Published in final edited form as:

Clin Cancer Res. 2011 October 1; 17(19): 6250–6261. doi:10.1158/1078-0432.CCR-11-0397.

Metabolic Imaging: A link between Lactate Dehydrogenase A, Lactate and Tumor Phenotype

Inna Serganova^{#1}, Asif Rizwan^{#2}, Xiaohui Ni^{#2}, Sunitha B. Thakur^{2,3}, Jelena Vider¹, James Russell², Ronald Blasberg^{1,3,5}, and Jason A. Koutcher^{2,3,4,5}

¹ Department of Neurology, Memorial Sloan Kettering Cancer Center, 415 E68 Street, New York, NY 10065, USA.

² Department of Medical Physics, Memorial Sloan Kettering Cancer Center, 415 E68 Street, New York, NY 10065, USA.

³ Department of Radiology, Memorial Sloan Kettering Cancer Center, 415 E68 Street, New York, NY 10065, USA.

⁴ Department of Medicine, Memorial Sloan Kettering Cancer Center, 415 E68 Street, New York, NY 10065, USA.

⁵ Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, 415 E68 Street, New York, NY 10065, USA.

These authors contributed equally to this work.

Abstract

Purpose—We compared the metabolic profiles and the association between LDH-A expression and lactate production in two isogenic murine breast cancer cell lines and tumors (67NR and 4T1). These cell lines were derived from a single mammary tumor and have different growth and metabolic phenotypes.

Experimental Design—LDH-A expression, lactate concentration, glucose utilization and oxygen consumption were measured in cells, and the potential relationship between tumor lactate levels (measured by magnetic resonance spectroscopic imaging (MRSI)) and tumor glucose utilization (measured by [¹⁸F] 2-deoxy-2-fluoro-D-glucose positron emission tomography ([¹⁸F]FDG-PET)) was assessed in orthotopic breast tumors derived from these cell lines.

Results—We show a substantial difference in LDH-A expression between 67NR and 4T1 cells under normoxia and hypoxia. We also show that small orthotopic 4T1 tumors generate tenfold more lactate than corresponding 67NR tumors. The high lactate levels in small primary 4T1 tumors are associated with intense pimonidazole staining (a hypoxia indicator). Less intense hypoxia staining was observed in the larger 67NR tumors, and is consistent with the gradual increase and plateau of lactate concentration in enlarging 67NR tumors.

Conclusions—Lactate-MRSI has a greater dynamic range than [^{18}F]FDG-PET and may be a more sensitive measure with which to evaluate the aggressive and metastatic potential of primary breast tumors.

Keywords

Breast cancer; 67NR; 4T1; LDH-A; LDH-B; lactate; hypoxia; MRSI; SelMQC; FDG/PET; metastases

INTRODUCTION

Metabolic changes in primary tumors have a significant impact on tumor progression and on the development of the metastatic phenotype (1, 2). The accumulation of lactate in tumor cells was first described by Warburg and is associated with aerobic glycolysis (3). Clinical studies showed that high-lactate levels (with median concentrations above 8 mM) are associated with the subsequent development of metastases (4) and include primary cervical, head and neck, and rectal cancers (5-7). The recent coupling between metabolic and genetic variations in cancer cells has stimulated renewed interest in the role of cellular metabolism (2, 8, 9). In cancer patients, serum total lactate dehydrogenase (LDH) levels are often increased, and the gene for *LDH-A* protein, is often upregulated in tumors (10, 11). These features have been linked to poor prognosis (11-15), and a greater metastatic potential has been reported in patients with high LDH serum levels (11, 14). Since LDH-A protein is required for the maintenance and progression of many tumors (10, 16), it is also becoming a potential target for cancer therapy (16-18). Many cancers, particularly those originating in the breast and ovary, are highly heterogeneous, representing a large array of diseases with different etiologies (19) and with distinct genetic and phenotypic signatures (20). The metabolic response of individual tumor cells within a tumor is dependent on the environmental conditions (nutrient depletion, hypoxia, acidity, specific stromal cell components, etc.) encountered within the local tumor microenvironment, as well as specific oncogenic and/or tumor suppressor mutations of the tumor cell itself (9).

We investigated the relationship between LDH-A expression and lactate production in two isogenic breast cancer lines (67NR and 4T1). These two cell lines were derived from a single mammary tumor that developed spontaneously in a BALB/c mouse. Each subclone was shown to have different phenotypic properties (21-23). 67NR cells form primary tumors, but do not metastasize. 4T1 cells are able to complete all steps leading to distant metastases, and efficiently form macroscopic nodules in the lung (21) and other organs (22, 24). We have shown a substantial variation in LDH-A expression between these two cell lines under normoxia and hypoxia that reflect LDH enzyme activity and lactate concentrations in tumors. Consistent with other reports (25, 26), only minor changes in LDH-B expression were observed. We also studied the potential correlation between glucose utilization, as measured by ^{18}F -2-deoxy-2-fluoro-D-glucose uptake and positron emission tomography ([^{18}F]FDG-PET), and lactate production, as measured by magnetic resonance spectroscopic imaging (MRSI), in growing orthotopic tumors.

We consider lactate dehydrogenase to be a critical branch point in metabolism. It is involved in the metabolism of the two major nutrients, glucose and glutamine, as well as in determining tumor pH and the activity of the TCA cycle (27). We hypothesized that tumor lactate levels monitored by MRSI will reflect LDH-A enzymatic activity and tumor phenotype. We show that lactate-MRSI measurements have a greater dynamic range than concurrent [^{18}F]FDG-PET measurements. We suggest that lactate-MRSI is a more sensitive measure than [^{18}F]FDG-PET, and could be used in the clinic to evaluate the aggressive potential of primary breast tumors, since this imaging technology has been applied in human tumors.

MATERIALS AND METHOD

Cell culture and growth

Two isogenic tumorigenic cell lines (67NR and 4T1), derived from a spontaneous breast tumor in a BALB/c mouse (provided by Fred Miller; Karmanos Cancer Institute) were studied. Cells were grown in complete DMEM containing 10% FCS with 2 mM L-glutamine, penicillin/streptomycin. Cells were plated at a density of 5×10^6 cells. Fourteen hours later, plates were placed under normal or hypoxic (1%) conditions to assess cell growth. During days 2 – 6, cells were trypsinized and resuspended in 1 ml of medium. Cells were counted using Countess Automated Cell Counter (Invitrogen).

Assessment of LDH-A mRNA expression

For all mRNA and proteins assays, we used cells growing for 48 hours (exponential growth phase). RNA was isolated using a “RNeasy” total RNA isolation kit (Qiagen), following the manufacturer's protocol. The presence of LDH-A and β -actin mRNAs was assessed using a Qiagen OneStep RT-PCR kit. The mouse LDH-A cDNAs was amplified using the oligonucleotides 5'-CCTGTGGCTGGGCTCTTGGC-3' and 5'-AGCCGGCTCTCCCCCTCTTG-3'. The level of β -actin transcript was used as an internal control and amplified using the oligonucleotides 5'-CCTAAGGCCAACCGTGAAAAGATG-3' and 5'-GGGTGTAAACGCAGCTCAGTAAC-3'.

Western blotting

Breast cancer cell pellets underwent protein extraction using 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). In equivalent amounts (5-20 $\mu\text{g}/\text{well}$), the 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 and were immunoblotted with anti-LDH-A antibody (#2012, Cell Signaling Technology) at a 1:1,000 dilution, anti-HXKII antibody (c-14, sc-6521, Santa Cruz Biotechnology) at a 1:200 dilution, anti-PKM2 antibody (#3198, Cell Signaling Technology) at a 1:1,000 dilution, anti-LDHB antibody (#1974-1, Eptomics) at a 1:1,000 dilution. Bound primary antibodies were visualized with appropriate horseradish

peroxidase-conjugated secondary antibodies (1:2,000) using enhanced chemiluminescence reagent (Western Lightning-ECL). Immunoblots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) and reprobed with anti- β -actin antibody (Abcam) at a 1:5,000 dilution.

LDH-activity

Total LDH activity of 67NR and 4T1 cells was assessed using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Diagnostics). Different numbers of cells were plated in 96-well plates and incubated (37°C, 5% CO₂, 90% humidity) for 3-6 hours, lysed and LDH activity was measured as described by the manufacturer.

Glucose utilization

Glucose utilization of 67NR and 4T1 cells was assessed using the Glucose Assay Kit (MBL International). The glucose concentration in growth medium was measured and compared with a control medium at days 2 and 5.

L- Lactate

Lactate production by 67NR and 4T1 cells during growth was assessed by measuring the culture medium lactate using a L-Lactate Assay Kit (Eton Bioscience).

Oxygen consumption assay

Oxygen consumption was measured using the Oxylite system (Oxford Optronics, Oxford, UK)(28). Cells were cultured in 75 cm² flasks to approximately 90% confluence. Cells were trypsinized to prepare single cells in the growth media media containing 25 mM HEPES. Cells were suspended in 5 ml medium, and incubated in sealed Reacti-vials (Pierce Scientific) at 37°C, with continuous stirring. The Oxylite probe was introduced into the cells mix using a 19 G needle to pierce the rubber septum. Measurements were recorded over 30-60 minutes. Medium pH was measured before and after the experiment. To demonstrate a maximal inhibition of the electron transfer chain (ETC), an inhibitor of the respiratory chain, rotenone was added to cells for 30 minutes at concentrations of 1 or 10 μ M. All experiments were repeated at least 3 times with cells from independent cultures.

Animal model

The animal protocol was approved by the MSKCC's IACUC. 1×10^6 67NR and 4T1 cells were injected into the 4th right mammary fat pad of athymic nu/nu female mice (NCI). Tumors were categorized into three groups: small (<150 mm³), medium (150-400 mm³), and large (>400 mm³). Tumor volume (V) was calculated from caliper-measurements, where $V = (\pi/6) * x * y * z$ where x, y, and z are three orthogonal diameters. A total of 20 mice (eight 67NR and twelve 4T1) were used in the imaging experiments.

In vivo lactate detection

The lactate level in the tumor was detected using magnetic resonance spectroscopic imaging (MRSI). Tumor bearing mice were anesthetized with Isoflurane (1.5%) combined with oxygen. MRSI experiments were performed on a 7T Bruker Biospec Spectrometer using

home-built 2-turn solenoid coils (7 mm and 12 mm in diameters). The coil was positioned in the center of the platform and matched with the isocenter of the magnet. Scout images acquired with the Bruker Para-Vision Tripilot sequence ensured that the tumor was in the center of the magnet. The body temperature of the mice was monitored and maintained at 35°C using a rectal temperature probe and warm air was directed on the animal (MR-compatible, small rodent Heater System, SA Instruments). The lactate signal was detected using the Selective Multiple Quantum Coherence (SelMQC) editing sequence (29, 30). Spectra of both the whole tumor and a 5-mm thick center slice in the coronal plane were acquired with TR = 2 sec, number of excitations = 8, 512 data points and spectral width of 2510 Hz. Two dimensional chemical shift imaging (2D CSI) of the localized, 5-mm thick coronal slice was performed with parameters: matrix size 16 X 16 and field of view (FOV) = 24 mm (1.5 × 1.5 mm in plane resolution). The voxel volume of the CSI was 11.25 mm³ and total 2D-CSI acquisition time was 70 minutes. The 2D CSI was co-registered with a T₂-weighted image using a Multi-Slice Multi-Echo sequence (MSME). The parameters for the MSME included a slice thickness of 1 mm, FOV of 24 mm, 2217 ms repetition time, 40 ms echo time, matrix size 512 X 256, number of average = 8. Multi-slice images of the tumor in the coronal plane were also acquired using Rapid Acquisition with Relaxation Enhancement (RARE) sequence with a slice thickness of 1 mm. The volumes of the tumor in the central slice for CSI 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.

MR Data Processing

The 1-dimensional (1D) lactate spectra from the 5-mm thick center slice were processed by a 1D Fourier transform, similar to our previous methodology (31). The absolute magnitude of the echo signal from the slice was fitted by a home-written program using Matlab® (The MathWorks) and normalized to the slice volume. Lactate spectra quantitation was performed by the phantom replacement technique (32), using a 10 mM lactate/H₂O cylinder. The concentration of *in vivo* tumor lactate, C, can be calculated from the phantom solution of known lactate concentration C^{ref} as:

$$C = C^{ref} \times f_{T_1, T_2} \times \frac{A}{A^{ref}} \quad (1)$$

where A and A^{ref} are the measured areas under the fitted peaks in tumor and in the reference phantom, respectively. The relaxation times, T₁ and T₂, were measured using modified T₁-Sel-MQC and T₂-Sel-MQC sequences which have been described elsewhere (30). The correction factor for T₁ and T₂ differences between phantom and *in vivo* data is:

$$f_{T_1, T_2} = \exp \left[TE \left(\frac{1}{T_{2 \text{ vivo}}} - \frac{1}{T_{2 \text{ phantom}}} \right) \right] \times \frac{[1 - \exp(-TR/T_{1 \text{ phantom}})]}{[1 - \exp(-TR/T_{1 \text{ vivo}})]} \quad (2)$$

The T₁ value for the 67NR and 4T1 are 1.61s and 1.86s respectively. The T₂ value for the 67NR and 4T1 tumors are 0.21s and 0.34s respectively. For the lactate phantom, the T₁ is 1.3s and T₂ is 0.5s. The 2-dimensional chemical shift imaging (CSI) data from the tumor and the phantom were processed voxel by voxel by the 3DiCSI software package (courtesy of Truman Brown, Ph.D., Columbia University). The spectra from the 2D-CSI were

superimposed on the corresponding T₂-weighted MSME image using the 3DiCSI software. The spectrum from each tumor voxel was extracted for further processing by software written using Matlab®. Voxel volume was selected as 11.25 mm³ for both tumor and phantom. Also the maximum concentration of lactate in a voxel was identified (hot spot for lactate) and recorded.

MicroPET

[¹⁸F]FDG-PET imaging was performed after lactate MRS imaging, as previously described (33). [¹⁸F]FDG (specific activity >11 Ci/mmol, average purity 99%) was provided by IBA Molecular (Somerset, NJ, USA). An experimentally determined system calibration factor was used to convert voxel count rate to radioactivity concentrations, expressed as % injected dose/cc (%ID/cc). Region-of-interest (ROI) analysis of the reconstructed images was performed using ASIPro software (Siemens Medical Solutions) to determine the average and maximum tumor radioactivity values. A partial volume correction was applied to the ROI-measured radioactivity data (**Fig. S1**).

Immunohistochemical staining

Selected animals were injected with Pimonidazole hydrochloride (hypoxyprobe-1, HPI) and Hoechst 33342 (Sigma-Aldrich) at 60 and 40 mg/kg, respectively. These agents were dissolved in PBS and administered by way of the tail vein. Pimonidazole hydrochloride was administered one hour pre sacrifice and Hoechst 33342 was administered 5 minutes pre sacrifice (34). Tumors were immediately frozen at -80°C for immunohistochemistry. 10-μm thick adjacent frozen sections from the estimated location of the MRS slice were obtained using a Microm HM500 cryostat microtome (Microm International GmbH). The sections were imaged for Hoechst (blood perfusion) and for Pimonidazole (hypoxia) detection. The sections were then stained with CD31 antibody (endothelial cell marker) and adjacent tumor sections were processed for H&E staining. Images of the sections were processed using Matlab® to estimate the fraction of tumor necrosis (31).

Statistical analyses

All results are expressed as mean values ± standard deviation (SD). Statistical significance was determined by a two-tailed Student T-test; a P value of <0.05 was considered significant.

RESULTS

Metabolic characterization of 67NR and 4T1 murine breast cancer cell lines

The metabolic features of two isogenic tumor cell lines, with different phenotypic growth and metastatic characteristics, were compared and related to LDH-A mRNA expression and protein levels. Semi-quantitative RT-PCR and immunoblotting for LDH-A showed that non-metastatic 67NR cells express lower levels of LDH-A in comparison with highly aggressive, metastases prone 4T1 cells during exponential growth under normal incubation conditions (**Fig. 1A, B**). To confirm that LDH-A expression levels correlate with the functional activity of LDH as an enzymatic complex, we performed an enzymatic assay in viable 67NR and

4T1 cancer cells (**Fig. 1C**). LDH activity was approximately five-fold higher in 4T1 cells, compared to 67NR cells.

We also assessed the expression levels of two other glycolytic enzymes, HXKII (hexokinase II) and PKM2 (pyruvate kinase muscle isozyme 2) (**Fig. 1B**). HXKII and PKM2 are overexpressed in 4T1 cells compared to 67NR cells; the relative intensity of the immunoblot bands was measured and demonstrated a three- and two-fold difference, respectively (data not shown).

A significant difference in acidification of the incubation medium and lactate production was also observed between the two cell lines (**Figs. 1D, 1G**). At day 5 of cell growth, the medium pH of 4T1 was 6.9 ± 0.1 , whereas the medium pH of 67NR cells was 7.4 ± 0.1 . Glucose utilization by 67NR and 4T1 cells was also significantly different (**Fig. 1F**); 4T1 cells exhausted the supply of glucose in the medium within 5 days of incubation, whereas 67NR cells utilized only 50% of the available glucose during this period. These differences are significant at day 5 of cells growth and cannot be explained by differences in cell numbers.

Many advanced cancers that reflect the Warburg phenotype have high glucose uptake with conversion of glucose to lactate. Cells with this phenotype consume available oxygen at a rate dictated by their oxidative capacity, and this consumption rate can be measured. Interestingly, 4T1 cells had a three-fold higher rate of oxygen consumption than 67NR cells. The inhibition of the mitochondrial respiratory chain complex I by 1 μ M rotenone was ~90% in 4T1 cells and 100% in 67NR cells (**Fig. 1E**). Rotenone at 10 μ M completely inhibited oxygen consumption in both cell lines.

***In vivo* lactate detection with ^1H -MRSI using a SelMQC sequence**

Since 67NR and 4T1 cancer cells show significant differences in the expression of HXKII, PKM2 and LDH-A, as well as glycolytic flux and mitochondrial respiration, we decided to assess whether they exhibited a different metabolic phenotype *in vivo* as well. Lactate concentration in 67NR and 4T1 tumors was monitored by MRSI using the SelMQC sequence during tumor growth (the tumor growth profiles are shown in **Fig. S2**). The two-dimensional chemical shift images (2D-CSI) of nonmetastatic 67NR and metastatic-prone 4T1 tumors are compared at three different tumor volumes (**Fig. 2A, 2B**). Note that the distribution of lactate signal varies spatially across both 67NR and 4T1 tumors, reflecting the heterogeneity of tumor metabolism. A difference in total LDH-A expression is also present in 67NR and 4T1 tumors, as assessed by Western blotting (**Fig. 2C**).

Small ($<150\text{ mm}^3$) 67NR tumors showed very low levels of lactate (average lactate $0.9 \pm 1.9\text{ mM}$) (**Fig. 3A, 3C**). Lactate levels in 67NR tumors larger than 150 mm^3 gradually rose and reached a plateau during tumor growth, with an average concentration of $5.5 \pm 1.8\text{ mM}$. Small ($<150\text{ mm}^3$) 4T1 tumors have significantly higher lactate concentrations ($9.3 \pm 2.7\text{ mM}$) than small 67NR tumors ($p=0.0001$). In contrast to 67NR tumors, the lactate signal in 4T1 tumors decreased rapidly, as tumor size increased (**Fig. 3B, 3C**). Expressed differently, primary 4T1 tumors (6-8 days after orthotopic implantation when metastases are first detected; mean volume $=73 \pm 14\text{ mm}^3$) have very high lactate levels ($11.0 \pm 1.5\text{ mM}$).

Plotting tumor lactate concentrations vs. time after orthotopic implantation yielded lactate concentration profiles (**Fig. 3D**) similar to that obtained when plotted vs. tumor volume (**Fig. 3A, 3B**).

An examination of the H&E sections of different size 67NR and 4T1 tumors showed that necrosis became visible in 4T1 tumors even at 100 mm³ in size, whereas necrotic areas were essentially absent in large (>400 mm³) 67NR tumors (**Fig. S3**). The decrease in lactate signal as 4T1 tumors increase in size (**Fig. 3B**) may be explained in part by the increase in tumor necrosis. We analyzed the H&E sections of five large 4T1 (450 mm³, 530 mm³, 670 mm³, 770 mm³, and 860 mm³) tumors that had MRSI/Lactate measurements just prior to sacrifice. The fraction of viable-appearing tissue in these large 4T1 tumors was calculated from the H&E images of these tumors using Matlab® (described in Methods). When the lactate signal was corrected for the viable tissue volume, the average lactate concentration in those large 4T1 tumors was calculated to be 7.3±1.4 mM.

[¹⁸F]fluoro-2-deoxy-D-glucose (FDG) microPET imaging

[¹⁸F]FDG microPET scans were performed following lactate ¹H-MRSI imaging on the same tumor bearing animals. Small 67NR tumors (<150 mm³) had an average partial volume-corrected [¹⁸F]FDG uptake of 6.2±1.5 %ID/cc, which slowly declined as the tumors increased in size (**Fig. 4A, 4C**). The partial volume-corrected [¹⁸F]FDG uptake of small 4T1 tumors was 6.8±2.2 %ID/cc, which decreased rapidly with tumor growth, approaching a plateau value of ~3 %ID/cc as the tumors increased in size (**Fig. 4B, 4C**). All 4T1 tumors exhibited more intense FDG accumulation along the periphery during the late stages of the growth (**Fig. 4D**), reflecting lower metabolism in central tumor areas with greater necrosis. MRSI also showed heterogeneity of lactate levels in medium and large 4T1 tumors, but there was not a strong segregation between the periphery vs. central core.

Comparison of growth profiles, LDH-A and LDH-B expression under normoxic and hypoxic conditions

4T1 cells grow more rapidly than 67NR cells during first 4 days of culture and then plateau, whereas 67NR cells continue exponential cell growth through day 7 (**Fig. 5A**). Since we determined that 4T1 cells have a higher rate of oxygen consumption compared with 67NR cells (**Fig. 1E**), we conducted experiments to study the effect of hypoxia (1% oxygen) on cell growth. We found that the growth of 4T1 cells under hypoxic conditions declined after day 3 (**Fig. 5A**), suggesting that 4T1 cells are very sensitive to hypoxia. In contrast, 67NR cells were less affected by hypoxia and continued to grow slowly after day 3 (**Fig. 5A**).

We also assessed the impact of hypoxia (1% of oxygen) on LDH-A and LDH-B expression levels by immunoblotting. Hypoxia had little effect on LDH-B levels in the two cell lines, consistent with previous published studies (25, 26); the major change was found in LDH-A expression (**Fig. 5**). Importantly, 4T1 cells had a >10-fold higher expression of LDH-A than 67NR cells under normoxic culture conditions, and both cell lines increase LDH-A expression in response to hypoxia (based on the β -actin normalized intensity of the bands) (**Fig. 5B, 5C**). 67NR cells have very low levels of LDH-A under normoxia, but an 18-fold

up-regulation was observed under hypoxia (**Fig. 5B, 5C**). Note that the LDH-A protein levels were similar in both cell lines under hypoxia.

The differential expression of LDH-A protein in 4T1 and 67NR cells under normoxic and hypoxic conditions in cell culture is consistent with the variation and difference in lactate levels measured by MRSI in small 4T1 and medium-to-large 67NR orthotopically growing tumors (**Fig. 3**). In support of this explanation, tumor samples were collected and processed after administration of Pimonidazole hydrochloride and Hoechst 33342 (hypoxia and the blood perfusion probes, respectively) (**Fig. 6**). Consistent with the *in vitro* results, small (100 mm³) 4T1 tumors demonstrate small zones of necrosis (or impending necrosis) associated with pimonidazole (hypoxia) and attenuated perfusion staining. The intensity of pimonidazole staining was significantly higher in all 4T1 samples compared to 67NR tumors. The visible, but less intense, hypoxia staining was observed only in the larger 67NR tumor samples. These results could partially explain the gradual increase and plateau level (5.5±1.8 mM) of lactate in medium and large sizes 67NR tumors, as a result of increasing hypoxia that develops in the enlarging tumors (>150 mm³) (**Fig. 3A**). Moreover, the development of significant necrosis observed in all enlarging 4T1 tumors (**Fig. S3**) may explain the lower lactate accumulation in comparison to small 4T1 tumors.

DISCUSSION

Glucose and glutamine are the major carbon sources for rapidly proliferating tumors, providing precursors for nucleic acids, proteins and lipids, as well as metabolic-reducing capability (NADPH). Pyruvate is largely derived from both glucose and glutamine metabolism; it can be converted to lactate by the lactate dehydrogenase (LDH) complex and/or enter the TCA cycle for conversion to CO₂ and ATP. The conversion of pyruvate to lactate is also catalyzed by LDH which is a reversible reaction that results in the formation of NAD⁺, which is necessary for further glycolysis. LDH isoenzymes are found in almost all eukaryotic cells and tissues, reflecting the importance of this metabolic step. As a consequence of increased glucose and glutamine metabolism, tumors secrete lactate, alanine and NH₄⁺. When oxygen is available, the accumulated and exogenous lactate can be reutilized and converted back to pyruvate, where it is further oxidized to CO₂ and H₂O in the TCA cycle, generating ATP and NAD⁺ (35). Recently, it was shown that LDH-A is required for the maintenance and progression of many tumors (10, 16, 17), but the mechanisms by which LDH-A facilitates tumor progression are poorly understood.

We studied two isogenic cell lines (67NR and 4T1) originating from the same mammary tumor (21). Both cell lines generate orthotopic breast tumors with different growth and metastatic profiles (21): 67NR cells form primary tumors, but no metastases; whereas 4T1 cells generate tumors that rapidly complete all steps leading to macroscopic nodules in lungs within 6-8 days of orthotopic implantation. In this study, we show that these cell lines and their corresponding orthotopic tumors have different metabolic profiles as well.

Significant differences in the gene expression pattern of 4T1 and 67NR cells have been described (22, 24, 36), and these differences contribute to the distinct metabolic and phenotypic behaviors of 4T1 and 67NR cells and tumors. A substantial number of highly

expressed genes in 4T1 cells/tumors are associated with cell adhesion, migration, angiogenesis, extracellular matrix modification, cytoskeleton function, cell proliferation, apoptosis, survival, inflammation, immune response and cellular metabolism. 4T1 cells also displayed elevated levels of *Gadd45*, *Pfkfb3*, *Vegfc*, *Flt1*; some of these genes are known to be regulated by hypoxia and glucose deprivation. This gene-expression profile suggests that 4T1 cells are in a stress-related state, with high metabolic requirements that are inadequately supplied by the vasculature leading to hypoxia and glucose deprivation.

Our additional analysis of published data (22, 36) identified several other important modulators of tumor metabolism. Carbonic anhydrase isoform-12 (CA12) in 4T1 cells is expressed a 6.7-fold above that in 67NR cells. CA12 is a transmembrane enzyme that maintains normal intracellular pH, and is known to be upregulated by HIF-1. Furthermore, a 2.3-fold elevation of MYC expression was observed in 4T1 cells compared to non-metastatic 67NR cells (22). This raises the interesting possibility of a link between glucose and glutamine metabolism in these cells, since Myc: a) induces mitochondrial biogenesis in proliferating cells (37), b) stimulates mitochondrial glutamine metabolism (38), and c) up-regulates *LDH-A* (39). In addition to data from gene expression analyses, comprehensive proteomics profiling across these cell lines has been performed (40). The majority of the detected changes in protein expression are associated with metabolism-related proteins (>40%), including the expression of LDH-A (40).

A recently completed metabolomic analysis of the same cells using liquid chromatography-tandem mass spectroscopy (LC-MS/MS) confirmed the above observations (41). The metastatic capacity of 4T1 cells was shown to be associated with altered glycolysis, pentose phosphate pathway and fatty acid synthesis, as well as decreased GSH/GSSG redox pool. Additionally 4T1 cells have shown enrichment of tricarboxylic acid (TCA cycle) intermediates (citrate, isocitrate and malate) (41), which correlates with a 3.2-fold higher expression of malic enzyme in 4T1 cells (36). This data coincides with a recent study (42) showing that breast cancer cells with the potential to form brain metastases, may use aerobic glycolysis coupled to the TCA cycle and oxidative phosphorylation to generate energy for cell growth. However it is unclear, whether the increase TCA cycle activity is driven primarily by glucose or by glutamine metabolism, and additional studies will be required to answer this question.

The metabolism of both glucose and glutamine involves lactate dehydrogenase (LDH). LDH is a tetrameric enzyme, containing two major subunits (A and B) coded by two different genes (*LDH-A* and *LDH-B*), which may form five isozymes (43). All five isozymes can catalyze the forward and backward conversion of pyruvate and lactate. LDH-A (LDH-5, M-LDH, or A4) kinetically favors the conversion of pyruvate to lactate, while LDH-B (LDH-1, H-LDH, or B4) predominantly converts lactate to pyruvate, which will be further oxidized through the TCA cycle (44). The LDH-A and LDH-B subunits and their ratio are a very important in the formation and function of the tetrameric enzyme, and the sub-unit composition impacts on the kinetics and the direction of the LDH regulated reaction.

We found nearly equal levels of LDH-A and LDH-B expression in 4T1 cells using immunoblot analysis. The LDH-A/LDH-B ratio was 1.0 and 1.4 in 4T1 cells under

normoxia and hypoxia, respectively. In 67NR cells the corresponding LDH-A/LDH-B ratio variation was much greater under normoxia and hypoxia, ranging from 0.11 to 1.66, respectively. The LDH isozymes of 4T1 and 67NR cells have the capacity to catalyze pyruvate-lactate reactions in both directions, with a tendency toward lactate formation under hypoxic conditions. Moreover, these results (reflecting a change in the LDH-A/LDH-B tetrameric enzyme ratio) are consistent with the oxygen consumption experiments, where high mitochondrial TCA cycle activity was observed in 4T1 cells; and with the *in vivo* MRSI experiments showing high lactate and early development of necrosis in small 4T1 tumors. The hypoxia-induced change in the LDH-A/LDH-B tetrameric enzyme ratio of 67NR cells was more dramatic and consistent with the appearance of lactate in medium- and large-size in 67NR tumors. Under hypoxia, 67NR cells significantly upregulate LDH-A and change the composition of the LDH enzymatic complex with the preference toward lactate formation (44).

The higher LDH-A expression in 4T1 cells compared to that in 67NR cells under normal oxygen conditions, is reflected in higher LDH enzymatic activity, higher lactate production, greater generation of hydrogen ions and a greater consumption of glucose by 4T1 cells. In contrast with a traditional Warburg phenotype, 4T1 cells consume oxygen significantly faster compared with 67NR cells, demonstrating high mitochondrial respiration in these metastatic-prone cancer cells. In the last several years a number of publications have shown that oncogenes such as c-Myc (37) and mutant H-Ras (45) increase mitochondrial metabolism which correlated with metastatic potential. Importantly, active oxidative phosphorylation could be very essential for the *in vivo* growth of highly glycolytic tumors, which could be a step to recycle secreted lactate to fuel mitochondrial activity (46), or be an adaptation to other metabolic stress (47, 48). Additionally, the anchorage-independent growth phenotype, which is an important signature of metastatic tumors, is also associated with the activated mitochondrial biogenesis (49).

Several non-invasive imaging techniques can be used for the assessment of tumor metabolism. MRI/MRS provides a unique ability to non-invasively obtain structural as well as metabolic information from soft tissues. [¹⁸F]FDG/PET is primarily used in tumor diagnosis, to stage the extent of disease and to monitor the response to therapy (50); the magnitude of FDG uptake has also been shown to reflect, in part, the rate of glycolysis (50). The capability of MRSI to non-invasively obtain metabolic information is a valuable asset in the metabolic profiling of tumors, and can provide molecular signatures of specific biological processes in discrete anatomical structures using natural metabolites and stable (non-radioactive) isotopes (¹³C, ¹⁵N etc.). Using SelMQC transfer in combination with chemical shift imaging (CSI) for *in vivo* lactate detection, we have shown striking differences and changes in tumor lactate levels during orthotopic 67NR and 4T1 breast tumors growth. A tenfold higher level of lactate was measured in small (<150 mm³) 4T1 tumors compared to small 67NR tumors (**Fig. 3**). Lung metastases are known to develop early (~6-8 days after orthotopic implantation of 4T1 cells; **Fig. S4**) (21) and at approximately the same time that 4T1 tumor lactate levels are very high (11.0±1.5 mM, 8 days after implantation; **Fig. 3D**). The correspondence of high lactate levels in small 4T1

tumors and concurrent development of lung metastases is of particular interest, and suggests that high levels of lactate may be associated with the initiation of metastases.

Differences in lactate production and microenvironment acidity have been previously shown to differentiate metastatic and non-metastatic human xenografts (51, 52). The high lactate concentration (~10 mM; **Fig. 3D**) measured in small 4T1 tumors, concurrent with the development of lung metastases (day 6-8; **Fig. S4**), is consistent with clinical studies (4, 6) where high-lactate levels (with median concentrations above 8 mM) were associated with the subsequent development of metastases (4).

In comparison to tumor lactate levels, there were only small changes in [¹⁸F]FDG accumulation during 4T1 and 67NR tumor growth. At the time metastases were developing from small orthotopic 4T1 tumors, there was only a 1.1-fold difference in [¹⁸F]FDG accumulation between 4T1 and 67NR tumors. In contrast there was a 10-fold difference in lactate levels in the same tumors, at the time when there was little or no tumor necrosis. The subsequent decline in lactate concentration and [¹⁸F]FDG accumulation during 4T1 tumor growth may reflect the effects of tumor necrosis. Since tumor lactate concentration and [¹⁸F]FDG accumulation are likely to be related in a glycolytic phenotype, we compared the ratio of tumor lactate concentration and tumor [¹⁸F]FDG accumulation, sequentially during tumor growth. Despite a fair amount of scatter, 67NR and 4T1 tumors showed marked differences in their lactate-to-FDG ratio profiles when the tumors were small (**Fig.S5**), whereas, both tumors approached a similar plateau ratio (1.0-1.1 mM/%ID/cc) as they increased in size.

Cellular oxygen consumption is one of the determinants of intracellular oxygen levels (53). Under conditions of high oxygen demand, cells can become hypoxic due to high oxygen consumption (54). This is reflected in the metabolic profiles observed for small 4T1 and 67NR tumors, and is very similar to those observed in the cell culture experiments. It suggests that the *in vitro* cell culture environment may better reflect the *in vivo* environment of small tumors. Thus, *in vitro* cell cultures may be better models for small well-perfused tumors, prior to the development of significant ischemia, hypoxia and necrosis. In culture, 4T1 cells produce lactate and consume significantly more glucose and oxygen in comparison to 67NR cells. The appearance of necrotic zones, even in small 4T1 tumors at the time early metastases are developing, is likely to reflect an oxygen requirement for efficient 4T1 cell metabolism and may contribute to hypoxia-induced cell death in these tumors. Moreover, the significant necrosis observed in all enlarging 4T1 tumors (**Fig. S3**) is associated with lower levels of lactate production in medium and large size 4T1 tumors. The intensity of the pimonidazole staining was also significantly higher in all 4T1 samples when compared with 67NR tumors. Less intense hypoxia staining was observed only in the larger 67NR tumors, and is consistent with the gradual increase and plateau of lactate concentration in medium and large sizes 67NR tumors.

This study presents several important findings: (1) the expression of LDH-A and production of lactate in 4T1 breast cancer cells and small orthotopic tumors are higher than that in isogenic 67NR cells and tumors; (2) changes in LDH-A level under hypoxic conditions could explain the formation of lactate in larger 67NR tumors; (3) MRSI was considerably

better than [^{18}F]FDG/PET in identifying significant differences in the metabolic phenotype of small 4T1 tumors (high lactate production and high glucose utilization) compared to 67NR tumors, and these differences were most prominent during early tumor growth when 4T1 metastases were developing; (4) lactate-MRSI has a greater dynamic range than [^{18}F]FDG-PET and may be a more sensitive measure with which to evaluate the aggressive potential of primary breast tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mihaela Lupu for excellent technical assistance; Dr. Sean Carlin, for providing some material and valuable discussions; Drs. Pat Zanzonico, Peter Smith-Jones for providing data for partial volume corrections; Dr. Steven Larson for help and generous support. This work was supported in part by funds: BC060114, CA098505, CA115675, CA86438, CA94060, U24CA83084, DOD W81XWH-09-1-0042 (for SBT).

REFERENCES

1. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. *Nat Rev Cancer*. 2008; 8:56–61. [PubMed: 18059462]
2. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*. 2004; 4:891–9. [PubMed: 15516961]
3. Warburg O. On respiratory impairment in cancer cells. *Science*. 1956; 124:269–70. [PubMed: 13351639]
4. Walenta S, Mueller-Klieser WF. Lactate: mirror and motor of tumor malignancy. *Semin Radiat Oncol*. 2004; 14:267–74. [PubMed: 15254870]
5. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfor K, Rofstad EK, et al. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res*. 2000; 60:916–21. [PubMed: 10706105]
6. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW, et al. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. *Int J Radiat Oncol Biol Phys*. 2001; 51:349–53. [PubMed: 11567808]
7. Walenta S, Chau TV, Schroeder T, Lehr HA, Kunz-Schughart LA, Fuerst A, et al. Metabolic classification of human rectal adenocarcinomas: a novel guideline for clinical oncologists? *J Cancer Res Clin Oncol*. 2003; 129:321–6. [PubMed: 12827509]
8. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci*. 2010; 35:427–33. [PubMed: 20570523]
9. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009; 324:1029–33. [PubMed: 19460998]
10. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*. 2006; 9:425–34. [PubMed: 16766262]
11. Koukourakis MI, Giatromanolaki A, Sivridis E, Bougioukas G, Didilis V, Gatter KC, et al. Lactate dehydrogenase-5 (LDH-5) overexpression in non-small-cell lung cancer tissues is linked to tumour hypoxia, angiogenic factor production and poor prognosis. *Br J Cancer*. 2003; 89:877–85. [PubMed: 12942121]
12. Koukourakis MI, Giatromanolaki A, Simopoulos C, Polychronidis A, Sivridis E. Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. *Clin Exp Metastasis*. 2005; 22:25–30. [PubMed: 16132575]
13. Koukourakis MI, Giatromanolaki A, Sivridis E, Gatter KC, Harris AL, Tumour Angiogenesis Research G. Lactate dehydrogenase 5 expression in operable colorectal cancer: strong association

- with survival and activated vascular endothelial growth factor pathway--a report of the Tumour Angiogenesis Research Group. *J Clin Oncol*. 2006; 24:4301–8. [PubMed: 16896001]
14. Ryberg M, Nielsen D, Osterlind K, Andersen PK, Skovsgaard T, Dombernowsky P. Predictors of central nervous system metastasis in patients with metastatic breast cancer. A competing risk analysis of 579 patients treated with epirubicin-based chemotherapy. *Breast Cancer Res Treat*. 2005; 91:217–25. [PubMed: 15952055]
 15. Koukourakis MI, Giatromanolaki A, Sivridis E, Gatter KC, Trarbach T, Folprecht G, et al. Prognostic and predictive role of lactate dehydrogenase 5 (LDH5) expression in colorectal cancer patients treated with PTK787/ZK 222584 (Vatalanib) anti-angiogenic therapy. *Clin Cancer Res*. 2011
 16. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci U S A*. 2010; 107:2037–42. [PubMed: 20133848]
 17. Seth P, Grant A, Tang J, Vinogradov E, Wang X, Lenkinski R, et al. On-target inhibition of tumor fermentative glycolysis as visualized by hyperpolarized pyruvate. *Neoplasia*. 2011; 13:60–71. [PubMed: 21245941]
 18. Xie H, Valera VA, Merino MJ, Amato AM, Signoretti S, Linehan WM, et al. LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. *Mol Cancer Ther*. 2009; 8:626–35. [PubMed: 19276158]
 19. Nevins JR, Potti A. Mining gene expression profiles: expression signatures as cancer phenotypes. *Nat Rev Genet*. 2007; 8:601–9. [PubMed: 17607306]
 20. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell*. 2006; 127:679–95. [PubMed: 17110329]
 21. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res*. 1992; 52:1399–405. [PubMed: 1540948]
 22. Tao K, Fang M, Alroy J, Sahagian GG. Imagable 4T1 model for the study of late stage breast cancer. *BMC Cancer*. 2008; 8:228. [PubMed: 18691423]
 23. Heppner GH, Miller FR, Shekhar PM. Nontransgenic models of breast cancer. *Breast Cancer Res*. 2000; 2:331–4. [PubMed: 11250725]
 24. Eckhardt BL, Parker BS, van Laar RK, Restall CM, Natoli AL, Tavarua MD, et al. Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. *Mol Cancer Res*. 2005; 3:1–13. [PubMed: 15671244]
 25. Firth JD, Ebert BL, Ratcliffe PJ. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. *J Biol Chem*. 1995; 270:21021–7. [PubMed: 7673128]
 26. Ebert BL, Gleadle JM, O'Rourke JF, Bartlett SM, Poulton J, Ratcliffe PJ. Isoenzyme-specific regulation of genes involved in energy metabolism by hypoxia: similarities with the regulation of erythropoietin. *Biochem J*. 1996; 313(Pt 3):809–14. [PubMed: 8611159]
 27. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell*. 2008; 13:472–82. [PubMed: 18538731]
 28. Urano M, Chen Y, Humm J, Koutcher JA, Zanzonico P, Ling C. Measurements of tumor tissue oxygen tension using a time-resolved luminescence-based optical oxylite probe: comparison with a paired survival assay. *Radiat Res*. 2002; 158:167–73. [PubMed: 12105986]
 29. He Q, Shungu DC, van Zijl PC, Bhujwala ZM, Glickson JD. Single-scan in vivo lactate editing with complete lipid and water suppression by selective multiple-quantum-coherence transfer (Sel-MQC) with application to tumors. *J Magn Reson B*. 1995; 106:203–11. [PubMed: 7719620]
 30. Muruganandham M, Koutcher JA, Pizzorno G, He Q. In vivo tumor lactate relaxation measurements by selective multiple-quantum-coherence (Sel-MQC) transfer. *Magn Reson Med*. 2004; 52:902–6. [PubMed: 15389963]
 31. Yaligar J, Thakur S, Carlin S, Thaler H, Lupu M, Wang Y, et al. Lactate Imaging and DCE-MRI as surrogate markers of prostate tumor aggressiveness. *NMR Biomed*. Revisions requested.
 32. Danielsen ER, Michaelis T, Ross BD. Three methods of calibration in quantitative proton MR spectroscopy. *J Magn Reson B*. 1995; 106:287–91. [PubMed: 7719627]

33. Moroz M, Kochetkov T, Cai S, Wu J, Shamis M, Nair J, et al. Imaging colon cancer response following treatment with AZD1152: a preclinical analysis of [18F]fluoro-2-deoxyglucose and 3'-deoxy-3'-[18F]fluorothymidine imaging. *Clin Cancer Res.* 2011 in press.
34. Carlin S, Khan N, Ku T, Longo VA, Larson SM, Smith-Jones PM. Molecular targeting of carbonic anhydrase IX in mice with hypoxic HT29 colorectal tumor xenografts. *PLoS One.* 2010; 5:e10857. [PubMed: 20523727]
35. Granchi C, Bertini S, Macchia M, Minutolo F. Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. *Curr Med Chem.* 2010; 17:672–97. [PubMed: 20088761]
36. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell.* 2004; 117:927–39. [PubMed: 15210113]
37. Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, et al. Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. *Mol Cell Biol.* 2005; 25:6225–34. [PubMed: 15988031]
38. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A.* 2008; 105:18782–7. [PubMed: 19033189]
39. Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A.* 1997; 94:6658–63. [PubMed: 9192621]
40. Ho J, Kong JW, Choong LY, Loh MC, Toy W, Chong PK, et al. Novel breast cancer metastasis-associated proteins. *J Proteome Res.* 2009; 8:583–94. [PubMed: 19086899]
41. Lu X, Bennet B, Mu E, Rabinowitz J, Kang Y. Metabolomic changes accompanying transformation and acquisition of metastatic potential in a syngeneic mouse mammary tumor model. *J Biol Chem.* 2010; 285:9317–21. [PubMed: 20139083]
42. Chen EI, Hewel J, Krueger JS, Tiraby C, Weber MR, Kralli A, et al. Adaptation of energy metabolism in breast cancer brain metastases. *Cancer Res.* 2007; 67:1472–86. [PubMed: 17308085]
43. Everse J, Kaplan NO. Lactate dehydrogenases: structure and function. *Adv Enzymol Relat Areas Mol Biol.* 1973; 37:61–133. [PubMed: 4144036]
44. Stambaugh R, Post D. Substrate and product inhibition of rabbit muscle lactic dehydrogenase heart (H4) and muscle (M4) isozymes. *J Biol Chem.* 1966; 241:1462–7. [PubMed: 4287748]
45. Telang S, Lane AN, Nelson KK, Arumugam S, Chesney J. The oncoprotein H-RasV12 increases mitochondrial metabolism. *Mol Cancer.* 2007; 6:77. [PubMed: 18053146]
46. Sonveaux P, Vegran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest.* 2008; 118:3930–42. [PubMed: 19033663]
47. Chen V, Shtivelman E. CC3/TIP30 regulates metabolic adaptation of tumor cells to glucose limitation. *Cell Cycle.* 2010; 9:4941–53. [PubMed: 21150275]
48. Wellen KE, Thompson CB. Cellular metabolic stress: considering how cells respond to nutrient excess. *Mol Cell.* 2010; 40:323–32. [PubMed: 20965425]
49. Mori S, Chang JT, Andrechek ER, Matsumura N, Baba T, Yao G, et al. Anchorage-independent cell growth signature identifies tumors with metastatic potential. *Oncogene.* 2009; 28:2796–805. [PubMed: 19483725]
50. Mankoff DA, Eary JF, Link JM, Muzi M, Rajendran JG, Spence AM, et al. Tumor-specific positron emission tomography imaging in patients: [18F] fluorodeoxyglucose and beyond. *Clin Cancer Res.* 2007; 13:3460–9. [PubMed: 17575208]
51. Schornack PA, Gillies RJ. Contributions of cell metabolism and H⁺ diffusion to the acidic pH of tumors. *Neoplasia.* 2003; 5:135–45. [PubMed: 12659686]
52. Jordan BF, Black K, Robey IF, Runquist M, Powis G, Gillies RJ. Metabolite changes in HT-29 xenograft tumors following HIF-1 α inhibition with PX-478 as studied by MR spectroscopy in vivo and ex vivo. *NMR Biomed.* 2005; 18:430–9. [PubMed: 16206237]
53. Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1 α . *Science.* 2003; 302:1975–8. [PubMed: 14671307]

54. Aragonés J, Schneider M, Van Geyte K, Fraisl P, Dresselaers T, Mazzone M, et al. Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nat Genet.* 2008; 40:170–80. [PubMed: 18176562]

TRANSLATIONAL RELEVANCE

Using sub-clones derived from a single spontaneous murine breast tumor, we show that the more aggressive clone (4T1, with a high propensity to metastasize to lung and other organs) produces higher levels of lactate in small tumors that are associated with higher *LDH-A* expression and a higher intensity of pimonidazole staining, when compared to the nonmetastatic 67NR clone. Lactate-MRSI, but not [^{18}F]FDG-PET imaging, was able to identify significant differences in the metabolic phenotype between these two orthotopic tumors. There was a 10-fold higher level of lactate in small 4T1 tumors compared to 67NR tumors, at the time 4T1 lung metastases were developing. In contrast, there was only a 1.1-fold difference in [^{18}F]FDG accumulation in the same tumors. These studies demonstrate a much greater dynamic range for tumor lactate measurements compared to measurements of [^{18}F]FDG accumulation in these tumor models.

Figure 1. Metabolic features of isogenic 67NR and 4T1 breast cancer cells

LDH-A mRNA expression in 67NR and 4T1 cells by the semi-quantitative RT-PCR (A). LDH-A, HKII, PKM2 proteins expression assessed by Western blotting (B). Total LDH enzyme activity in 67NR cells and 4T1 cells (C). Cell culture medium acidification; supernatant pH (D). Oxygen consumption of 67NR and 4T1 cells in normal growth media (DMEM HG+10%FCS+2mM L-Glutamine, 25 mM HEPES) and upon treatment with 1 μ M of rotenone (E) using the Oxylite system. Glucose utilization; clearance of glucose from the culture medium (F). Lactate production; appearance of lactate in the culture medium (G).

Figure 2. Lactate CSI Spectra

Different size 67NR non-metastatic tumors (**A**) and 4T1 metastasis-prone tumors (**B**) are shown. The lactate signal was detected using chemical shift imaging (CSI) and a Selective Multiple Quantum Coherence (SelMQC) editing sequence. LDHA protein expression assessed by Western blotting in different sizes of 67NR and 4T1 tumors (TV- tumor volume)(**C**).

Figure 3. MRSI measurements of lactate concentration in orthotopic 67NR and 4T1 breast tumors during tumor growth

Lactate concentration in 67NR tumors (solid circles, dashed line) (A), and in 4T1 tumors (solid squares, solid line) (B) plotted vs tumor volume. Average lactate concentration at three different tumor volumes (small <150 mm³, medium 150-400 mm³, large >400 mm³) (C). Values are the mean, $\pm$ SD, where * = P<0.05 and ** = P<0.01. Tumor lactate concentration plotted vs days post-orthotopic implantation (D).

Figure 4. PET/FDG measurements of [^{18}F]FDG accumulation in orthotopic 67NR and 4T1 breast tumors during tumor growth

[^{18}F]FDG accumulation in 67NR tumors (solid circles, dashed line) (**A**), and in 4T1 tumors (solid squares, solid line) (**B**) plotted vs tumor volume. Average [^{18}F]FDG accumulation at three different tumor volumes (small $<150 \text{ mm}^3$, medium $150\text{--}400 \text{ mm}^3$, large $>400 \text{ mm}^3$) (**C**). Values are the mean, $\pm$ SD. Representative [^{18}F]FDG microPET images of tumor-bearing mice (**D**); small and large 4T1 orthotopic tumors are visualized (white arrows). High radioactivity is seen in the cervical brown fat, heart and bladder. Values are color-coded to a range of values (%ID/cc).

Figure 5. The effect of hypoxia on the cells growth and LDH-A expression

Growth profiles of 67NR cells and 4T1 cells under normoxic (N, 21%) and hypoxic (H, 1%) conditions (A). Immunoblot of LDH-A and LDH-B subunits from 67NR and 4T1 cells exposed to normoxia and hypoxia for 24 hours (B). The β -actin normalized intensity of LDH-A and LDH-B levels in cells under normoxic and hypoxic conditions (C). The ratio of β -actin normalized LDH-A and LDH-B expression under normoxic and hypoxic conditions (D).

Figure 6. Immunohistochemical staining of 67NR and 4T1 tumors

Histology (H&E), endothelial cell density (CD31, red), blood flow (Hoechst 33342, blue) and hypoxia (Pimonidazole, green) are compared. Small 67NR tumors showed no necrosis, a uniform endothelial cell density and blood flow and no hypoxia. Medium size 67NR tumors showed some Pimonidazole staining. This small 4T1 tumor shows some central necrosis, a decrease in endothelial cell density and blood flow, with a corresponding zone of hypoxia surrounding the necrotic zone. These observations were amplified in medium size 4T1 tumors.