**Specific expression of lactate dehydrogenases in glioblastoma controls intercellular lactate transfer to promote tumor growth and invasion**

**Key words:** Glioblastoma, lactate dehydrogenases, energy metabolism, invasion, antiepileptic drug.

**Short title:** lactate dehydrogenases in GBM development

**Supplementary Figure Legends**

**Supplementary Figure 1: Expression of LDHA and LDHB in publicly available databases (Extended data Figure 1)**

**a.** Correlation between gene expression of LDHA and LDHB and cell localization based on single cell RNA sequencing data extracted from Darmanis et al.1 (1010 tumor cells and 62 periphery cells). **b.** *LDHA* and *LDHB* gene expression relative to HIF1A gene expression according to their anatomical origin (data extracted from Ivy Glioblastoma Atlas Project).LE, Leading Edge; IT, Infiltrating Tumor; CT, Cellular Tumor; CTpan, Cellular Tumor pseudopalisading cells around necrosis; CTmvp, Cellular Tumor microvascular proliferation. **c.** Survival analysis based on *LDHA* (*left*) or *LDHB* (*right*) gene expression level in glioblastoma (data extracted from TCGA).

**Supplementary Figure 2: Independent stem-like cell line BL13 confirms results obtained in P3 stem-like cells (Extended data Figure 2a-h)**

**a.** Western blot analysis of LDHA and LDHB from BL13 cells upon exposure to 21% or 0.1% O2 during 6, 24, 48 and 72 h. The graphs represent densitometry quantification of the immunoblots normalized to tubulin (*n* = 4). **b.** Lactate secretion of BL13 cells exposed to 21% or 0.1% O2 measured by bioluminescent assay using a pro-luciferin reductase substrate converted to luciferin in the presence of NADH (*n* = 3). **c, d.** Colorimetric assays related to the activity of (**c**) LDH enzymes (*n* = 4) or (**d**) immune-captured native LDHB enzyme (*n* = 3) in BL13 cells by measuring the absorbance at 450 nm. **e.** P3 spheroid cytotoxicity assay recorded over 24 h when incubated with or without lactic acid at different concentrations (1, 10, 20, 30, 40, 50, and 100 mM). Area of spheroids were measured at 0 and 24 h. Growth is represented as a percentage of spheroid area when compared to time 0 and viability is estimated with Live/Dead fluorescence ratio at 24 h and represented as a fitted curve (8–10 spheroids per condition, *n* = 3). Images of representative spheroids in each condition (in green, calcein ; in red, ethidium homodimer-1). Scale: 250 µm. **f.** Principal component analysis of morphologic data on P3 cells incubated 7 days with or without lactate (20 mM). Cell number and morphology were measured at 0 h, 24 h and 7 days. Images of representative adherent cells in each condition (in green, GFP; in red, nuclear Tomato). Scale: 40 µm. The graphs represent quantification of the cell number and elongated cells (Aspect ratio > 2,5; *n* = 3). **g.** P3 spheroid invasion in collagen I gel incubated 24 h at 21% O2 and treated with 20 mM pyruvate or 1.5 mM HCl. Invasion rate is expressed as a fold change of the control (6-8 spheroids per condition, *n* = 3). Data are represented as mean ± s.d. *n* represents independent experiments. *P* values were calculated using One-Way ANOVA following by Dunnett’s multiple comparisons test (**a**), unpaired t test (**b**, **c**, **d**), Kruskal-Wallis test following by Dunn’s multiple comparisons test (**e, g**); Two-Way ANOVA following by a Sidak’s multiple comparisons test (**f**); \**P*<0.05 ; \*\**P*<0.01 ; \*\*\**P*<0.001 ; ns not significant; exact *P* values are provided in the Source Data.

**Supplementary Figure 3: Metabolic tracing using [13C3]lactate (Extended data Figure 2i)**

P3 cells were infused during 0, 1, 2, 4, 6 and 24 h with [13C3]lactate at a concentration of 5 mM. Metabolites from cell extracts (endometabolome) or cell medium (exometabolome) measured by gas chromatography-mass spectrometry. **a.** Metabolite abundance of some intermediates of metabolic pathway of interest. **b.** Amount of labeled isotopes relative to the total amount of this element, expressed as a percentage (fractional contribution). **c.** Quantification of the [13C3]lactate carbon incorporation into intermediates of the carbon metabolism (isotopologue contribution).

**Supplementary Figure 4: LDHA/B KO in BL13 cells and bevacizumab treatment in P3 tumors (Extended data Figure 3)**

**a.** Western blot analysis of LDHA and LDHB from BL13 cells knockout by Crispr-Cas9 lentiviral vectors against LDHA, LDHB or both, and upon exposure to 21% O2. **b, c.** Colorimetric assays related to the activity of (**b**) LDH enzymes (*n* = 4) or (**c**) immune-captured native LDHB enzyme (*n* = 3) by measuring the absorbance at 450 nm in KO P3 cells. **d.** Schematic representation of the intracellular lactate level monitoring with a fluorescent biosensor. The presence of the lactate changes the conformation of the biosensor and fluorescence emission. Known as an accelerated-exchange transport (trans-acceleration), oxamate was used to quickly release the lactate out of the cells for the determination of the lactate basal level. Then, diclofenac was used to block the lactate transporter for the quantification of the lactate production rate. **e.** Cells incubated during 48 h at 21% or 0.1% O2, labeled with Annexin V-FITC and analyzed by cytometry. Table of statistical comparisons **f.** BL13 spheroid invasion in collagen I gel incubated 24 h at 21 or 0.1 % O2. Invasion rate is expressed as a fold change of the control (6-8 spheroids per condition, *n* = 4). Images of representative invasive sgControl or sgLDHA/B spheroids. Scale: 100 µm. **g.** Supernatants were collected from each cell line and analyzed by using ELISA to detect VEGF. **h.** Kaplan-Meier survival curves complement of **Figure 3i** where xenotransplanted mice with LDHA/B KO P3 spheroids were treated by bevacizumab. **i.** Kaplan-Meier survival curves of xenotransplanted mice with BL13 cells KO for LDHA/B (red) or control (blue) (*n* = 10 mice per group). Data are represented as mean ± s.d (**b, c, e, g**). *n* represents independent experiments. *P* values were calculated using Two-way ANOVA following by Tukey’s multiple comparisons test.

**Supplementary Figure 5: Detailed metabolograms (Extended data Figure 4c)**

Circular metabologram illustrating metabolic and transcriptomic differences in metabolite pathways between LDH KO P3 cells. The metabologram is divided in two parts, the left corresponds to metabolomic analysis and the right to the transcriptomic analysis. The outer circle corresponds to the log2 fold change for each metabolite (*left*) and transcripts (*right*). The central circle displays the average fold change of all analytes. Metabolites and gene names were added into these metabolograms.

**Supplementary Figure 6: RNAseq/metabolomics profiles of P3 sgControl adaptations to hypoxia or basal differences between P3 sgControl and P3 sgLDHA/B cells (Extended data Figure 4)**

Metabolic changes of central 13-labeled-carbon metabolism when knock out P3 cells are infused with [13C6]glucose in 1% O2. Metabolites are labelled with colored-oval and enzyme transcripts with colored-square whose colour corresponds to the log2 fold changes between **a.** sgCont 0 h and sgCont 48 h (blue, increase in sgCont 0 h; red, increase in sgCont 48 h; gray, not measured or not computable) or **b.** sgCont 0 h and sgLDHA/B 0 h (blue, increase in sgCont 0 h; red, increase in sgLDHA/B 0 h; gray, not measured or not computable). For details, see also **Figure 4**. **c.** *Left:* Volcano plots for visualizing gene expression in described comparisons. *Right:* Enrichment analysis usingGene Ontology with filtered terms “biological process” and “cellular component”.

**Supplementary Figure 7: Metabolic tracing using [13C6]glucose (Extented data Figure 4)**

P3 sgControl, sgLDHA, sgLDHB and sgLDHA/B were infused during 0, 24 and 48 h at 0.1% O2 with [13C6]glucose. Metabolites from cell extracts (endometabolome) or cell medium (exometabolome) measured by gas chromatography-mass spectrometry. Abundance and isotopolog contribution of all metabolite isotopes and fractional contribution of direct isotopes from glucose metabolism are showed.

**Supplementary Figure 8: uncropped western-blots related to Figures 2, 3, and 5.**

**Supplementary Tables:**

**Title:** Reagents list

**Description:** List of the antibodies, reagents and products used

**Title:** Source Data Fig2

**Description:** Data and statistical analysis of the graphics in the Figure2: densitometry analysis (sheet 1), lactate secretion (sheet 2), LDHs activity (sheet 3), LDHB activity (sheet 4), spheroid growth (sheet 5), spheroid invasion (sheets 6 and 7), oxygraphy (sheet 8), and change in the metabolite abundance (sheet 9).

**Title:** Source Data Fig3

**Description:** Data and statistical analysis of the graphics in the Figure3: lactate secretion (sheet 1), intracellular lactate measurements (sheet 2), spheroid growth (sheet 3), spheroid invasion (sheet 4), mice-implanted tumor quantification (sheets 5 and 6), and survival curves (sheet 7).

**Title:** Source Data Fig4

**Description:** Data and statistical analysis of the graphics in the Figure4: exometabolome measurements (sheet 1).

 **Title:** Source Data Fig5

**Description:** Data and statistical analysis of the graphics in the Figure5: densitometry analysis (sheet 1), mitochondrial morphology and mass (sheet 2), oxygraphy (sheet 3), and survival curves (sheet 4).

**Title:** Source Data Fig6

**Description:** Data and statistical analysis of the graphics in the Figure6: intracellular lactate measurements (sheet 1), oxygraphy (sheet 2), spheroid growth (sheet 3), spheroid invasion (sheet 4), survival (sheet 5), and mice-implanted tumor quantification (sheet 6).

**Title:** Source Data RNAseq

**Description:** Human genes classified with DESeq2 in P3 sgControl, sgLDHA, sgLDHB and sgLDHA/B cell lines at 21% or 0.1% O2. Two-class comparisons using the R package DESeq2 were performed with P3 sgControl and sgLDHA (sheets 1 and 2), sgControl and sgLDHB (sheets 3 and 4) and sgCont and sgLDHA/B (sheets 5 and 6) genes.

**Title:** Source Data Supp Fig2

**Description:** Data and statistical analysis of the graphics in the Supp Figure2: densitometry analysis (sheet 1), lactate secretion (sheet 2), LDHs activity (sheet 3), LDHB activity (sheet 4), spheroid growth and viability (sheet 5), cellular morphology (sheet 6), and spheroid invasion (sheet 7).

**Title:** Source Data Supp Fig4

**Description:** Data and statistical analysis of the graphics in the Supp Figure4: LDHs activity (sheet 1), LDHB activity (sheet 2), Annexin-V quantification (sheet 3), spheroid invasion (sheet 4), ELISA VEGF analysis (sheet 5), survival curves (sheets 6 and 7).

**Supplemental Experimental Procedures**

**TCGA Glioblastoma cohort**

The TCGA Glioblastoma (GBM) RNAseqV2 normalized data (level 3, log2(x+1) transformed RSEM normalized count, version 2017-10-13), the associated clinical data and complementary clinical data from GDC pancan were downloaded from the xenabrowser website datapages (<https://xenabrowser.net/datapages/>).
For the genes LDHA and LDHB, primary tumor samples from the GBM cohort were split into three groups of equivalent size defined by the level of their expression. Overall survival (in months) was used to estimate survival distributions using the Kaplan–Meier method and the three distributions were compared using the log‐rank test.

**Ivy-GAP cohort**

The expression of  HIF1A, LDHA and LDHB were downloaded from the IVY-GAP website (<https://glioblastoma.alleninstitute.org/rnaseq/search/index.html>) with associated clinical data. Spearman correlation was computed between HIF1A and LDHA and between HIF1A and LDHA for all samples and for each anatomic structure separately.

**RNA sequencing**

* **Sample preparations and RNA sequencing**

P3 sgControl, sgLDHA, sgLDHB and sgLDHA/B were cultivated under 0.1% or 21% oxygen for 48h. Total RNA was extracted from fresh frozen cells with the Qiagen RNeasy Mini Kit according to the manufacturer’s protocol. Quality and quantity of RNA was checked using a Fragment Analyzer (Agilent) with the company’s Standard Sensitivity RNA Kit (DNF-471). Libraries were prepared using the TruSeq stranded mRNA Kit (Illumina). All barcoded samples were pooled and sequenced together in 75nt paired-end mode with an Illumina NextSeq500 in 2 runs to reach sufficient coverage. Runs were demultiplexed with bcl2fastq (v2.20.0.422, Illumina).

* **Bioinformatics analysis**

Quality of obtained fastq files was initially checked by FastQC v0.11.4 2 followed by adapter removal and quality trimming using Trim Galore v0.4.2. Mapping of reads to the human reference genome (GRCh38 Ensembl release 95) was done using STAR v2.5.3a with standard settings3 and duplicates were marked using Picard tools v1.141.

Quality analysis of mapped reads was done using RSeQC v3.0.0 4 to analyze read distributions across gene bodies. Raw read counts per gene were determined by counting gene-specific reads in exons of protein-coding genes using FeatureCounts v1.5.3 5. Finally, a gene expression data matrix was created by removing genes without any reads and lowly expressed genes (less than 1 read per million in more than 50% of samples) followed by cyclic loess normalization resulting in normalized log2-counts per million for 14,111 protein-coding genes that were measured in each sample.

The R package DESeq26 v1.22.2 was used to identify differentially expressed genes. Enrichment analysis was performed using the ClusterProfiler7 R package v3.10.1. Gene Ontology (GO) terms enrichment analysis was visualized using Bubble Chart to Compare Annotations (BACA) using the p-value threshold at 0.01. Only GO terms from Biological Pathways level 5 were used for this analysis. GO terms were clustered to get annotation clusters with a similarity of genes greater than 0.85. PathView R package v.1.22.3 was used to visualize KEGG metabolic pathways.

**Metabolomics Supplemental Method 1: [13C]6 Glucose – 13C isotopic profiling**

* **Sample preparations**

For cells in suspension (fast filtration method), 1 mL of cell culture are dropped on a filter (Sartolon Polyamide 0.2μm) in order to eliminate cultivation medium. The filter is then rinsed with washing solution, quickly removed from the filtration unit, putted on an aluminum foil and frozen in liquid nitrogen. Every filter is then extracted into a centrifuge tube containing the 5 mL of cold sampling solution (see table below). The centrifuge tubes are then vortexed and placed 1 hour at -20°C. After one hour, the tubes are centrifuged 5 min at 2000 g and the supernatant is putted in a new tube for evaporation.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Types of metabolites** | **Sampling solution composition** | **Temperature of extraction** | **Duration of extraction** | **Evaporation** | **Solution of resuspension** |
| Central metabolites | ACN(4)/MeOH(4)/ H2Omq (2) 125 mM formic acid  | -20°C | 1 h minimum | Speedvac | Water |
| Intracellular amico acids | ACN(4)/MeOH(4)/ H2Omq (2) 125 mM formic acid  | -20°C | 1 h minimum | Speedvac | Water |
| Coenzyme A | ACN(4)/MeOH(4)/ H2Omq (2) 125 mM formic acid  | -20°C | 1 h minimum | Speedvac | 2% methanol, 98% water, 25 mM formic acid, pH adjusted at 3.5  |

For cell supernatants, the sampling procedure is based on the separation of the cells from the medium thanks to the combination of filtration and centrifugation. This give access only to the exometabolome content for metabolomics or fluxomics studies. The supernatant can be stored at -80°C before shipment and analysis evaporated. Then samples are prepared either for NMR or MS analysis manually or automatically using a robotic station.

To control the quality of the analysis, blank samples are done for each type of samples (culture medium, conditions, treatment…). These blank samples are obtained from “cell-free” culture made in parallel and sampled in the same way and time as the culture with cells.

* **Nuclear Magnetic Resonance (NMR) profiling**

The acquisition of 1D 1H NMRAvance II 800MHz equipped with a 5mm CQPCI Z-Gradient cryoprobe. Following parameters were used for the acquisition: Pulse program: zgpr30; Pulse angle 30°; Time Domaine (TD) 64k; Number of dummy scan: 4; Number of scan: 32; Acquisition time: 2.04 sec; Pulse P1 length: 7.70 µsec; Pulse P1 power: -12.39 dB; Pulse P9 power: 43.79 dB; Acquisition temperature: 280°K. Raw data obtained after acquisition are FID. A Fourier transform were applied for each spectrum with a specific smoothing (efp with LB = 0.3 and SI = 128K). Phase and baseline correction were also performed using automatic tools form TopSpin 3.5 software before the automatic integration of specific signals belonging to exo-metabolites present in the samples. Absolute quantification of metabolites of interest was performed using the internal standard TSP-d4 as reference. The quality of the analysis is based on the good resolution of the spectrum: width at half height for TSP-d4 signal < 2.5Hz.

* **Liquid chromatography / Mass Spectrometry analysis**

Central metabolites were separated on an ionic chromatography column IonPac AS11 (250 × 2 mm i.d.; Dionex, CA, USA). Solvent used was KOH at a flow rate of 350 μL/min. Solvent was varied as follows: 0 min: 2 %, 2 min: 2 %, 10 min: 5 %, 16 min: 35 %, 20 min: 100 % and 24 min: 100%. The column was then equilibrated for 6 min at the initial conditions before the next sample was analyzed. The volume of injection was 15 μL. High-resolution experiments were performed with an ICS5000+, ion chromatography system (Dionex, CA, USA) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization probe. MS analyses were performed in negative FTMS mode at a resolution of 60 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 350 °C, the source heater temperature, 300 °C, the sheath gas flow rate, 50 a.u. (arbitrary unit), the auxiliary gas flow rate, 5 a.u., the S-Lens RF level, 60 %, and the source voltage, 3.5 kV.

Amino acids were separated on a PFP column (150 × 2.1 mm i.d., particle size 5 µm; Supelco Bellefonte, PEN, USA). Solvent A was 0.1 % formic acid in H20 and solvent B was 0.1 % formic acid in acetonitrile at a flow rate of 250 µL/min. Solvent B was varied as follows: 0 min: 2 %, 2 min: 2 %, 10 min: 5 %, 16 min: 35 %, 20 min: 100 % and 24 min: 100%. The column was then equilibrated for 6 min at the initial conditions before the next sample was analyzed. The volume of injection was 5 µL. High-resolution experiments were performed with a Vanquish HPLC system coupled to an Orbitrap Qexactive+ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization probe. MS analyses were performed in positive FTMS mode at a resolution of 70 000 (at 400 m/z) in full-scan mode, with the following source parameters: the capillary temperature was 320 °C, the source heater temperature, 300 °C, the sheath gas flow rate, 40 a.u. (arbitrary unit), the auxiliary gas flow rate, 10 a.u., the S-Lens RF level, 40 %, and the source voltage, 5 kV.

All the metabolites were determined by extracting the exact mass with a tolerance of 5 ppm. For central metabolites isotopic profile analysis and amino acids isotopic profile analysis, their concentrations have to be included in the dynamic range of the method, respectively. This range was determined during the method validation with the PA-PT sample and corresponds to the total area of the cluster / number of isotopologues with a bias of less than 5 %.

* **Data Quality**

Filters and supernatants were received on 03.04.19 and analyzed on 10.04.19 (MS) and 11.04.19 (NMR). All the acceptability criteria were satisfied, values meet MetaToul’s acceptance as shown in the table below.

|  |  |  |
| --- | --- | --- |
|  |  | **Acceptability criteria** |
| MS calibration ≤ 7 days | Day of injection (11.04.19) | **Passed** |
| Bias on Pascal Triangle sample ≤ 5% | PEP -1.4% maxFruBP -2.5% maxATP 2.4% max | **Passed****Passed****Passed** |

* **Data extraction and Quality**

The data extraction of the raw mass spec data files yielded information that could loaded into a relational database. Peaks were identified using IsoCor peak integration software8.

* **Normalization**

Data correction was performer to correct variation resulting from the difference of cell number into each condition. For abundance interpretation, sample P3 LDHA/B at 24 h and 48 h were 2-times concentrated.

**Metabolomics Supplemental Method 2: [13C]6 Lactate – 13C isotopic profiling**

* **Sample preparations**

Metabolite extraction was performed using 80% methanol and 0.2% of myristic acid d27 (internal standard). After 5 minutes of incubation cells were scraped and collected in a new tube. Following a centrifugation at 20’000 G for 10 minutes at 4°C, the supernatant was transferred to a new vial for MS analysis. Pellet was used for protein quantification.

* **Liquid chromatography / Mass Spectrometry analysis**

10 µl of each sample was loaded into a Dionex UltiMate 3000 LC System (Thermo Scientific Bremen, Germany) equipped with a C-18 column (Acquity UPLC -HSS T3 1. 8 µm; 2.1 x 150 mm, Waters) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) operating in negative ion mode. A step gradient was carried out using solvent A (10 mM TBA and 15 mM acetic acid) and solvent B (100% methanol). The gradient started with 0% of solvent B and 100% solvent A and remained at 0% B until 2 min post injection. A linear gradient to 37% B was carried out until 7 min and increased to 41% until 14 min. Between 14 and 26 minutes the gradient increased to 100% of B and remained at 100% B for 4 minutes. At 30 min the gradient returned to 0% B. The chromatography was stopped at 40 min. The flow was kept constant at 250 uL/min at the column was placed at 25°C throughout the analysis. The MS operated in full scan mode (m/z range: [70–1050]) using a spray voltage of 3.2 kV, capillary temperature of 320 °C, sheath gas at 10.0, auxiliary gas at 5.0. The AGC target was set at 3e6 using a resolution of 140.000, with a maximum IT fill time of 512ms. Data collection was performed using the Xcalibur software (Thermo Scientific). The data analyses were performed by integrating the peak areas (El-Maven – Polly - Elucidata).

**ELISA VEGF**

Supernatants were collected from all cell lines and a VEGF Human ELISA Kit (Thermo Fisher) and procedure was followed based on manufacturer instructions.

**Supplementary references**

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