two home built coils (2-turn solenoid coils, 7 mm and 12 mm in diameters) were used. MRSI spectra were quantitated by means of the phantom replacement technique to estimate the *in vivo* lactate concentration. The coil was positioned in the center of the platform and matched with the isocenter of the magnet. Tumor bearing mice were anesthetized with Isoflurane (1.5%) combined with oxygen were inserted into the magnet with care taken to ensure that the tumor was in the center of the magnet. Scout images were acquired with the Bruker Para-Vision Tripilot sequence. The body temperature of the mice was monitored and maintained at 35^{o}C (MR-compatible, small rodent Heater System, SA Instruments). The lactate signal was detected using the Selective Multiple Quantum Coherence (SelMQC) editing sequence (13). Spectra of a 5-mm thick slice were acquired with TR (Repetition Time) = 2 sec, TE (Echo Time)=0.189s, 128 excitations, 1024 data points and spectral width of 12 ppm. Two dimensional chemical shift imaging (2D CSI) of the localized, 5-mm thick coronal slice was performed with the following parameters: matrix size 16 X 16 and field of view (FOV) = 24 mm (1.5 x 1.5 mm in plane resolution). T2-weighted sagittal MR images were collected (slice thickness = 1 mm, number of slices = 10, FOV =24 mm,

TR = 2s, TE = 40 ms, matrix= 512 X 256, number of acquisitions = 4). The volumes of the tumor in the slices were calculated by drawing ROI (regions of interest) in the T2 weighted image to find the tumor area and then multiplying the area by the slice thickness (1mm)(8).

MR Data Processing. The 1-dimensional (1D) lactate spectra from the slice were processed by a 1D Fourier transform, similar to our previous methodology (8). The magnitude of the echo signal was fit by a home-written program (Matlab® -The Math Works) and normalized to the slice volume. Lactate spectral quantification was performed by the phantom replacement technique using a 10 mM lactate phantom. The concentration of *in vivo* tumor lactate, C, can be calculated from the formula as:

$$C = C^{ref} \times f_{T_1, T_2} \times \frac{A}{A^{ref}}$$

where, $\mathbf{C^{ref}}$ is the known lactate concentration in the phantom and **A** and $\mathbf{A^{ref}}$ are the measured areas under the fitted peaks in tumor and in the reference phantom, respectively. The correction factor for T1 and T2 differences between phantom and in vivo data is $f_{T_1, T_2} = \exp\left[TE\left(\frac{1}{T_{2\ vivo}} - \frac{1}{T_{2\ phantom}} \right) \right] \times \frac{\left[1 - \exp\left(-TR/T_{1\ phantom}\right)\right]}{\left[1 - \exp\left(-TR/T_{1\ vivo}\right)\right]}$

The relaxation times, T1 and T2, were measured using modified T1-Sel-MQC and T2-Sel-MQC sequences which have been described elsewhere (14).

Visualization of lung metastases by India ink. Selected mice were sacrificed and injected through the trachea with 1 ml of 15% India ink. The normal lung becomes deep black due to the uptake of the India ink. The stained lungs were excised and washed

with PBS. The lungs were then fixed in 5 mL of Fekete's solution for 24 h (15). The tumor tissue emerged as white nodules on a black normal lung background and was photographed.

***Ex vivo* preparation of Frozen Sections.** Selected mice were sacrificed for histopathological examination of the primary tumors and the lungs with metastatic tumors. Frozen tissue samples were cut in sets of 10 contiguous 10 μm-thick sections and processed for H&E staining as described previously (8).

Cell and Tumor doubling-time calculation. The expansion of cells in culture and tumors *in vivo* initially follow exponential growth and can be described by $V = Vo \exp(t/\tau)$. The relationship between exponential time constant "t" and doubling time "t^d" can be found from: $2V_o = V_o \exp(t^d/\tau)$; thus, the doubling time can be calculated by $t^d = \tau \ln(2)$, where τ is estimated from an exponential fit of the growth profile.

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