The SIRT1/HIF2α Axis Drives Reductive Glutamine Metabolism under Chronic Acidosis and Alters Tumor Response to Therapy

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Abstract

Extracellular tumor acidosis largely results from an exacerbated glycolytic flux in cancer and cancer-associated cells. Conversely, little is known about how tumor cells adapt their metabolism to acidosis. Here, we demonstrate that long-term exposure of cancer cells to acidic pH leads to a metabolic reprogramming toward glutamine metabolism. This switch is triggered by the need to reduce the production of protons from glycolysis and further maintained by the NAD+-dependent increase in SIRT1 deacetylase activity to ensure intracellular pH homeostasis. A consecutive increase in HIF2α activity promotes the expression of various transporters and enzymes supporting the reductive and oxidative glutamine metabolism, whereas a reduction in functional HIF1α expression consolidates the inhibition of glycolysis. Finally, in vitro and in vivo experiments document that acidosis accounts for a net increase in tumor sensitivity to inhibitors of SIRT1 and glutaminase GLS1. These findings highlight the influence that tumor acidosis and metabolism exert on each other. Cancer Res; 74(19): 5507–19. ©2014 AACR.

Introduction

Acidosis and cycling hypoxia are two well-known physico-chemical properties of the tumor microenvironment (1, 2). The release of lactate (the end product of glycolysis) together with a proton by hypoxic tumor cells but also the hydration of CO2 into bicarbonate and proton (by tumor cells that have access to O2) contribute to the acidification of the tumor microenvironment. Large amounts of lactate/H+ are also released by tumor cells exhibiting aerobic glycolysis (the so-called Warburg effect; refs. 3 and 4). Extracellular pH (pHe) has actually been determined in a wide variety of cancers to be significantly more acidic than in normal tissues, with values ranging from 5.9 to 7.2 (5, 6). Acidosis is known to contribute to the genetic instability of tumor cells (7) and to profoundly alter their transcriptomic profile (8), leading to phenotypes that are particularly suited for survival and growth in an acidic environment. A low pHe has for instance a wide impact on cancer progression by promoting tumor cell migration, invasion, and metastasis (9–11) and by stimulating angiogenesis (12–14). Although tumor metabolic peculiarities directly account for the acidification of the tumor microenvironment, whether acidosis may itself influence metabolism remains elusive. One may however reason that when the extracellular acidic stress increases or persists, reducing the source of H+ by shifting the metabolic preference may also represent an option for tumor cells. Interestingly, although glucose metabolism is generally viewed as the main contributor to acidic tumor pHe, glutamine metabolism is less prone to H+ production. Glutamine can for instance support anaplerosis/cataplerosis without the need for tricarboxylic acid (TCA) cycle to be coupled to OXPHOS and may thus supply tumor cells with ATP as well as a large variety of biosynthetic precursors and redox equivalents without producing carbonic acid (15–17).

Although a few studies have addressed the effects of acute changes in pHe on tumor metabolism (8, 18), interpretation is usually complicated by the overall tumor cell death associated with the rapid exposure of tumor cells to an acidic pH. In this study, we postulated that long-term selection of tumor cells able to survive and proliferate under acidic conditions could offer more relevant models to get insights on the influence of acidosis on tumor metabolism. We found that despite a similar proliferation rate in tumor cells adapted to acidic pH and in parental cells (maintained at pH 7.4), a metabolic shift from a largely glycolytic metabolism toward the reductive glutamine metabolism was actually observed in response to chronic acidotic conditions. The capacity of different tumor cells to develop resistance to the intracellular acidification was associated with an increase in SIRT1-driven protein deacetylation leading to a reduction in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-14-0705 ©2014 American Association for Cancer Research.
HIF1α activity and abundance concomitantly to a net increase in HIF2α activity and expression. Major targets of the latter were identified, including the glutamine transporter SLC1A5 and the glutaminase isoform GLS1, both supporting the preferential glutamine metabolism and a corresponding shift in the response to therapeutic interventions.

Materials and Methods

Cell culture
All cell lines were acquired in the last 3 years from ATCC where they are regularly authenticated by short tandem repeat profiling. Cells were stored according to the supplier’s instructions and used within 6 months after resuscitation of frozen aliquots. SiHa cervix cancer cells, FaDu pharynx squamous cell carcinoma cells, and HCT-116 colon cancer cells were maintained in DMEM supplemented with 25 mmol/L of both PIPES and HEPES to adjust pH to 7.4 or 6.5.

Transfection
Tumor cells were transfected with 50 nmol/L siRNA using Lipofectamine RNAiMAX (Life Technologies). Sequences are described in Supplementary Data. Plasmids encoding Flag-tagged wild-type or mutant H363Y SIRT1 were obtained from Addgene and were transfected with Lipofectamine 2000 (Life Technologies).

Metabolic profiling
Cells were cultured for 24 hours in DMEM supplemented with either 10 mmol/L d-[U-13C]glucose and 2 mmol/L unlabeled l-glutamine, or 10 mmol/L unlabeled d-glucose and 2 mmol/L l-[U-13C]glutamine (CLM-1822; Cambridge Isotope Laboratories). Mass spectrometry measurements were performed by the University of Michigan Metabolomics Resource Core (19). Glucose and lactate concentrations were measured using enzymatic assays (CMA Microdialysis AB) and a CMA 600 analyzer (Aurora Borealis). Glutamine concentration was determined with a colorimetric assay from BioAssay Systems. Intracellular NAD+ and NADH contents were determined by using a dedicated Quantification Kit (Sigma-Aldrich). To measure amino acid uptake, SiHa cells were incubated at 37°C in a Krebs solution containing either 2 μmol/L l-glutamine + l-[3H]-glutamine or 0.8 μmol/L l-leucine + l-[3H]-leucine. Radioactivity was determined in a microplate counter (PerkinElmer Topcount). Oxygen-consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XF96 plate reader.

Western blot analysis
Immunoprecipitation and Western blotting experiments were carried out as reported elsewhere (20); antibodies are listed in Supplementary Data.

Intracellular pH measurements
Cells were loaded with the pH-sensitive dye carboxy-SNARF-1-AM for 30 minutes. After washing, cells were exposed to media buffered at pH 7.4 or 6.5, and SNARF-1 fluorescence was recorded at 580 and 642 nm after excitation at 485 nm, in a Victor X4 fluorescence microplate reader (PerkinElmer).

In vivo experiments
All the experiments involving mice received the approval of the university ethic committee (approval ID 2012/UCL/MD005) and were carried out according to national animal care regulations. Seven-week-old female NMRI nude mice were purchased from Elevage Janvier and 2 × 10⁶ native tumor cells (pH 7.4) or pH 6.5–adapted tumor cells were injected subcutaneously in the left and right flanks, respectively. When tumors reached a mean diameter of 5 mm, BPTES (10 mg/kg) or EX-527 (2 mg/kg) were daily injected intraperitoneally.

Statistical analysis
Results are expressed as mean ± SEM of at least three independent experiments. Two-tailed unpaired Student t test, one-way or two-way ANOVA tests (Bonferroni’s post hoc test) were used where appropriate.

Results

Chronic acidosis causes a shift from glucose to glutamine metabolism in three different tumor cell lines
To study the metabolic adaptation of cancer cells to an acidic environment, we cultured several tumor cell lines in a medium buffered at pH 6.5. We first observed that a 72-hour incubation in the acidic medium led to a significant inhibition of tumor cell growth (see 6.5/acute condition in Fig. 1A). We then maintained tumor cells in this medium (repeatedly renewed) for 8 to 10 weeks until they completely recovered and grew at the same rate as parental cells (see 6.5 vs. 7.4 conditions in Fig. 1A). We carried out this procedure on three different cell lines, namely SiHa, FaDu, and HCT-116 cancer cells, with similar results (Fig. 1A).

We then investigated possible changes in the consumption of either glucose or glutamine in tumor cells adapted to acidic pH 6.5 (called 6.5/tumor cells here below) versus parental cells maintained at pH 7.4 (i.e., 7.4/tumor cells). Interestingly, glutamine consumption was increased (Fig. 1B) whereas glucose consumption and lactate secretion were dramatically reduced (Fig. 1C and D) in the three cell lines chronically cultured at pH 6.5 (see also Supplementary Fig. S1A–S1C). Preference for glutamine over glucose for 6.5/tumor cells was confirmed by culturing tumor cells in media deprived of either fuel. Glutamine deprivation significantly reduced the growth of the 3 6.5/tumor cell lines whereas glucose deprivation was more detrimental for the corresponding parental 7.4/tumor cells (Fig. 1E and Supplementary Fig. S1D). Similarly, inhibition of glucose metabolism by 2-deoxyglucose (2-DG) reduced parental cell growth, by about 50%, whereas it did not or barely affect the growth of 6.5/tumor cells (Fig. 1F and Supplementary Fig. S1E). Conversely, inhibition of glutamine metabolism by using l-γ-glutamyl-p-nitroanilide (GPN), an inhibitor of SLCA15 (ASCT2), the main glutamine transporter in carcinoma cells (21, 22) or by using BPTES, a glutaminase...
(GLS1)-selective inhibitor (23) reduced the growth of 6.5/tumor cells compared with parental cells (Fig. 1F and Supplementary Fig. S1E).

**Chronic acidosis enhances both glutamine-fueled respiration and reductive glutamine metabolism**

Using radiolabeled glutamine, we then confirmed that SiHa/6.5 cells displayed a significantly higher rate of glutamine uptake than parental cells (Fig. 2A). Immunoblotting experiments also showed that the expression of both SLC1A5 and GLS1 was significantly increased (P < 0.01, n = 3; Fig. 2B). Expression of glutaminase GLS2 and glutamine synthetase GS was not altered (Fig. 2B). Inhibition of SLC1A5 expression by gene silencing reduced SiHa/6.5 cell growth by about 50% whereas a limited 20% reduction was found in SiHa/7.4 (Fig. 2C), confirming the results with the pharmacologic inhibitor of glutamine influx, GPNA (Fig. 1F).

Figure 1. Chronic acidosis results in a metabolic switch from glucose to glutamine utilization. A, proliferation of SiHa, FaDu, and HCT-116 cells under acute (gray) or chronic (dashed) acidic pH conditions versus neutral, physiologic pH (black). B, glutamine consumption; C, glucose consumption; D, lactate secretion (during 24 hours) by parental and pH 6.5–adapted tumor cells. Cell growth after 72-hour incubation (E) in media containing either glucose or glutamine or both fuels and following treatment with 10 mmol/L 2-DG, 2 mmol/L GPNA, or 10 μmol/L BPTES (F). Data represent means ± SEM of three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
We also found that SiHa/6.5 cells exhibited higher oxygen consumption rate (OCR) at basal level but also after exposure to glutamine (vs. SiHa/7.4 cells; Fig. 2D); both basal and stimulated OCR were inhibited by mitochondrial respiratory chain inhibitors, rotenone and antimycin A (Fig. 2D). We also found that DM-α-ketoglutarate (αKG), a cell-permeable analog of αKG, could partially restore the extent of SiHa/6.5 cell growth to the level observed in the presence of glutamine (Fig. 2E).

We also incubated cells with [U-13C5]glutamine and analyzed the incorporation in various metabolites using gas chromatography–mass spectrometry. We found that SiHa/6.5 displayed an increase in reductive glutamine metabolism (Fig. 2F), as documented by increases in the m + 3 mass isotopomers of malate and fumarate (Fig. 2G) and in the m + 5 mass isotopomer of citrate (vs. m + 4 citrate; Fig. 2G and H). Interestingly, we also found that isocitrate dehydrogenase-1 (IDH1), the enzyme identified as the main support to glutamine reductive carboxylation (24), was overexpressed in SiHa/6.5 (Fig. 2F). Also, IDH1 gene silencing strongly inhibited the growth of SiHa/6.5 whereas it did not affect SiHa/7.4 cells (Fig. 2G).

We finally examined the glutamine-dependent capture of major amino acids (25). We found that leucine uptake was increased in SiHa/6.5 (Supplementary Fig. S2A). In addition, knocking down the expression of SLC7A5, 1 of the two subunits composing the large neutral amino acids transporter LAT1, significantly reduced the growth of SiHa/6.5 but not of SiHa/7.4 cells (Supplementary Fig. S2B).

**Glycolysis inhibition only partly mimics acidosis-triggered increase in glutamine metabolism**

Using [U-13C5]glucose to probe glucose fate (Fig. 3A), we also showed that the glycolytic flux was strongly reduced in SiHa/6.5 as documented by a decrease in the m + 3 mass isotopomers of pyruvate and lactate (Fig. 3B), confirming the data obtained in Fig. 1D. Reduced glucose uptake in SiHa/6.5 cells also affected oxidative metabolism, as m + 2 mass isotopomers of glucose-derived TCA cycle intermediates, including αKG, succinate or malate, were decreased (Supplementary Fig. S3A). Moreover, the contribution of glucose to cell respiration was significantly reduced in SiHa/6.5 cells despite a basal increase in OCR (Supplementary Fig. S3B). Interestingly, we observed a concomitant decrease in the expression of the glucose transporter GLUT1 and the monocarboxylate transporter MCT4, known to mediate glucose uptake and lactate release, respectively (Fig. 3C).

We then determined if glycolysis inhibition was the trigger of the enhanced glutaminolysis observed in acidic conditions. To address this question, we exposed SiHa cells to the glycolysis inhibitor 2-DG for several weeks in order to isolate tumor cells independent of glucose uptake for growth (SiHa/2-DGR; Fig. 3D). We could document in SiHa/2-DGR cells, a decrease in ECAR, at basal level, but also after glucose treatment, as for SiHa/6.5 cells (Fig. 3E). Of note, an almost complete inhibition of both lactate release and glucose consumption was observed (Supplementary Fig. S3C), indicating that there was no major derivation of glucose toward other pathways. SiHa/2-DGR cells also exhibited an increase in glutamine consumption (vs. SiHa/7.4), although to a lower extent (P < 0.001) than SiHa/6.5 cells (Fig. 3F). When SiHa/2-DGR cells were placed at pH 6.5, however, glutamine consumption reached a similar level to that observed in SiHa/6.5 (Fig. 3F).

**MCT1 and SIRT1 contribute to the maintenance of the intracellular pH in tumor cells chronically exposed to acidosis**

Interestingly, we observed that control SiHa/7.4 cells placed in a medium buffered at pH 6.5 exhibited a rapid and prolonged decrease in intracellular pH (Fig. 4A). By contrast, when SiHa/6.5 cells were transferred from a medium at pH 7.4 to pH 6.5, they rapidly recovered from the initial pH fall to a neutral and even slightly alkaline pH (Fig. 4A).

Among the variety of mechanisms to maintain pHi in response to changes in pHe, MCTs have been shown to play key roles particularly at low pHe values (26–28). Because we showed that MCT4 expression was reduced at pH 6.5, we examined the expression of MCT1, a more ubiquitous MCT isoform supporting transport of H+/lactate and ketone bodies. We found that MCT1 was significantly more abundant in SiHa/6.5 cells together with c-myc, a recently elicited trigger of MCT1 expression (Fig. 4B; ref. 29). Furthermore, silencing MCT1 reduced the growth of SiHa/6.5 cells up to 30% whereas this treatment had no effect in SiHa/7.4 cells (Fig. 4C). Interestingly, accumulation of ketones was detected in the extracellular medium of cultured SiHa/6.5 cells (Fig. 4D), supporting the hypothesis that acetate could be the counteranion cotransported with H+ through MCT1. Indeed, a recent study reported that the dynamic acetylation and deacetylation of histones together with flux of acetate anions and protons in and out of the cell through the MCTs provided a mechanism for cells to modulate their pHi (30). We reasoned that among the different histone deacetylases, the NAD+-dependent sirtuins were the most likely to be involved in this regulation because in parallel, we showed that NAD+ concentration was significantly increased.

![Figure 2](image-url) Oxidative and reductive glutamine metabolism pathways are both increased under chronic acidosis. A, [3H]-glutamine 6-minute uptake measured in SiHa/7.4 and SiHa/6.5 cells. B, representative immunoblotting for the indicated regulators of glutamine metabolism (n = 3). C, SiHa cell growth (72 hours) following transfection with control or SLC7A5 siRNA; silencing was validated by immunoblotting. D, OCR in SiHa/7.4 and SiHa/6.5 cells under basal conditions and after treatment with 2 mmol/L glutamine and 1 μmol/L rotenone/antimycin A. E, SiHa/6.5 cell growth after 72-hour incubation in medium with or without 2 mmol/L glutamine or with 1 mmol/L dimethyl αKG (and no glutamine). F, carbon atom transition map emphasizing the reductive carboxylation of [U-13C5]glutamine (dashed). G and H, relative abundance of reductive carboxylation-specific mass isotopomers of citrate (M5), malate (M3), and fumarate (M3; Q) and of citrate mass isotopomers in SiHa/7.4 and SiHa/6.5 cells cultured for 24 hours in the presence of [U-13C5]glutamine (h, I, representative IDH1 immunoblotting (n = 3). J, SiHa cell growth (72 hours) following transfection with control or IDH1 siRNA. Silencing was validated by immunoblotting. Data represent means ± SEM of three independent experiments. **, P < 0.01; *** , P < 0.001.
Figure 3. Glycolytic pathway is inhibited under chronic low pH conditions. A, carbon atom transition map depicting oxidation of [U-13C6]glucose. B, relative abundance of glycolysis-specific mass isotopomers in SiHa cells cultured for 24 hours in the presence of [U-13C6]glucose. C, representative MCT4 and GLUT1 immunoblotting (n = 2). D, cell growth of parental, acidic pH-adapted and 2-DG-resistant SiHa cells after 72 hours incubation with 10 mmol/L 2-DG. E, ECAR in SiHa cells under basal conditions and after treatment with 10 mmol/L glucose and 10 mmol/L 2-DG. F, glutamine consumption (during 24 hours) in the indicated SiHa cells; note that SiHa/2-DGR at pH 6.5 correspond to SiHa/2-DGR acutely exposed to pH 6.5. Data represent means ± SEM of three independent experiments. ns, nonsignificant; *, P < 0.01; ***, P < 0.001.
increased in SiHa/6.5 cells (vs. SiHa/7.4; Fig. 4E) but also in FaDu/6.5 and HCT-116/6.5 cells (Supplementary Fig. S4A). We next examined the effects of siRNA-targeting MCT1 as well as SIRT1, the major stress-sensitive NAD$^+$-dependent protein deacetylase, on the rate of pH recovery of SiHa/6.5 cells when transferred from pH 7.4 to 6.5. We found that both treatments prevented the restoration of pH$_i$ (vs. sham SiHa/6.5 cells; Fig. 4F).

Figure 4. MCT1 and SIRT1 contribute to the maintenance of the intracellular pH in tumor cells exposed to chronic acidosis. A, intracellular pH measurements in SiHa cells incubated at pH 7.4 for 5 minutes and then changed to pH 6.5 for 90 minutes. B, representative MCT1 and c-myc immunoblotting ($n = 3$). C, SiHa cell growth (72 hours) following transfection with control or MCT1 siRNA; silencing is validated by immunoblotting. D, ketones measurement in conditioned medium after 72 hours; b.d.l., below detection level. E, NAD$^+$ and total NAD$^+$-NADH contents in indicated SiHa cells. F, intracellular pH measurements in SiHa/6.5 cells following transfection with control, MCT1, or SIRT1 siRNA, incubated at pH 7.4 for 5 minutes and then changed to pH 6.5 for 90 minutes. Data represent means ± SEM of three independent experiments. *** $P < 0.001$. 

Acidosis-Triggered Reprogramming of Tumor Metabolism

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SIRT1 supports the adaptation of tumor cells to chronic acidosis through changes in HIF1α and HIF2α acetylation and abundance

To further validate the role of SIRT1, we first showed that EX-527, a cell-permeable, selective inhibitor of SIRT1 (31) inhibited the growth of SiHa/6.5 but not of SiHa/7.4 cells (Fig. 5A) and that overexpression of a deacetylase-defective mutant of SIRT1 (H363Y) acting as a dominant-negative protein led to similar results (Fig. 5B). We then performed SIRT1 knockdown and knock-in experiments. We found that SIRT1 silencing blocked proliferation of SiHa/6.5 cells (Fig. 5C) and that reexpression of wild-type SIRT1 but not mutated SIRT1 (see immunoblots in Fig. 5E) restored both cell proliferation (Fig. 5C) and glutamine consumption (Fig. 5D). Similar results were obtained with FaDu/6.5 and HCT-116/6.5 cells (Supplementary Fig. S4B). Restoration of the glutamine metabolism was further supported by the reexpression of GLS1 and SLC1A5 in wild-type SIRT1 but not mutated-SIRT1 knock-in cells (Fig. 5E). Also, in these knock-in experiments, the glucose-to-lactate metabolism was found to be regulated in an opposite fashion to that of glutamine (Supplementary Fig. S4C).

SIRT1 has been described to deacetylate both HIF1α and HIF2α, altering their activity in opposing directions (32, 33), deacetylation suppressing HIF1α activity but promoting the activity of HIF2α. The use of antibodies directed against acetylated lysines to probe immunoprecipitated HIF1α and HIF2α (see Supplementary Fig. S4D and S4E) confirmed that both HIF isoforms were deacetylated in SiHa/6.5 cells (vs. SiHa/7.4 cells; Fig. 5F). The SIRT1 H363Y mutant (but not wild-type SIRT1) and the SIRT1 inhibitor EX-527 significantly increased the extent of HIF acetylation (Supplementary Fig. S4D and S4E). Interestingly, although SIRT1 abundance was not modified in SiHa/6.5 and SiHa/7.4, HIF1α expression was decreased and HIF2α expression was increased in SiHa/6.5 (vs. SiHa/7.4; Fig. 5G), indicating that activity but also expression of HIF could be altered in response to acidic pH. We also examined the protein levels of different cancer cell metabolism regulators, the expression of which were reported to be HIF1α or HIF2α dependent. Using siRNA-based strategies, we found that the HIF2α targets SLC7A5 and GLS1 were upregulated in SiHa/6.5 in a strictly HIF2α-dependent manner (Fig. 5H). By contrast, the HIF1α target MCT4 was downregulated in SiHa/6.5 in agreement with the decrease in HIF1α expression observed in these cells (Fig. 5H). Finally, we found that although SiHa/7.4 cell growth was strongly dependent on HIF1α expression (Fig. 5I), SiHa/6.5 cell growth was reduced by about 70% when HIF2α was silenced but remained unaffected when HIF1α was silenced (Fig. 5I).

Acidosis-triggered metabolic shift toward glutamine metabolism is reversible and parallels the opposite alterations in HIF1α and HIF2α abundance

We finally explored the reversibility of the metabolic reprogramming resulting from chronic tumor cell exposure to acidic pH. SiHa/6.5 cells were cultured at pH 7.4 for 24 to 96 hours. SiHa/6.5 cells restored their original metabolism, with a progressive increase in glucose consumption (and lactate release; Fig. 6A) and a reduction in glutamine uptake (Fig. 6B). A similar reversibility of the metabolic phenotype was observed with FaDu/6.5 and HCT-116/6.5 cells, although with a much faster kinetics than in SiHa cells (Supplementary Fig. S5A and S5B). Reversal to the original glucose metabolism was associated with a decrease in intracellular NAD+ content after 48-hour incubation at pH 7.4 (Fig. 6C). Moreover, when SiHa/6.5 cells were cultured at pH 7.4, acetylation of both HIF1α and HIF2α was progressively increased, in agreement with a decrease in NAD+–dependent SIRT1 activity (Fig. 6D). Importantly, the abundance of HIF1α and HIF2α also progressively increased and decreased with the incubation time at pH 7.4, respectively, to reach basal levels similar to parental cells continuously maintained at pH 7.4 (Fig. 6D). Proteins under the control of HIF2α, including SLC1A5, MCT1, and IDH1 (see above), remarkably followed the same pattern of reduced expression whereas the expression of MCT4, a bona fide HIF1α target, increased in parallel to the increase in HIF1α abundance (Fig. 6D).

Inhibition of SIRT1-driven glutamine metabolism delays the growth of tumors arising from acidic pH-adapted cancer cells

To explore the in vivo relevance of the SIRT1-mediated shift in glutamine metabolism, we first looked at the effect of BPTES on the growth of SiHa/6.5 xenograft in mice. SiHa cells were chosen for these in vivo experiments as they exhibited the slower phenotype reversibility and were thus more prone to maintain their in vitro phenotype after injection (see Fig. 6A and B). Interestingly, daily administration of 10 mg/kg BPTES significantly reduced the growth of SiHa/6.5 tumors but not that of SiHa/7.4 tumors (Fig. 7A and B). We also treated SiHa tumor-bearing mice with EX-527 (2 mg/kg) and found that daily treatment with this SIRT1 inhibitor also exclusively impaired SiHa/6.5 tumor growth (72 hours) following treatment with 1 μmol/L SIRT1 inhibitor EX-527 (A), transfection with wild-type or catalytically impaired H363Y mutant SIRT1 (B), and transfection with control or SIRT1 siRNA alone or together with wild-type (WT) or H363Y (MUT) SIRT1 (knock-in; C). D, glutamine uptake; E, representative SIRT1, HIF1α, and HIF2α immunoblotting (n = 3). H, expression of glycolysis-associated (HIF1α and MCT4) and glutaminolysis-associated proteins (HIF2α, SLC7A5, GLS1) in SiHa cells following transfection with control, HIF1α, or HIF2α siRNA. I, SiHa cell growth (72 hours) following transfection with control, HIF1α, or HIF2α siRNA. Immunoblot experiments were repeated 2–3-fold with similar results and bar graphs represent means ± SEM of three independent experiments. ns, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
growth (Fig. 7C). Interestingly, tumor xenografts derived from SiHa/6.5 cells displayed the same phenotype as observed in vitro (i.e., SLC1A5, MCT1, and IDH1 overexpression, associated with MCT4 downregulation; Fig. 7D).

Discussion

In this study, we have characterized the metabolic reprogramming occurring in tumor cells chronically exposed to acidic pH and documented an associated shift in the sensitivity to therapeutic modalities. We used tumor cells maintained for several weeks at pH 6.5 to avoid the confusing effects of a rapid change in extracellular pH (see Figs. 1A and 4A). The chronic adaptation to acidic pH led to a cell phenotype with the exact same proliferation rate as that of parental cells maintained at pH 7.4 but exhibiting dramatic alterations in the cellular metabolic preferences. This metabolic shift was shared by tumor cells of distinct origins, thereby excluding clonal selection of cells harboring specific mutations patterns. The acidification-triggered alterations in the metabolic profile include a dramatic reduction in the use of glucose and thus in the glycolytic flux, an increase in the reductive metabolism of glutamine together with an increase in glutamine-fueled OXPHOS. Reductive metabolism of αKG was recently reported to represent a glucose-independent manner to synthesize AcCoA for lipid synthesis in an IDH1-dependent manner (24). Although this was so far proposed to occur under hypoxia (thus in a context of OXPHOS inhibition), our data support a
model where (i) glutamine-dependent reductive carboxylation occurs in the cytosol as supported by the upregulation of the cytosolic isoform IDH1 (but not the mitochondrial IDH2) and (ii) concomitantly, glutamine supports mitochondrial cell respiration through the fueling of oxidative TCA cycle. Importantly, through the identification of the mechanisms supporting this metabolic conversion toward the preferential use of glutamine, we also provide evidence that the modalities inhibiting proliferation at acidic or physiological pH are different and may thus influence the therapeutic outcomes for patients treated with drugs targeting tumor metabolism.

McBrian and colleagues (30) reported that deacetylation of chromatin functions as a rheostat to regulate pHi when cells are exposed to an acidic extracellular pH. We report here that in addition to a global unspecific deacetylation process, the NAD⁺-dependent histone deacetylase SIRT1 drives this phenomenon in an unexpected specific manner. SIRT1-mediated deacetylation of both HIF1α and HIF2α is actually observed in cells chronically exposed to acidic pH and accounts for a reduction in HIF1α activity and a net increase in HIF2α activity. Although the latter promotes the expression of the glutamine transporter ASCT2 and the glutaminase enzyme GLS1, the reduction in HIF1α activity is associated with a reduction in MCT4, the bona fide monocarboxylate transporter responsible for lactate/H⁺ release. By contrast, MCT1, another MCT isoform is upregulated in accordance with the role of this transporter in the efflux of protons with acetate as the counter-ion. Altogether, these alterations in the expression of key metabolic actors resulting from deacetylation of the HIF protein family support the preferential use of glutamine and the reduced glucose metabolism in tumor cells adapted to the acidic pH environment. Interestingly, although tumor cell clones resistant to 2-DG (SiHa/2-DGR) led to a reduction in the glycolytic flux to the same extent as tumor cells adapted to acidosis (Fig. 3E), glutamine consumption was significantly less increased in SiHa/2-DGR than in SiHa/6.5 (Fig. 3F). Moreover, acidification of SiHa/2-DGR led to an increment in glutamine consumption (Fig. 3F), indicating that glycolysis inhibition alone is not sufficient to explain the metabolic shift observed in response to long-term acidosis. A model of two
parallel pathways reinforcing each other can therefore be proposed (Supplementary Fig. S6). From one hand, acidification of the extracellular tumor environment accounts for the blockade of lactate/H⁺ efflux and thus of glycolysis (in order to limit the production of H⁺), leading to an increase in NAD⁺ that activates SIRT1. From the other hand, the extra pool of protons that enter the cells are handled by acetate that acts as a counteranion to transport H⁺ out of the cells (Supplementary Fig. S6). Altogether, the continual loss of free acetate in cells exposed to acidic pHe together with the increased activity of SIRT1 concur to influence the acetylation and thus the activity of HIF1α and HIF2α as emphasized above.

In our study, the regulation of either HIF isoform according to the extracellular pH conditions can be documented in terms of both acetylation and abundance. The opposite effects of HIF1α versus HIF2α deacetylation are in adequation with previous reports. Indeed, SIRT1 was reported to form a complex with HIF2α and to deacetylate lysine residues in the N-TAD region, leading to an increase in HIF2α transcriptional activity (33), whereas similar deacetylation of lysine residues in HIF1α was associated with the blockade of p300 recruitment and consecutive HIF1α transcriptional repression (32). Changes in the HIF abundance were not reported in these two studies focused on the regulation of HIF activity by SIRT1 under hypoxia. In our models of cells adapted to acidic pH under normoxic conditions, the reduction in HIF1α abundance and the elevation of the HIF2α expression level further reinforce the opposite changes resulting from the deacetylation of either transcription factor. This mode of regulation supports a major role of HIF2α to drive the glutamine metabolism in replacement of the preferred glucose metabolism observed at neutral pH.

In conclusion, we report here how acidosis occurring in the tumor microenvironment may dramatically influence the tumor metabolic preferences and thereby directly modulate sensitivity (and resistance) to therapeutic modalities. In particular, our data suggest that the efficacy of treatments targeting acidosis-associated metabolic pathways using inhibitors of sirtuins and glutaminase (34–36) may have been underestimated to date by using cancer cell lines poorly reflecting the status of acidic tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Corbet, O. Feron
Development of methodology: C. Corbet, C. N. Draoui, F. Polet, A. Pinto, O. Riant, O. Feron
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Grant Support
This work was supported by grants from the Fonds national de la Recherche Scientifique (FRS-FNRS), the Belgian Foundation Against Cancer, the J. Maisin Foundation, the interuniversity attraction pole (IUAP) research program #P7/03 from the Belgian Science Policy Office (Belspo), and an Action de Recherche Concertée (ARC 14/19).

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Received March 11, 2014; revised June 28, 2014; accepted July 14, 2014; published OnlineFirst August 1, 2014.

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Received March 11, 2014; revised June 28, 2014; accepted July 14, 2014; published OnlineFirst August 1, 2014.
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Cyril Corbet, Nihed Draoui, Florence Polet, et al.


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