PFKFB3-mediated Pro-glycolytic Shift in Hepatocellular Carcinoma Proliferation

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SUMMARY

The metabolic regulator PFKFB3 closely modulates lactate flux in hepatocellular carcinoma (HCC). Targeted inhibition was effective in preventing a switch to high-grade HCC, a process trackable with in vivo h13C magnetic resonance imaging. This offers a potential new theragnostic strategy for HCC.

BACKGROUND & AIMS: Metabolic reprogramming, in particular, glycolytic regulation, supports abnormal survival and growth of hepatocellular carcinoma (HCC) and could serve as a therapeutic target. In this study, we sought to identify glycolytic regulators in HCC that could be inhibited to prevent tumor progression and could also be monitored in vivo, with the goal of providing a theragnostic alternative to existing therapies.

METHODS: An orthotopic HCC rat model was used. Tumors were stimulated into a high-proliferation state by use of off-target liver ablation and were compared with lower-proliferating controls. We measured in vivo metabolic alteration in tumors before and after stimulation, and between stimulated tumors and control tumors using hyperpolarized 13C magnetic resonance imaging (MRI) (h13C MRI). We compared metabolic alterations detected by h13C MRI to metabolite levels from ex vivo mass spectrometry, mRNA levels of key glycolytic regulators, and histopathology.

RESULTS: Glycolytic lactate flux increased within HCC tumors 3 days after tumor stimulation, correlating positively with tumor proliferation as measured with Ki67. This was associated with a shift towards aerobic glycolysis and downregulation of the pentose phosphate pathway detected by mass spectrometry. MRI-measured lactate flux was most closely coupled with PFKFB3 expression and was suppressed with direct inhibition using PFK15.

CONCLUSIONS: Inhibition of PFKFB3 prevents glycolytic-mediated HCC proliferation, trackable by in vivo h13C MRI. (Cell Mol Gastroenterol Hepatol 2022;1– –; https://doi.org/10.1016/j.jcmgh.2022.09.009)

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This is attributed to the necessity of cancers to maintain viability and build new biomass in a nutrient-poor environment.6 Amino acids for protein synthesis, nucleic acid for DNA duplication, and lipids for cell membrane synthesis are all significantly altered to serve HCC proliferation and metastasis.5,7 Metabolic pathways may offer targets for HCC tracking or therapy, but it remains unclear what the best diagnostic or treatment approaches are.

The tendency of cancer cells to favor aerobic glycolysis, known as the Warburg effect, is relatively inefficient but provides energy rapidly, resulting in high lactate production.8-10 Pyruvate is located at an intersection of key pathways of metabolism. Intracellular pyruvate can be converted to acetyl Co-A to enter tricarboxylic acid cycle, lactate (in part due to the Warburg effect), or alanine via transamination. Hyperpolarized $^{13}$C magnetic resonance imaging ($^{13}$C MRI) enables in vivo real-time high-resolution imaging of the uptake and metabolism of $^{13}$C-labeled tracer molecules including pyruvate.11 Altered glycolysis was shown in McA-RH7777 hepatomas using in vivo $^{13}$C MRI.12 Latent HCC, in response to chemoembolization, has also demonstrated increased glycolytic dependency and lactate flux relative to alanine.13-15 supporting the notion that glycolysis plays a crucial role in HCC treatment response.

The purpose of our study was to identify targetable factors in the glycolytic modulation of HCC that could also be tracked and monitored in vivo with $^{13}$C MRI, a metabolic imaging modality which is clinically available.11,14,15 To achieve this we used an orthotopic HCC model and exploited a phenomenon whereby tumor proliferation and aggression is stimulated by use of off-target hepatic radiofrequency ablation (RFA).16-18

This effect has been linked to hypoxia-inducible factor 1-alpha (HIF-1a), which is known to transactivate several genes encoding glucose transporters and glycolysis including 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3), HK2, GLUT1, LDHA, and MCT4.19-23 This allows a controlled model using the same cell line and baseline tumors to generate high-grade and low-grade disease states.

We observed that N1S1 tumors with greater proliferation exhibited higher lactate flux as measured by $^{13}$C MRI. We then identified the glycolytic regulator PFKFB3 as having the greatest correlation with lactate flux and proliferation. Lastly, we show suppression of N1S1 proliferation and lactate flux with targeted PFKFB3 inhibition. Our results show that glycolytic activity and suppression with PFKFB3 targeting are both trackable in vivo with $^{13}$C MRI.

**Results**

**Off-target Ablation Stimulates N1S1 Proliferation**

Off-target hepatic RFA has been previously linked to increased growth of untreated and distal tumors,24 and recurrent HCC in patients following RFA demonstrate higher grade disease associated with lower overall survival.25 We found that the tumor volume change following RFA stimulation was significantly greater than in a control group (stimulation 1.7 ± 0.42 vs control 0.89 ± 0.21; P = .029; n = 4) (Figure 1). This is concordant with studies on other tumor models.26

**Increased Glycolysis in Stimulated N1S1 HCC is Detectable in Vivo by $^{13}$C MRI and Correlates With Greater Tumor Proliferation**

To understand the early changes of metabolism in tumor following stimulation, we measured metabolic alteration in rat liver in vivo at baseline and 3 days following off-target RFA (stimulated, high-grade HCCs) or a sham RFA (control, low-grade HCC) with $^{13}$C MRI. Example images of $^{13}$C-pyruvate and $^{13}$C-lactate signal within tumors are shown in Figure 2, A. $^{13}$C MRI was successful in all 12 rats/tumors at both baseline (before RFA or sham) and post-stimulation (after RFA or sham) timepoints with the exception of a single control rat post sham, where the spectroscopic data was only partially captured and could not be reliably compared with the other data; this MRI data point was therefore excluded from analyses. In tumors, the maximum signal of $^{13}$C-pyruvate was approximately 6 seconds after injection, whereas the maximum $^{13}$C-lactate peak occurred at approximately 12 seconds (Figure 2, B). $^{13}$C lactate flux was greater in N1S1 tumors compared with normal livers (lactate to pyruvate ratio [LPR] 0.51 ± 0.17 vs 0.25 ± 0.048; P = .0006; n = 11) (Figure 2, C), as well as $^{13}$C-bicarbonate flux (bicarbonate to pyruvate ratio [BPR] 0.03 ± 0.02 vs 0.002 ± 0.01; P = .0029).

**Figure 1. RFA of the liver promotes off-target HCC tumor growth.** The volume change between baseline and 7 days post-stimulation/control ($\Delta$Volume = Volume after - Volume before) in both stimulated (STI) and control/sham (Ctrl) group. The comparison was performed with a Mann-Whitney test.

**Abbreviations used in this paper:** ANOVA, analysis of variance; APR, alanine to pyruvate ratio; BPR, bicarbonate to pyruvate ratio; h13C MRI, hyperpolarized $^{13}$C magnetic resonance imaging; HCC, hepatocellular carcinoma; HIF-1a, hypoxia-inducible factor 1-alpha; LPR, lactate to pyruvate ratio; mRNA, messenger RNA; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PPP, pentose phosphate pathway; RFA, radiofrequency ablation; ROI, region of interest.

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$P = .0152; n = 11$) (Figure 3, A), whereas $^{13}\text{C}$-alanine flux was lower (alanine to pyruvate ratio [APR] $0.16 \pm 0.05$ vs $0.27 \pm 0.07; P = .0008; n = 11$) (Figure 3, B). To track the evolution of the glycolytic state of N1S1 tumors following stimulation from off-target hepatic RFA, we compared $^{13}\text{C}$-lactate, $^{13}\text{C}$-alanine, and $^{13}\text{C}$-bicarbonate flux in HCC tumor before (baseline) and 3 days after stimulation with $^{13}\text{C}$ MRI. Tumor $^{13}\text{C}$-lactate flux was increased in the stimulated tumors 3 days post-stimulation (LPR $0.47 \pm 0.16$ vs $0.74 \pm 0.12; P = .007; n = 6$) but not in controls ($0.55 \pm 0.18$ vs $0.56$).
Figure 3. $^{13}$C alanine and $^{13}$C bicarbonate flux in N1S1 tumor. A–B, Bicarbonate flux (BPR) (A) and alanine flux (APR) (B). T, tumor, NL, normal liver. C–D, APR in tumor (C) and normal liver (D) from baseline to 3 days post-stimulation/control. E–F, BPR in tumor (E) and normal liver (F) from baseline to 3 days post-stimulation/control. The $P$ value was evaluated by paired t test. G–H, Correlation between Ki67 expression and post-stimulation APR (G) or post-stimulation $\Delta$APR (post-stimulation APR – baseline APR) (H). I–J, Correlation between tumor volume change $\Delta$Volume and post-stimulation APR (I) or post-stimulation $\Delta$APR (J).

Ki67 expression of the background hepatic parenchyma between the RFA-stimulated or control group, confirming that the RFA-stimulation specifically affected tumor proliferation rather than globally. Mean tumor volumes did not differ at 3 days, although Ki67 expression in N1S1 tumors was positively associated with tumor volume (Figure 4, B–C). We then compared tumor $^{13}$C-lactate and $^{13}$C-alanine flux with Ki67 expression to see if imaging biomarkers were associated with tumor proliferation. Ki67 expression trended positively with $^{13}$C-lactate flux measured in stimulated tumors (Figure 5, A), whereas there was no correlation between Ki67 expression and $^{13}$C-alanine flux (Figure 3, G). When comparing tumor Ki67 expression with the net change in lactate or alanine flux ($\Delta$LPR = post-stimulation LPR – baseline LPR, and $\Delta$APR = post-stimulation APR – baseline APR), Ki67 expression positively correlated with $\Delta$LPR (Figure 5, B) and negatively with $\Delta$APR (Figure 3, H). Ki67 expression was also positively correlated with lactate flux within the liver parenchyma (Figure 3, C–D). $^{13}$C-bicarbonate flux was no different between baseline and stimulated N1S1 tumors or within the liver parenchyma (Figure 3, E–F). Lactate flux as measured using $K_{PL}$ also showed increased lactate flux in the stimulated tumors compared with controls (0.03 ± 0.01 vs 0.04 ± 0.01; $P = .03$; n = 6) (Figure 2, F). No change was seen in tumor $R_{1eff}$ (Figure 2, G).

Next, we compared the proliferation of implanted N1S1 liver tumors 3 days following stimulation. Ki67 expression was significantly greater in the stimulated group relative to control (stimulated 54.13 ± 10.14 vs control 32.82 ± 13.24; $P = .0152$; n = 6) (Figure 4, A). There was no difference in $^{13}$C lactate flux in tumor, NL, normal liver. C–D, APR in tumor (C) and normal liver (D) from baseline to 3 days post-stimulation/control. E–F, BPR in tumor (E) and normal liver (F) from baseline to 3 days post-stimulation/control. The $P$ value was evaluated by paired t test. G–H, Correlation between Ki67 expression and post-stimulation APR (G) or post-stimulation $\Delta$APR (post-stimulation APR – baseline APR) (H). I–J, Correlation between tumor volume change $\Delta$Volume and post-stimulation APR (I) or post-stimulation $\Delta$APR (J).
Figure 4. Stimulated normal liver by stimulation promotes HCC tumor proliferation. A, Ki67 expression in orthotopic tumors was examined by immunohistochemistry in both stimulated (STI) and control/sham (Ctrl) group. Representative images were provided as indicated. B, The volume change between baseline and 3 days post-stimulation/control (ΔVolume = Volume after - Volume baseline) in both stimulated and control/sham group. Analyses were performed using the Mann-Whitney test. C, Correlation of Ki67 expression and volume change.

Quantitative Metabolome Profiling of N1S1 Matches in vivo h13C MRI

We performed quantitative metabolome profiling of post-stimulated N1S1 tumors using liquid chromatography-mass spectroscopy to compare the steady-state lactate and alanine levels with their respective flux observed with h13C MRI. Two hundred ninety-one metabolites were detected. Multivariate analysis showed a clear separation of metabolic features for N1S1 tumors in the stimulated vs control groups (Figure 6, A). The Mann-Whitney U test was used to identify metabolites that were significantly altered in stimulated tumors as compared with control, with a heat map was generated (Figure 6, B). Fifty-one metabolites levels were significantly altered in stimulated tumors as compared with control, with 14 upregulated and 37 downregulated (Figure 7, A). Lactate levels in the stimulated tumors were elevated relative to the control group, consistent with h13C MRI (stimulated 1.5E-08 vs control 1.4E-08; P = .05; n = 9) (Figure 7, B). No difference was seen in alanine levels between stimulated and control groups (stimulated 4.0E-07 vs control 3.6E-07; P = .13; n = 9) (Figure 7, C).

Enrichment analysis was performed with Metaboanalyst 5.0 to differentiate metabolites between stimulated tumors and controls, linking to the Kyoto Encyclopedia of Genes and Genomes metabolism pathway maps. Glycolysis/gluconeogenesis, the pentose phosphate pathway (PPP), and arginine biosynthesis were identified as major pathways (Table 1). In the glycolysis pathway, the expression levels of all intermediate metabolites of glycolysis were decreased in stimulated tumors, whereas glycolysis-produced lactate was increased (Figure 7, D). In the PPP, a metabolic pathway parallel to glycolysis, 26,29 7 involved metabolites were decreased in stimulated tumors compared with controls (Figure 7, E). Collectively, when stimulated, the glycolysis pathway was upregulated in N1S1 tumors, while shunting metabolism towards PPP, concurrent with our observations from h13C MRI.

Overexpression of PFKFB3 Confers Tumor Aggression Via Glycolysis

The switch from PPP to glycolysis is a common response mechanism to hypoxia in tumor cells and normal cells.20,31
Off-target hepatic RFA induces inflammation at the periablational liver parenchyma, resulting in a global proliferative effect. To elucidate the mechanisms underlying this metabolic response in tumors, we assessed the messenger RNA (mRNA) expression of several genes involved in pyruvate and lactate metabolism and transport from the harvested tumors (Figure 8, A). The expression of HIF-1α was significantly increased within the hepatic tissue along the rim of the ablation, also known as the periablation zone (Figure 8, B), simulating hypoxic-like induction. Indeed, the expression of glycolysis-related genes GLUT1, PKM, and HK2 were significantly elevated at the periablation zone and within N1S1 tumors following stimulation, concordant with HIF-1α expression (Figure 8, C–E). In addition, GTP1 (gene, alanine aminotransferase 1) expression was markedly decreased at the periablation zone, corresponding to deceased tumor alanine flux within stimulated tumors as measured by h13C MRI (Figure 8, F). The expression of PFKFB3 was also assessed by immunohistochemical staining (Figure 9, B), showing greater

**Figure 5.** Correlation between tumor growth and 13C-lactate flux post-stimulation. A–B, There is positive correlation between Ki67 expression and post-stimulation LPR and ΔLPR (post-stimulation LPR – baseline LPR). C–D, There is positive correlation between Ki67 expression and post-stimulation KPL and ΔKPL (post-stimulation KPL – baseline KPL). E–F, There is positive correlation between tumor volume change ΔVolume and post-stimulation LPR and ΔLPR. G–H, There is positive correlation between tumor volume change ΔVolume and post-stimulation KPL and ΔKPL.

**Figure 6.** Metabolic profiles of N1S1 tumors. A, Multivariate analysis of metabolomic data using 3D PLS-DA model. B, Total heat map demonstrating contrasting metabolic profiles of N1S1 tumors of control vs post-stimulated groups.
PFKFB3 production within tumors following stimulation as compared with control (Figure 9, C). Notably, PFKFB3 expression was markedly high at the RFA periablational zone, while absent within normal liver parenchyma (Figure 9, D). PFKFB3 expression in N1S1 tumors was positively correlated with Ki67 expression (Figure 9, E). We then compared \(^{13}\text{C}\) lactate flux with tumor PFKFB3 expression to see if the imaging biomarkers were associated with PFKFB3 expression. Tumor PFKFB3 expression was positively correlated with both \(K_{\text{PL}}\) and \(\Delta K_{\text{PL}}\). A similar trend was seen with LPR, although not statistically significant (Figure 9, F–I).

**PFKFB3 Inhibition With PFK15 Suppresses Glycolytic Alteration in N1S1 Tumor**

With the observed correlation between Ki67 expression and PFKFB3 in N1S1, we tested the reliance of PFKFB3 for N1S1 proliferation by assessing response of tumors from heat treatment in vitro or off-target hepatic RFA in vivo in combination with PFK15, a highly selective small molecule inhibitor of PFKFB3.\(^{33,34}\)

To mimic the marginal zone of RFA treatment in vitro, we tested the response of N1S1 cells in response to heat stress in vitro. Annexin V-PI staining showed that N1S1 cells exposed to 45 °C for 5 minutes resulted in 80.9% survival...
after 24 hours without signs of apoptosis, vs 34.3% at 50 °C, and 0% at 55 °C (Figure 10, A). Three to 10 days following heat treatment, the proliferation rate of N1S1 within the 50 °C-treated group was significantly greater compared with the controls at 37 °C (2-way analysis of variance [ANOVA], 48 hours P = .0004; 72 hours P < .0001) (Figure 10, B). Heat-treated N1S1 cells in vitro demonstrated increased cellular proliferation measured by MTT assay without PFK15 or at low doses (1 nmol/L and 2 nmol/L), with greater proliferation at 45 °C and 50 °C as compared with controls at 37 °C (2-way ANOVA, 1 nmol/L 37 °C vs 45 °C P = .02; 37 °C vs 50 °C P = .002; 2 nmol/L 37 °C vs 50 °C P = .008). However, at effective concentrations of PFK15 at 10 nmol/L, N1S1 proliferation was suppressed at all 3 temperatures (Figure 10, C).

In vivo h13C MRI showed the LPR and KPL were elevated after stimulation, whereas the increase of LPR and KPL was inhibited by PFK15 administration (Figure 11, A–B). PFK15 suppressed the elevation of PFKFB3 in N1S1 tumors following stimulation in both mRNA level and protein level (Figure 11, C–D). Taken together, these results suggest that PFK15 can suppress glycolysis upregulation, supporting the notion that PFKFB3 may be a treatment target for HCC.

**Discussion**

Metabolic reprogramming is a characteristic feature of cancer cells, and in cancers, metabolic pathways may undergo alterations constantly. Enhancement of glycolysis and lactate accumulation in the interstitial space primarily serves the purpose of building and preserving a protumorigenic environment, suggesting that lactate signaling maybe serve as an early indicator for disease progression. Prior studies have supported the safety and feasibility of in vivo h13C MRI, with the real-time pyruvate, lactate, and alanine signal detectable in different tissues, including prostate, liver, kidney, and brain. In this study, we used h13C MRI to assess HCC metabolism and found a strong correlation between measured in vivo lactate flux and tumor proliferation before detectable volumetric change. h13C MRI may therefore provide a noninvasive way to obtain earlier insight into transformation of tumors into a higher grade.

Using a model of stimulated HCC, we found that increased lactate flux in HCC tumors was detectable in vivo by h13C MRI 3 days after stimulation, correlating with proliferation as detected by Ki67 expression, ahead of

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**Table 1. Altered Metabolic Pathways Between RFA Tumor Group and Control Tumor Group**

<table>
<thead>
<tr>
<th>Metabolite set</th>
<th>P value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPP</td>
<td>1.04E-07</td>
<td>8.71E-06</td>
</tr>
<tr>
<td>Arginine biosynthesis</td>
<td>6.38E-04</td>
<td>2.56E-02</td>
</tr>
<tr>
<td>Glycolysis/glucogenesis</td>
<td>9.15E-04</td>
<td>2.56E-02</td>
</tr>
</tbody>
</table>

FDR, ; PPP, pentose phosphate pathway; RFA, radiofrequency ablation.

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**Figure 8. mRNA expression of glycolysis-related genes in stimulated/non-stimulated N1S1 tumors and liver tissue.** A, Representative gene expression patterns in tumor (T), liver tissue adjacent to RFA/sham site (periablational) (P), and normal liver (N). B–F, Relative HIF-1α (B), GLUT1 (C), PKM (D), HK2 (E), and GPT1 (F) gene expression levels within tumor, periablational, and normal liver, in stimulated (STI) and control (Ctrl) groups. Densitometry quantification of band intensity is presented as a percentage of relative densitometry normalized to the CYP A gene. Analyses were performed using 2-way ANOVA.
changes in tumor volume which are detectable only at a later time. Meanwhile, the effective lactate relaxation ($R_{1\text{eff}}$), a product of MR parameters such as flip angle and $T_1$ relaxation, remained unchanged, confirming that the changes in measured lactate flux were not due to technical variations such as surface coil inhomogeneity.

The metabolic phenotype of cancer cells changes in response to shifting levels of oxygen and nutrients during growth.38,39 Glycolysis and PPP are parallel metabolic pathways that catalyze glucose and support cell survival and proliferation. In this study, we showed that tumor aggressiveness was associated with a shift towards aerobic glycolysis and downregulation of PPP in N1S1 HCC tumors. Hypoxia is known to induce a similar shift towards glycolysis in a broad spectrum of cancer and nonneoplastic cells.9,40 This shift, coupled with increased glucose uptake, is used by proliferating cells to convert macromolecules needed to construct new cells.9,40

Glucose metabolism consists of highly interconnected pathways, and the one of the key junction biomolecules between glycolysis and PPP is glucose-6-phosphate. Upon entering cells, glucose is rapidly phosphorylated to glucose-6-phosphate and subsequently metabolized towards glycolysis or the PPP. PFKFB3 is involved in this commitment towards glycolytic flux. Its product, fructose-2,6-bisphosphate, is a known potent allosteric activator of 6-phosphofructo-1-kinase, which in turn catalyzes commitment to glycolysis.41 PFKFB3 overexpression has been detected in several different tumor cells as well as various cells within the tumor microenvironment, and plays an important part in oncogenesis and the survival and proliferation of cancer cells.42,43 In our study, PFKFB3 expression was increased in stimulated N1S1 tumors. PFKFB3 expression was also associated with lactate flux as measured by $^{13}$C MRI, suggesting $^{13}$C MRI may guide the use of PFKFB3 inhibitors as an alternative or adjunct therapy. This is supported by our in vitro and in vivo studies where PFKFB was directly inhibited with PFK15, resulting in the suppression of proliferation and MRI-measured lactate flux in stimulated tumors. PFKFB3 may therefore be a potential therapeutic...
target in HCCs where glycolysis is the primary metabolic driver. However, glycolytic suppression may be compensated effectively by alternate pathways. Indeed, the PPP and glycolysis share glucose-6-phosphate such that increased glycolysis during reperfusion concomitantly leads to decreased dependency on the PPP.\textsuperscript{30} PFKFB3 inhibition has also been shown to redirect glucose from the glycolysis pathway to the PPP, which can lead to treatment resistance.\textsuperscript{14} Future studies are needed to see if this occurs and if concurrent PPP blockade may enhance therapeutic response.

Interestingly, alanine flux detected by $^{13}$C MRI was significantly lower in HCC tumor compared with normal liver, with a further significant decrease following stimulation. Our MRI results are concordant with a previous $^{13}$C MRI study, which reported absence of alanine flux in normal liver tissues, significantly increased alanine flux in precancerous tissues, and decreased alanine flux in established liver tumors.\textsuperscript{45} However, our measured change in tumor alanine flux following stimulation did not correlate with Ki67 expression and was not matched by the ex vivo metabolomics analysis. This can be explained by the fact that alanine levels measured by $^{13}$C MRI reflect conversion from injected pyruvate taken into the tumor cells, whereas the absolute value of alanine detected by mass spectroscopy does not discriminate the source, which can be exogenous (eg, muscle). This notion is supported by studies that have shown that exogenous alanine may be used as an alternative metabolic pathway.\textsuperscript{46}

In this study, we showed that N1S1 HCC proliferation is associated with a shift towards aerobic glycolysis and downregulation of PPP via up-regulation of PFKFB3 expression, with the corresponding glycolytic changes detectable by $^{13}$C MRI.

### Materials and Methods

#### N1S1 Cell Line

The rat hepatocellular carcinoma cell line N1S1 (ATCC, CRL-1604\textsuperscript{TM}, male) was obtained from ATCC (Manassas, VA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO2 and grown in Iscove’s Modified Dulbecco’s Medium (GIBCO) and 1% antibiotic-antimycotic (100×) (GIBCO).

#### Animal Models

Animal studies were performed under a protocol approved by the institutional animal care and use committee, in accordance with the guidelines of the Institute of Laboratory Animal Resources, National Research Council. The 2 rats were housed in a temperature-controlled (22 ± 1°C) animal facility with a 12-hour light/dark cycle. The general health of the rats was monitored daily for any signs of suffering or abnormal behavior.

A total of 15 adult female Fischer (CDF) rats (Charles River, Germantown, MD) weighing 120 to 150 g were used in this study. N1S1 cells (100/50 μl) were implanted into left hepatic lobe of rats. When tumors reached 8 to 10 mm,
12 rats were randomized into sham (control) group vs stimulated group (6 each).

All rats underwent laparotomy, which was performed under anesthesia using aseptic techniques. The right hepatic lobe was exposed, and a radiofrequency probe was placed on the liver surface. RFA (stimulated) or sham (control) was then performed at the surface of the right hepatic lobe (see next section). h13C MRI was performed prior to RFA/sham (baseline) and 72 hours post-RFA/sham.

All tumors were harvested immediately after the final h13C MRI study (ie, 3 days post-stimulation/sham) and stored at −80 °C for histologic, metabolomic, and mRNA analysis.

Radiofrequency Ablation and Sham Procedures

A monopolar RFA generator (500 kHz RF generator, Radionics, Model CC-1) was used to energize the probe tip at 70 °C for 5 minutes while it rested on the surface of the right hepatic lobe. In sham procedures performed on control rats, all the steps of RFA were performed, including resting of the probe on the liver surface for 5 minutes, except the probe was not energized.

Hyperpolarized 13C MRI

Rats were anesthetized with isoflurane (4% induction, 2.5%–3% maintenance) with oxygen (700 mL/minute) and the breathing rate was maintained at 80 ± 15 breaths per minute. Core body temperature was maintained at 36 ± 1 °C with a feedback-controlled warm air circulator equipped with a fiber-optic temperature probe. The lateral tail vein was cannulated with a 24-gauge flexible catheter connected to an 84-cm extension tube with 0.5 mm inside diameter (SAI Infusions, Lake Villa, IL). Hyperpolarized 13C pyruvate solution was prepared by dynamic nuclear polarization using a commercial polarizer (Hypersense, Oxford Instruments Molecular Biotools). [1-13C] pyruvic acid was combined with 15 mM OX063 radical (GE Healthcare, London, UK) and 1 mM gadoteridol (ProHance, Bracco, Milan, Italy) and polarized for 40 minutes or more at 1.4 K and 100 mW microwave power. The hyperpolarized material was then dissolved in saline containing 50 mM TRIS and 125 mg/L EDTA and adjusted to physiological pH with sodium hydroxide to obtain a 96 mM hyperpolarized pyruvate solution. A total of 2.5 mL was administered via tail vein as a bolus injection lasting approximately 10 seconds. Imaging was performed on a 9.4-T small animal MRI (Biospec 94/20, Bruker) with an 84-mm quadrature proton coil and an in-house built transmit/receive 13C surface coil built on a 65-mm cylindrical form with an axial length of 60 mm and a circumferential width of 50 cm. Animals were carefully situated with tumors within 5 mm of the center of the coil during imaging to minimize the effects of the surface coil sensitivity profile. 13C data were acquired in the axial plane across the middle of the tumor using an echo planar spectroscopic imaging non-gated sequence with a 4° tip angle.
Sixty-four repetitions were acquired with 2.16 seconds temporal resolution. Echoplanar spectroscopic imaging was initiated immediately prior to infusion of pyruvate solution to enable measurement of the entire time course of the pyruvate and lactate/alanine signals. A [1-13C] acetate phantom was placed adjacent to the animal on the opposite side of the surface coil. The chemical shift of acetate is approximately 0.5 ppm from lactate, enabling ready identification of pyruvate and lactate. The chemical shift assignments were confirmed by observing the large intravascular 13C signal, which at early time points is dominated by pyruvate.

Conventional 1H MRI was performed every 5 days to track tumor growth using the same magnet, with an 84 mm quadrature coil. T2-weighted axial, coronal, and sagittal sequences were acquired using the rapid imaging with refocused echoes sequence with the following parameters: 6 cm field of view, 128 × 128 matrix, echo train length of 8, TR 1245 ms, TE 11 ms, 2 mm slice thickness.

To quantify 13C-pyruvate to 13C-lactate, 13C-alanine, and 13C-bicarbonate flux, we integrated across each spectral peak and across all time points within a manually drawn region of interest using the 1H anatomical images as guidance. Lactate, alanine, and bicarbonate flux were measured as the ratio of the integrated signal of each species relative to pyruvate, expressed as LPR, APR, and BPR, respectively.

**MRI Reconstruction and Analysis**

Spectroscopic image data were separated into even and odd echoes, apodized with a 30-Hz line-broadening filter and reconstructed with standard Fourier methods to obtain pixel-by-pixel spectra with 2 kHz spectral width. Spectra from even and odd echoes were then combined after application of a first-order phase to account for the temporal offset between the starting points of the 2 echo trains.

Pixel-by-pixel magnitude spectra were computed, and metabolite signals were quantified by integrating their spectral peaks, after subtracting the mean magnitude noise from the spectra to eliminate spurious baseline signals.

Magnitude noise was estimated from a noise-only spectrum. Metabolite images were resliced to 128-by-128 matrices by zero fill interpolation and matched to the anatomical proton images. Regions of interest (ROIs) covering the tumors were drawn on the anatomical proton images, and metabolite signals were obtained from the sum of the pixel intensities over the ROIs in the resized 13C images. An ROI was drawn within the right hepatic lobe (the tumors having been implanted in the left lobe) to obtain background liver measurements. LPRs were computed using the time integrals of each metabolite’s signal, a measure that has been shown to reflect the rate of label exchange between pyruvate and lactate. This analysis was repeated to compute the APR and the BPR.

Kinetic modeling of lactate formation was performed fitting the measured lactate and pyruvate time courses to a simplified 2-site exchange model. In the model, the lactate signal \( L(t) \) is determined from the pyruvate signal \( P(t) \) in terms of a forward rate constant \( k_{PL} \) and an effective decay rate \( R_1^{off} \) according to

\[
\frac{dL(t)}{dt} = k_{PL}P(t) - R_1^{off}L(t)
\]

where \( R_1^{off} \) includes the effects of tracer outflow, T1 decay, and saturation by RF pulses. This equation was solved by standard methods using the measured pyruvate signal, and the parameters \( k_{PL} \) and \( R_1^{off} \) were determined by a least-squares fit of this solution to the measured lactate signal.

**Stimulated N1S1 and PFKFB3 Suppression With PFK15**

An additional 3 rats were implanted with N1S1 in the same fashion as detailed above, and when tumors reached 8 to 10 mm, these rats were treated with the same protocol as for the stimulated group (right hepatic lobe RFA) with the addition of treatment with PFK15 using a previously-established dose (Selleck, 25 mg/kg intraperitoneal). As with the other rats, h13C MRI was performed at baseline and again 72 hours post-stimulation.

**In Vitro Heat Treatment Assays**

Suspended N1S1 cells were collected in a 15 mL tube in 3 mL of medium (5 × 10⁵ cells), and immediately exposed to 37 °C, 45 °C, 50 °C, 55 °C, 60 °C, and 70 °C water-bath for 5 minutes. Cells were then seeded into 75-cm² cell-culture flasks in 18 mL of Iscove's Modified Dulbecco's Medium and maintained at 37 °C.

For cell death analysis, cells were harvested and washed once with phosphate buffered saline, and then resuspended in PI/Annexin-V solution (ab176749, Abcam) for apoptosis analysis. At least 10,000 cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed by using FlowJo software.

For in vitro proliferation measurements, N1S1 (10⁵ cells/well) from each of the different heat-treated groups were plated in 96-well cell culture plates. After 2 to 5 days, 10 μL MTT (5655, Sigma, 5 mg/mL, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added to each well, incubated for 4 hours at 37 °C, and 150 μL of DMSO (BP231-100, Fisher Scientific) were then added to each well to dissolve the crystal formazan dye. Absorbance was measured at 570 nm on an enzyme-linked immunosorbent assay reader. For relative quantification, the value of absorbance in each group was normalized to that in the 37 °C group.

**Quantitative Polar Metabolomic Profiling**

Metabolomics analyses were performed at the mass spectrometry (proteomics/metabolomics) core facility. Tumor xenografts were extracted immediately after the last h13C MRI examination and kept at liquid nitrogen until extraction, and metabolites were extracted according to an established protocol. Data were analyzed by R (RStudio software).
**Table 2. Primer Sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' to 3')</th>
<th>Reverse (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CypA</td>
<td>TATCTGCAGTGGCAAGACCTGAGTG</td>
<td>CTCTTGCTGGCTTGGCATTCC</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>TGTTGATGCTTATTATGGA</td>
<td>GTGCAAGTGTGAGGCTCCA</td>
</tr>
<tr>
<td>PKFB3</td>
<td>TTGCTACGTCACAGGCC</td>
<td>GCGGACTCCACAGACAGAA</td>
</tr>
<tr>
<td>GLUT1</td>
<td>GCTGTTGCTGTCCTCTCTAA</td>
<td>CGAGCAAGGCTTCTGCAAGG</td>
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<tr>
<td>PKM</td>
<td>TCATACAGAGAAATCGAGACCA</td>
<td>CCACGAGCTACATGTTCTCA</td>
</tr>
<tr>
<td>HK2</td>
<td>TCAGCCCAAGACTCTTTACCA</td>
<td>GAATTCACTTATCTGTTCAA</td>
</tr>
<tr>
<td>GPT1</td>
<td>AGGACACGCTTGTAGCGGAG</td>
<td>TGCAGCCATGAGCCTTAG</td>
</tr>
</tbody>
</table>

CYPΑ, Cyclophilin A; HIF-1α, hypoxia-inducible factor 1-alpha; PKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3.

**Total RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR (RT-qPCR)**

Frozen rat tissues (including tumor, ablation zone, and normal liver) were ground with a pestle in liquid nitrogen to obtain homogeneous tissue powder. Cells were collected and washed with phosphate buffered saline. Total RNA was extracted from the tissue powder or cell pellets using the RNeasy Mini kit (74134, Qiagen) and reverse transcribed into cDNA using iScript gDNA Clear cDNA Synthesis Kit (1725035, BIO-RAD). The level of mRNA expression was normalized to the housekeeping gene cyclophilin A. The mRNA expression of HIF-1α, GLUT1, HK2, PKFB3, PKM, and GPT1 were measured. The primer sequences are listed in Table 2.

**Histologic Analysis**

Liver tissue samples were fixed in 10% neutral formalin and paraffin-embedded section were stained with hematoxylin and eosin to analyze the histology of samples. Immunohistochemistry staining was performed on paraffin-embedded liver tissue sections. We used the following primary antibodies: PKFB3 (NOVUS Biologicals, NB2-75393, 1:200) and Ki67 (Cell Signaling, CST12202S, 1:1000). Staining was visualized using Goat anti-Rabbit IgG (Abcam, ab214880) conjugated with HRP polymer and 3,3-diaminobenzidine (Vector lab). Slides were scanned on an Axio Scan.Z1 (Zeiss) and the whole mount digitalized at 10× magnification. Three regions in each sample were randomly selected and analyzed using ImageJ.

**Statistical Analysis**

Statistical analysis was performed using Prism 9 (GraphPad). Comparisons between hyperpolarized 13C MRI and Ki67 expression were performed using 2-way ANOVA. Data are deemed to be statistically significant for p < .05. Errors are reported as standard error of means unless otherwise indicated.

**References**


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Qianhui Dou (Conceptualization: Supporting; Data curation: Lead; Formal analysis: Supporting; Funding acquisition: Supporting; Methodology: Supporting; Supervision: Supporting; Writing – review & editing: Supporting); Aaron K. Grant (Formal analysis: Supporting; Funding acquisition: Supporting; Methodology: Supporting; Supervision: Supporting; Writing – review & editing: Lead); Imad Nasser (Formal analysis: Supporting); Marwan Moussa (Methodology: Supporting; Resources: Supporting); Cody Callahan (Formal analysis: Supporting; Methodology: Supporting; Software: Supporting; Supervision: Supporting; Writing – review & editing: Supporting); Antoniu Groza (Formal analysis: Supporting; Methodology: Supporting; Software: Supporting); Qiong Peng (Formal analysis: Supporting; Funding acquisition: Supporting; Methodology: Supporting; Software: Supporting); Pedro Rocha (Formal analysis: Supporting; Methodology: Supporting; Software: Supporting); Patricia Coutinho de Souza (Funding acquisition: Supporting; Methodology: Supporting; Software: Supporting); Leo L. Tsai (Conceptualization: Lead; Formal analysis: Supporting; Funding acquisition: Supporting; Methodology: Supporting; Software: Lead; Supervision: Lead; Writing – review & editing: Lead).

Conflicts of interest
The authors disclose no conflicts.

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