Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α-ketoglutarate to citrate to support cell growth and viability

David R. Wise[a,b,1], Patrick S. Ward[a,b,1], Jessica E. S. Shay[b,c], Justin R. Cross[a], Joshua J. Gruber[b], Uma M. Sachdeva[b], Jesse M. Platt[b], Raymond G. DeMatteo[a], M. Celeste Simon[b,c], and Craig B. Thompson[a,2]

[a]Cancer Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065; and [b]Abramson Cancer Center, Perelman School of Medicine, and [c]Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA 19104

Contributed by Craig B. Thompson, October 27, 2011 (sent for review October 12, 2011)

Citrate is a critical metabolite required to support both mitochondrial bioenergetics and cytosolic macromolecular synthesis. When cells proliferate under normoxic conditions, glucose provides the acetyl-CoA that condenses with oxaloacetate to support citrate production. Tricarboxylic acid (TCA) cycle anaplerosis is maintained primarily by glutamine. Here we report that some hypoxic cells are able to maintain cell proliferation despite a profound reduction in glucose-dependent citrate production. In these hypoxic cells, glutamine becomes a major source of citrate. Glutamine-derived α-ketoglutarate is reductively carboxylated by the NADPH-linked mitochondrial isocitrate dehydrogenase (IDH2) to form isocitrate, which can then be isomerized to citrate. The increased IDH2-dependent carboxylation of glutamine-derived α-ketoglutarate in hypoxia is associated with a concomitant increased synthesis of 2-hydroxyglutarate (2HG) in cells with wild-type IDH1 and IDH2. When either starved of glutamine or rendered IDH2-deficient by RNAi, hypoxic cells are unable to proliferate. The reductive carboxylation of glutamine is part of the metabolic reprogramming associated with hypoxia-inducible factor 1 (HIF1), as constitutive activation of HIF1 recapitulates the preferential reductive metabolism of glutamine-derived α-ketoglutarate even in normoxic conditions. These data support a role for glutamine carboxylation in maintaining citrate synthesis and cell growth under hypoxic conditions.

Citrate plays a critical role at the center of cancer cell metabolism. It provides the cell with a source of carbon for fatty acid and cholesterol synthesis (1). The breakdown of citrate by ATP-citrate lyase is a primary source of acetyl-CoA for protein acetylation (2). Metabolism of cytosolic citrate by aconitase and IDH1 can also provide the cell with a source of NADPH for redox regulation and anabolic synthesis. Mammalian cells depend on the catabolism of glucose and glutamine to fuel proliferation (3). In cancer cells cultured at atmospheric oxygen tension (21% O_2), glucose and glutamine have both been shown to contribute to the cellular citrate pool, with glutamine providing the major source of the four-carbon molecule oxaloacetate and glucose providing the major source of the two-carbon molecule acetyl-CoA (4, 5). The condensation of oxaloacetate and acetyl-CoA via citrate synthase generates the 6 carbon citrate molecule. However, both the conversion of glucose-derived pyruvate to acetyl-CoA by pyruvate dehydrogenase (PDH) and the conversion of glutamine to oxaloacetate through the TCA cycle depend on NAD^+, which can be compromised under hypoxic conditions. This raises the question of how cells that can proliferate in hypoxia continue to synthesize the citrate required for macromolecular synthesis.

This question is particularly important given that many cancers and stem/progenitor cells can continue proliferating in the setting of limited oxygen availability (6, 7). Louis Pasteur first highlighted the impact of hypoxia on nutrient metabolism based on his observation that hypoxic yeast cells preferred to convert glucose into lactic acid rather than burning it in an oxidative fashion. The molecular basis for this shift in mammalian cells has been linked to the activity of the transcription factor HIF1 (8–10). Stabilization of the labile HIF1α subunit occurs in hypoxia. It can also occur in normoxia through several mechanisms including loss of the von Hippel-Lindau tumor suppressor (VHL), a common occurrence in renal carcinoma (11). Although hypoxia and/or HIF1α stabilization is a common feature of multiple cancers, to date the source of citrate in the setting of hypoxia or HIF activation has not been determined.

Here, we study the sources of hypoxic citrate synthesis in a glioblastoma cell line that proliferates in profound hypoxia (0.5% O_2). Glucose uptake and conversion to lactic acid increased in hypoxia. However, glucose conversion into citrate dramatically declined. Glutamine consumption remained constant in hypoxia, and hypoxic cells were addicted to the use of glutamine in hypoxia as a source of α-ketoglutarate. Glutamine provided the major carbon source for citrate synthesis during hypoxia. However, the TCA cycle-dependent conversion of glutamine into citric acid was significantly suppressed. In contrast, there was a relative increase in glutamine-dependent citrate production in hypoxia that resulted from carboxylation of α-ketoglutarate. This reductive synthesis required the presence of mitochondrial isocitrate dehydrogenase 2 (IDH2). In confirmation of the reverse flux through IDH2, the increased reductive metabolism of glutamine-derived α-ketoglutarate in hypoxia was associated with increased synthesis of 2HG. Finally, constitutive HIF1α-expressing cells also demonstrated significant reductive-carboxylation-dependent synthesis of citrate in normoxia and a relative defect in the oxidative conversion of glutamine into citrate. Collectively, the data demonstrate that mitochondrial glutamine metabolism can be rerouted through IDH2-dependent citrate synthesis in support of hypoxic cell growth.

Results

Some Cancer Cells Can Proliferate at 0.5% O_2 Despite a Sharp Decline in Glucose-Dependent Citrate Synthesis. At 21% O_2, cancer cells have been shown to synthesize citrate by condensing glucose-derived acetyl-CoA with glutamine-derived oxaloacetate through the activity of the canonical TCA cycle enzyme citrate synthase (4). In contrast, less is known regarding the synthesis of citrate by cells that can continue proliferating in hypoxia. The glioblastoma cell line SF188 is able to proliferate at 0.5% O_2 (Fig. 1*A*), a level of hypoxia that is sufficient to stabilize HIF1α (Fig. 1*B*) and predicted to limit respiration (12, 13). Consistent with previous observations in hypoxic cells, we found that SF188 cells demonstrated increased lactate production when incubated in hypoxia

Author contributions: D.R.W., P.S.W., J.E.S.S., J.R.C., M.C.S., and C.B.T. designed research; D.R.W., P.S.W., J.E.S.S., J.J.G., U.M.S., J.M.P., and R.G.D. performed research; D.R.W., P.S.W., J.E.S.S., J.R.C., M.C.S., and C.B.T. analyzed data; and D.R.W., P.S.W., and C.B.T. wrote the paper.

The authors declare no conflict of interest.

[1]D.R.W. and P.S.W. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: thompsonc@mskcc.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1117773108/-/DCSupplemental.

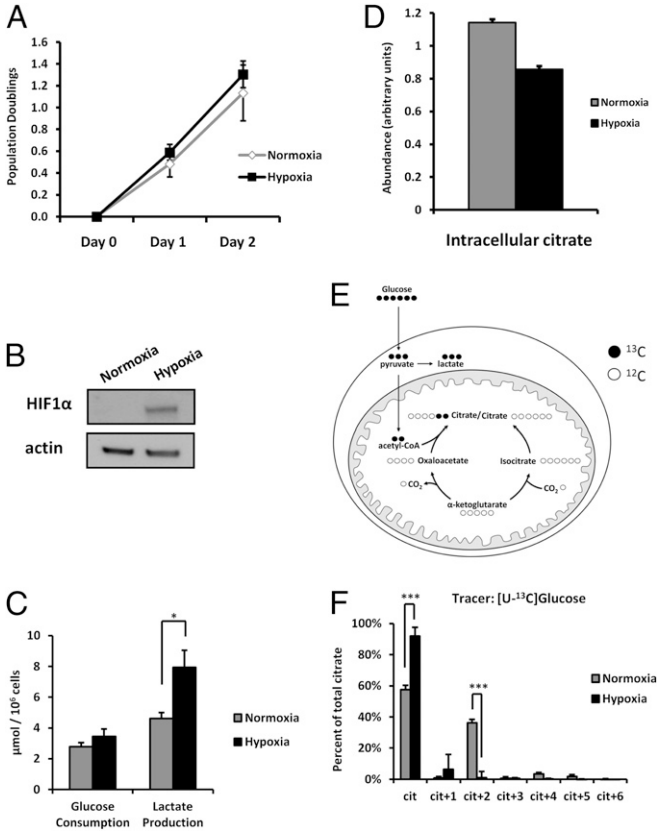

Fig. 1. SF188 glioblastoma cells proliferate at 0.5% O_2 despite a profound reduction in glucose-dependent citrate synthesis. (*A*) SF188 cells were plated in complete medium equilibrated with 21% O_2 (Normoxia) or 0.5% O_2 (Hypoxia), total viable cells were counted 24 h and 48 h later (Day 1 and Day 2), and population doublings were calculated. Data are the mean ± SEM of four independent experiments. (*B*) Western blot demonstrates stabilized HIF1α protein in cells cultured in hypoxia compared with normoxia. (*C*) Cells were grown in normoxia or hypoxia for 24 h, after which culture medium was collected. Medium glucose and lactate levels were measured and compared with the levels in fresh medium. (*D*) Cells were cultured for 24 h as in *C*. Intracellular metabolism was then quenched with 80% MeOH prechilled to −80 °C that was spiked with a ^{13}C-labeled citrate as an internal standard. Metabolites were then extracted, and intracellular citrate levels were analyzed with GC-MS and normalized to cell number. Data for *C* and *D* are the mean ± SEM of three independent experiments. (*E*) Model depicting the pathway for cit+2 production from [U-^{13}C]glucose. Glucose uniformly ^{13}C-labeled will generate pyruvate+3. Pyruvate+3 can be oxidatively decarboxylated by PDH to produce acetyl-CoA+2, which can condense with unlabeled oxaloacetate to produce cit+2. (*F*) Cells were cultured for 24 h as in *C* and *D*, followed by an additional 4 h of culture in glucose-deficient medium supplemented with 10 mM [U-^{13}C]glucose. Intracellular metabolites were then extracted, and ^{13}C-enrichment in cellular citrate was analyzed by GC-MS and normalized to the total citrate pool size. Data are the mean ± SD of three independent cultures from a representative of two independent experiments. *$P < 0.05$, ***$P < 0.001$.

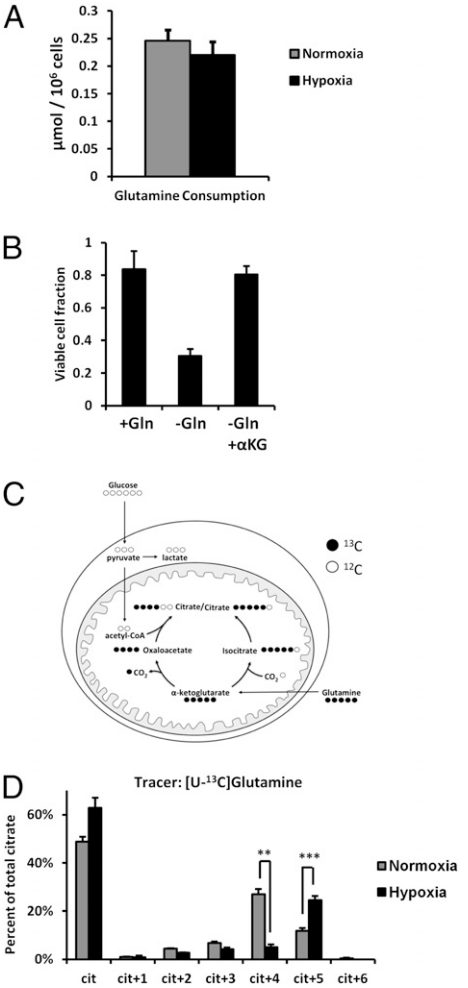

Fig. 2. Glutamine carbon is required for hypoxic cell viability and contributes to increased citrate production through reductive carboxylation relative to oxidative metabolism in hypoxia. (*A*) SF188 cells were cultured for 24 h in complete medium equilibrated with either 21% O_2 (Normoxia) or 0.5% O_2 (Hypoxia). Culture medium was then removed from cells and analyzed for glutamine levels which were compared with the glutamine levels in fresh medium. Data are the mean ± SEM of three independent experiments. (*B*) The requirement for glutamine to maintain hypoxic cell viability can be satisfied by α-ketoglutarate. Cells were cultured in complete medium equilibrated with 0.5% O_2 for 24 h, followed by an additional 48 h at 0.5% O_2 in either complete medium (+Gln), glutamine-deficient medium (−Gln), or glutamine-deficient medium supplemented with 7 mM dimethyl α-ketoglutarate (−Gln +αKG). All medium was preconditioned in 0.5% O_2. Cell viability was determined by trypan blue dye exclusion. Data are the mean and range from two independent experiments. (*C*) Model depicting the pathways for cit+4 and cit+5 production from [U-^{13}C]glutamine (glutamine+5). Glutamine+5 is catabolized to α-ketoglutarate+5, which can then contribute to citrate production by two divergent pathways. Oxidative metabolism produces oxaloacetate+4, which can condense with unlabeled acetyl-CoA to produce cit+4. Alternatively, reductive carboxylation produces isocitrate+5, which can isomerize to cit+5. (*D*) Glutamine contributes to citrate production through increased reductive carboxylation relative to oxidative metabolism in hypoxic proliferating cancer cells. Cells were cultured for 24 h as in *A*, followed by 4 h of culture in glutamine-deficient medium supplemented with 4 mM [U-^{13}C]glutamine. ^{13}C enrichment in cellular citrate was quantitated with GC-MS. Data are the mean ± SD of three independent cultures from a representative of three independent experiments. **$P < 0.01$.

(Fig. 1*C*), and the ratio of lactate produced to glucose consumed increased demonstrating an increase in the rate of anaerobic glycolysis. When glucose-derived carbon in the form of pyruvate is converted to lactate, it is diverted away from subsequent metabolism that can contribute to citrate production. However, we observed that SF188 cells incubated in hypoxia maintain their intracellular citrate to ∼75% of the level maintained under normoxia (Fig. 1*D*). This prompted an investigation of how proliferating cells maintain citrate production under hypoxia.

Increased glucose uptake and glycolytic metabolism are critical elements of the metabolic response to hypoxia. To evaluate the contributions made by glucose to the citrate pool under normoxia or hypoxia, SF188 cells incubated in normoxia or hypoxia were cultured in medium containing 10 mM [U-^{13}C] glucose. Following a 4-h labeling period, cellular metabolites were extracted and analyzed for isotopic enrichment by gas

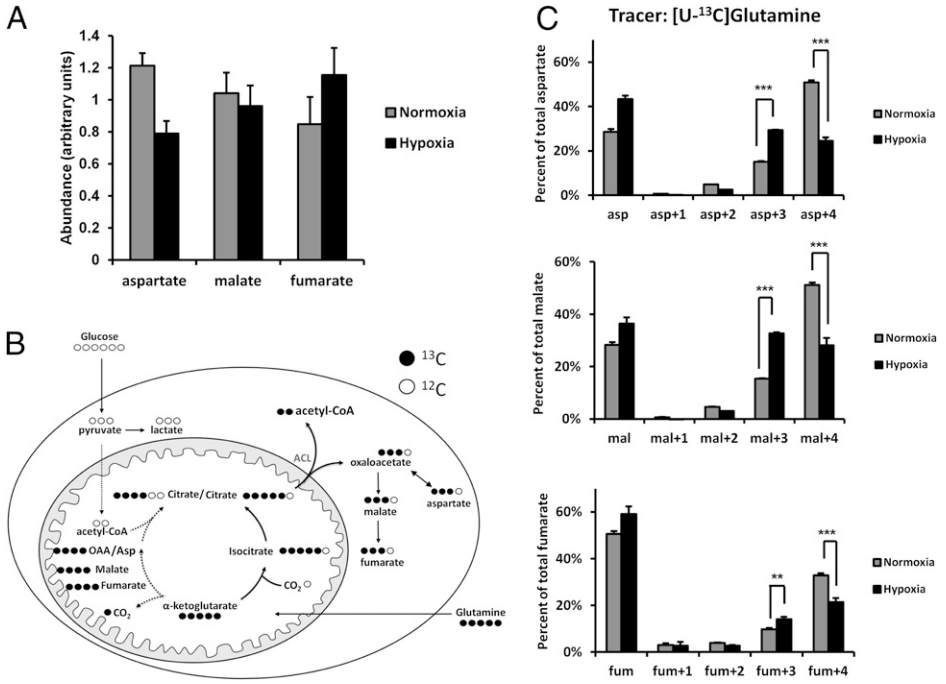

Fig. 3. Cancer cells maintain production of other metabolites in addition to citrate through reductive carboxylation in hypoxia. (*A*) SF188 cells were cultured in complete medium equilibrated with either 21% O_2 (Normoxia) or 0.5% O_2 (Hypoxia) for 24 h. Intracellular metabolism was then quenched with 80% MeOH prechilled to −80 °C that was spiked with a ^{13}C-labeled citrate as an internal standard. Metabolites were extracted, and intracellular aspartate (asp), malate (mal), and fumarate (fum) levels were analyzed with GC-MS. Data are the mean ± SEM of three independent experiments. (*B*) Model for the generation of aspartate, malate, and fumarate isotopomers from [U-^{13}C]glutamine (glutamine+5). Glutamine+5 is catabolized to α-ketoglutarate+5. Oxidative metabolism of α-ketoglutarate+5 produces fumarate+4, malate+4, and oxaloacetate(OAA)+4 (OAA+4 is in equilibrium with aspartate+4 via transamination). Alternatively, α-ketoglutarate+5 can be reductively carboxylated to generate isocitrate+5 and citrate+5. Cleavage of citrate+5 in the cytosol by ATP-citrate lyase (ACL) will produce oxaloacetate+3 (in equilibrium with aspartate+3). Oxaloacetate+3 can be metabolized to malate+3 and fumarate+3. (*C*) SF188 cells were cultured for 24 h as in *A*, and then cultured for an additional 4 h in glutamine-deficient medium supplemented with 4 mM [U-^{13}C]glutamine. ^{13}C enrichment in cellular aspartate, malate, and fumarate was determined by GC-MS and normalized to the relevant metabolite total pool size. Data shown are the mean ± SD of three independent cultures from a representative of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

chromatography-mass spectrometry (GC-MS). In normoxia, the major ^{13}C-enriched citrate species found was citrate enriched with two ^{13}C atoms (cit+2), which can arise from the NAD^+-dependent decarboxylation of pyruvate+3 to acetyl-CoA+2 by PDH, followed by the condensation of acetyl-CoA+2 with unenriched oxaloacetate (Fig. 1 *E* and *F*). Compared with the accumulation of cit+2, we observed minimal accumulation of cit+3 and cit+5 under normoxia. Cit+3 arises from pyruvate carboxylase (PC)-dependent conversion of pyruvate+3 to oxaloacetate+3, followed by the condensation of oxaloacetate+3 with unenriched acetyl-CoA. Cit+5 arises when PC-generated oxaloacetate+3 condenses with PDH-generated acetyl-CoA+2. The lack of cit+3 and cit+5 accumulation is consistent with PC activity not playing a major role in citrate production in normoxic SF188 cells, as reported (4).

In hypoxic cells, the major citrate species observed was unenriched. Cit+2, cit+3, and cit+5 all constituted minor fractions of the total citrate pool, consistent with glucose carbon not being incorporated into citrate through either PDH or PC-mediated metabolism under hypoxic conditions (Fig. 1*F*). These data demonstrate that in contrast to normoxic cells, where a large percentage of citrate production depends on glucose-derived carbon, hypoxic cells significantly reduce their rate of citrate production from glucose.

Glutamine Carbon Metabolism Is Required for Viability in Hypoxia. In addition to glucose, we have previously reported that glutamine can contribute to citrate production during cell growth under normoxic conditions (4). Surprisingly, under hypoxic conditions, we observed that SF188 cells retained their high rate of glutamine consumption (Fig. 2*A*). Moreover, hypoxic cells cultured in glutamine-deficient medium displayed a significant loss of viability (Fig. 2*B*). In normoxia, the requirement for glutamine to maintain viability of SF188 cells can be satisfied by α-ketoglutarate, the downstream metabolite of glutamine that is devoid of nitrogenous groups (14). α-ketoglutarate cannot fulfill glutamine's roles as a nitrogen source for nonessential amino acid synthesis or as an amide donor for nucleotide or hexosamine synthesis, but can be metabolized through the oxidative TCA cycle to regenerate oxaloacetate, and subsequently condense with glucose-derived acetyl-CoA to produce citrate. To test whether the restoration of carbon from glutamine metabolism in the form of α-ketoglutarate could rescue the viability defect of glutamine-starved SF188 cells even under hypoxia, SF188 cells incubated in hypoxia were cultured in glutamine-deficient medium supplemented with a cell-penetrant form of α-ketoglutarate (dimethyl α-ketoglutarate). The addition of dimethyl α-ketoglutarate rescued the defect in cell viability observed upon glutamine withdrawal (Fig. 2*B*). These data demonstrate that, even under hypoxic conditions, when the ability of glutamine to replenish oxaloacetate through oxidative TCA cycle metabolism is diminished, SF188 cells retain their requirement for glutamine as the carbon backbone for α-ketoglutarate. This result raised the possibility that glutamine could be the carbon source for citrate production through an alternative, nonoxidative, pathway in hypoxia.

Cells Proliferating in Hypoxia Preferentially Produce Citrate Through Reductive Carboxylation Rather than Oxidative Metabolism. To distinguish the pathways by which glutamine carbon contributes to citrate production in normoxia and hypoxia, SF188 cells were incubated in normoxia or hypoxia and cultured in medium containing 4 mM [U-^{13}C]glutamine. After 4 h of labeling, intracellular metabolites were extracted and analyzed by GC-MS. In normoxia, the cit+4 pool constituted the majority of the enriched citrate in the cell. Cit+4 arises from the oxidative metabolism of glutamine-derived α-ketoglutarate+5 to oxaloacetate+4 and its subsequent condensation with unenriched, glucose-derived acetyl-CoA (Fig. 2 *C* and *D*). Cit+5 constituted a significantly smaller pool than cit+4 in normoxia. Conversely, in hypoxia, cit+5 constituted the majority of the enriched citrate in the cell. Cit+5 arises from the reductive carboxylation of glutamine-derived α-ketoglutarate+5 to isocitrate+5, followed by the isomerization of isocitrate+5 to cit+5 by aconitase. The contribution of cit+4 to the total citrate pool was significantly lower in hypoxia than normoxia, and the accumulation of other enriched citrate species in hypoxia remained low. These data support the role of glutamine as a carbon source for citrate production in normoxia and hypoxia,

but through divergent metabolic pathways. In normoxia, oxidative metabolism of glutamine-derived α-ketoglutarate produces oxaloacetate, which can then condense with glucose-derived acetyl-CoA to generate citrate. In hypoxia, there is a relative increase in citrate that is generated independently of glucose via the reductive carboxylation of glutamine-derived α-ketoglutarate to isocitrate and subsequent isomerization to citrate.

Cells Proliferating in Hypoxia Maintain Levels of Additional Metabolites Through Reductive Carboxylation. Previous work has documented that, in normoxic conditions, SF188 cells use glutamine as the primary anaplerotic substrate, maintaining the pool sizes of TCA cycle intermediates through oxidative metabolism (4). Surprisingly, we found that, when incubated in hypoxia, SF188 cells largely maintained their levels of aspartate (in equilibrium with oxaloacetate), malate, and fumarate (Fig. 3*A*). To distinguish how glutamine carbon contributes to these metabolites in normoxia and hypoxia, SF188 cells incubated in normoxia or hypoxia were cultured in medium containing 4 mM [U-^{13}C]glutamine. After a 4-h labeling period, metabolites were extracted and the intracellular pools of aspartate, malate, and fumarate were analyzed by GC-MS.

In normoxia, the majority of the enriched intracellular aspartate, malate, and fumarate were the +4 species, which arise through oxidative metabolism of glutamine-derived α-ketoglutarate (Fig. 3 *B* and *C*). The +3 species, which can be derived from the citrate generated by the reductive carboxylation of glutamine-derived α-ketoglutarate, constituted a significantly lower percentage of the total aspartate, malate, and fumarate pools. By contrast, in hypoxia, the +3 species constituted a larger percentage of the total aspartate, malate, and fumarate pools than they did in normoxia. These data demonstrate that, in addition to citrate, hypoxic cells preferentially synthesize oxaloacetate, malate, and fumarate through the pathway of reductive carboxylation rather than the oxidative TCA cycle.

IDH2 Is Critical in Hypoxia for Reductive Metabolism of Glutamine and for Cell Proliferation. We hypothesized that the relative increase in reductive carboxylation we observed in hypoxia could arise from the suppression of α-ketoglutarate oxidation through the TCA cycle. Consistent with this, we found that α-ketoglutarate levels increased in SF188 cells following 24 h in hypoxia (Fig. 4*A*). Surprisingly, we also found that levels of the closely related metabolite 2-hydroxyglutarate (2HG) increased in hypoxia, concomitant with the increase in α-ketoglutarate under these conditions. 2HG can arise from the noncarboxylating reduction of α-ketoglutarate (Fig. 4*B*). Recent work has found that specific cancer-associated mutations in the active sites of either IDH1 or IDH2 lead to a 10- to 100-fold enhancement in this activity facilitating 2HG production (15–17), but SF188 cells lack IDH1/2 mutations. However, 2HG levels are also substantially elevated in the inborn error of metabolism 2HG aciduria, and the majority of patients with this disease lack IDH1/2 mutations. As 2HG has been demonstrated to arise in these patients from mitochondrial α-ketoglutarate (18), we hypothesized that both the increased reductive carboxylation of glutamine-derived α-ketoglutarate to citrate and the increased 2HG accumulation we observed in hypoxia could arise from increased reductive metabolism by wild-type IDH2 in the mitochondria.

In an experiment to test this hypothesis, SF188 cells were transfected with either siRNA directed against mitochondrial IDH2 (siIDH2) or nontargeting control, incubated in hypoxia for

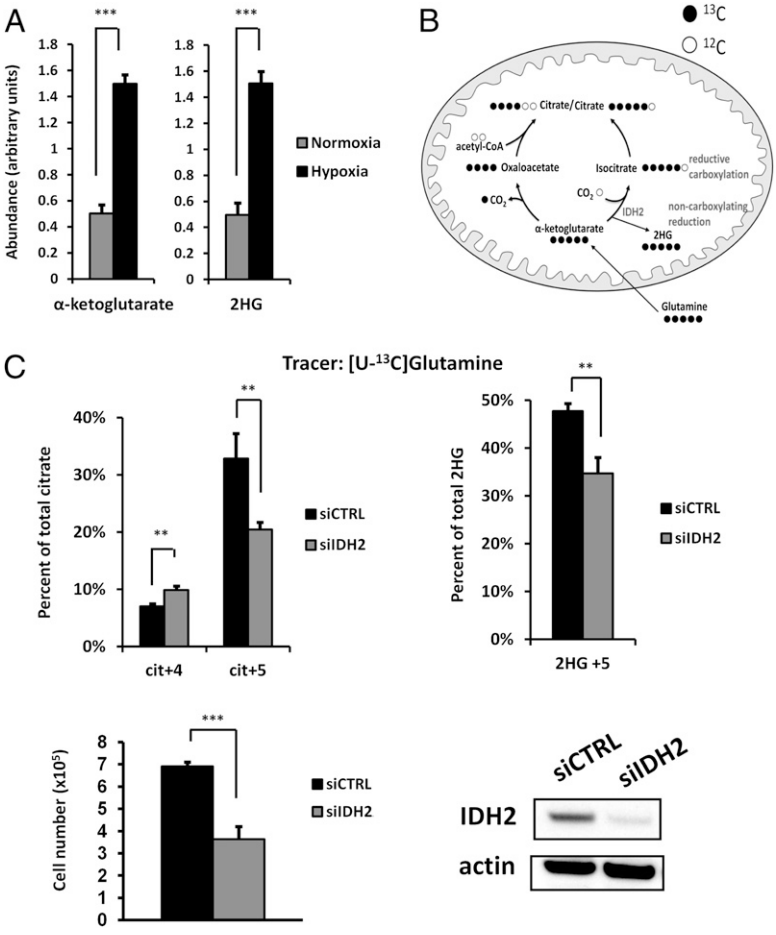

Fig. 4. Reductive carboxylation of glutamine-derived α-ketoglutarate to citrate in hypoxic cancer cells is dependent on mitochondrial IDH2. (*A*) α-ketoglutarate and 2HG increase in hypoxia. SF188 cells were cultured in complete medium equilibrated with either 21% O_2 (Normoxia) or 0.5% O_2 (Hypoxia) for 24 h. Intracellular metabolites were then extracted, cell extracts spiked with a ^{13}C-labeled citrate as an internal standard, and intracellular α-ketoglutarate and 2HG levels were analyzed with GC-MS. Data shown are the mean ± SEM of three independent experiments. (*B*) Model for reductive metabolism from glutamine-derived α-ketoglutarate. Glutamine+5 is catabolized to α-ketoglutarate+5. Carboxylation of α-ketoglutarate+5 followed by reduction of the carboxylated intermediate (reductive carboxylation) will produce isocitrate+5, which can then isomerize to cit+5. In contrast, reductive activity on α-ketoglutarate+5 that is uncoupled from carboxylation will produce 2HG+5. (*C*) IDH2 is required for reductive metabolism of glutamine-derived α-ketoglutarate in hypoxia. SF188 cells transfected with a siRNA against IDH2 (siIDH2) or nontargeting negative control (siCTRL) were cultured for 2 d in complete medium equilibrated with 0.5% O_2. (*Upper*) Cells were then cultured at 0.5% O_2 for an additional 4 h in glutamine-deficient medium supplemented with 4 mM [U-^{13}C]glutamine. ^{13}C enrichment in intracellular citrate and 2HG was determined and normalized to the relevant metabolite total pool size. (*Lower*) Cells transfected and cultured in parallel at 0.5% O_2 were counted by hemacytometer (excluding nonviable cells with trypan blue staining) or harvested for protein to assess IDH2 expression by Western blot. Data shown for GC-MS and cell counts are the mean ± SD of three independent cultures from a representative experiment. $^{**}P < 0.01$, $^{***}P < 0.001$.

2 d, and then cultured for another 4 h in hypoxia in media containing 4 mM [U-^{13}C]glutamine. After the labeling period, metabolites were extracted and analyzed by GC-MS (Fig. 4*C*). Hypoxic SF188 cells transfected with siIDH2 displayed a decreased contribution of cit+5 to the total citrate pool, supporting an important role for IDH2 in the reductive carboxylation of glutamine-derived α-ketoglutarate in hypoxic conditions. The contribution of cit+4 to the total citrate pool did not decrease with siIDH2 treatment, consistent with IDH2 knockdown specifically affecting the pathway of reductive carboxylation and not other fundamental TCA cycle-regulating processes. In confirmation of reverse flux occurring through IDH2, the contribution of 2HG+5 to the total 2HG pool decreased in siIDH2-treated cells. Supporting the importance of citrate production by IDH2-mediated reductive carboxylation for hypoxic cell proliferation, siIDH2-transfected SF188 cells displayed a defect in cellular accumulation in hypoxia. Decreased expression of IDH2 protein following siIDH2 transfection was confirmed by Western blot. Collectively, these data point to the importance of mitochondrial IDH2 for the increase in reductive carboxylation flux of glutamine-derived α-ketoglutarate to maintain citrate levels in hypoxia, and to the importance of this reductive pathway for hypoxic cell proliferation.

Reprogramming of Metabolism by HIF1 in the Absence of Hypoxia Is Sufficient to Induce Increased Citrate Synthesis by Reductive Carboxylation Relative to Oxidative Metabolism. The relative increase in the reductive metabolism of glutamine-derived α-ketoglutarate at 0.5% O_2 may be explained by the decreased ability to carry out oxidative NAD^+-dependent reactions as respiration is inhibited (12, 13). However, a shift to preferential reductive glutamine metabolism could also result from the active reprogramming of cellular metabolism by HIF1 (8–10), which inhibits the generation of mitochondrial acetyl-CoA necessary for the synthesis of citrate by oxidative glucose and glutamine metabolism (Fig. 5*A*). To better understand the role of HIF1 in reductive glutamine metabolism, we used VHL-deficient RCC4 cells, which display constitutive expression of HIF1α under normoxia (Fig. 5*B*). RCC4 cells expressing either a nontargeting control shRNA (shCTRL) or an shRNA directed at HIF1α (shHIF1α) were incubated in normoxia and cultured in medium with 4 mM [U-^{13}C]glutamine. Following a 4-h labeling period, metabolites were extracted and the cellular citrate pool was analyzed by GC-MS. In shCTRL cells, which have constitutive HIF1α expression despite incubation in normoxia, the majority of the total citrate pool was constituted by the cit+5 species, with low levels of all other species including cit+4 (Fig. 5*C*). By contrast, in HIF1α-deficient cells the contribution of cit+5 to the total citrate pool was greatly decreased, whereas the contribution of cit+4 to the total citrate pool increased and was the most abundant citrate species. These data demonstrate that the relative enhancement of the reductive carboxylation pathway for citrate synthesis can be recapitulated by constitutive HIF1 activation in normoxia.

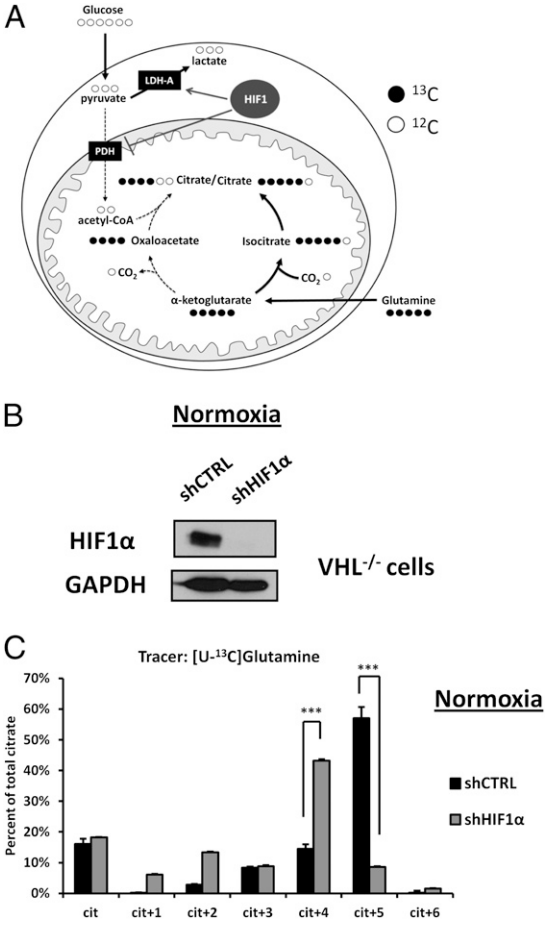

Fig. 5. Reprogramming of metabolism by HIF1 in the absence of hypoxia is sufficient to induce reductive carboxylation of glutamine-derived α-ketoglutarate. (*A*) Model depicting how HIF1 signaling's inhibition of pyruvate dehydrogenase (PDH) activity and promotion of lactate dehydrogenase-A (LDH-A) activity can block the generation of mitochondrial acetyl-CoA from glucose-derived pyruvate, thereby favoring citrate synthesis from reductive carboxylation of glutamine-derived α-ketoglutarate. (*B*) Western blot demonstrating HIF1α protein in RCC4 VHL$^{-/-}$ cells in normoxia with a nontargeting shRNA (shCTRL), and the decrease in HIF1α protein in RCC4 VHL$^{-/-}$ cells stably expressing HIF1α shRNA (shHIF1α). (*C*) HIF1-induced reprogramming of glutamine metabolism. Cells from *B* at 21% O_2 were cultured for 4 h in glutamine-deficient medium supplemented with 4 mM [U-^{13}C]glutamine. Intracellular metabolites were then extracted, and ^{13}C enrichment in cellular citrate was determined by GC-MS. Data shown are the mean ± SD of three independent cultures from a representative of three independent experiments. ***$P < 0.001$.

Discussion

Compared with glucose metabolism, much less is known regarding how glutamine metabolism is altered under hypoxia. It has also remained unclear how hypoxic cells can maintain the citrate production necessary for macromolecular biosynthesis. In this report, we demonstrate that in contrast to cells at 21% O_2, where citrate is predominantly synthesized through oxidative metabolism of both glucose and glutamine, reductive carboxylation of glutamine carbon becomes the major pathway of citrate synthesis in cells that can effectively proliferate at 0.5% O_2. Moreover, we show that in these hypoxic cells, reductive carboxylation of glutamine-derived α-ketoglutarate is dependent on mitochondrial IDH2. Although others have previously suggested the existence of reductive carboxylation in cancer cells (19, 20), these studies failed to demonstrate the intracellular localization or specific IDH isoform responsible for the reductive carboxylation flux. Recently, we identified IDH2 as an isoform that contributes to reductive carboxylation in cancer cells incubated at 21% O_2 (16), but remaining unclear were the physiological importance and regulation of this pathway relative to oxidative metabolism, as well as the conditions where this reductive pathway might be advantageous for proliferating cells.

Here we report that IDH2-mediated reductive carboxylation of glutamine-derived α-ketoglutarate to citrate is an important feature of cells proliferating in hypoxia. Moreover, the reliance on reductive glutamine metabolism can be recapitulated in normoxia by constitutive HIF1 activation in cells with loss of VHL. The mitochondrial NADPH/$NADP^+$ ratio required to fuel the reductive reaction through IDH2 can arise from the increased NADH/NAD^+ ratio existing in the mitochondria under hypoxic conditions (21, 22), with the transfer of electrons from NADH to $NADP^+$ to generate NADPH occurring through the activity of the mitochondrial transhydrogenase (23). Our

data do not exclude a complementary role for cytosolic IDH1 in impacting reductive glutamine metabolism, potentially through its oxidative function in an IDH2/IDH1 shuttle that transfers high energy electrons in the form of NADPH from mitochondria to cytosol (16, 24).

In further support of the increased mitochondrial reductive glutamine metabolism that we observe in hypoxia, we report here that incubation in hypoxia can lead to elevated 2HG levels in cells lacking IDH1/2 mutations. 2HG production from glutamine-derived α-ketoglutarate significantly decreased with knockdown of IDH2, supporting the conclusion that 2HG is produced in hypoxia by enhanced reverse flux of α-ketoglutarate through IDH2 in a truncated, noncarboxylating reductive reaction. However, other mechanisms may also contribute to 2HG elevation in hypoxia. These include diminished oxidative activity and/or enhanced reductive activity of the 2HG dehydrogenase, a mitochondrial enzyme that normally functions to oxidize 2HG back to α-ketoglutarate (25). The level of 2HG elevation we observe in hypoxic cells is associated with a concomitant increase in α-ketoglutarate, and is modest relative to that observed in cancers with IDH1/2 gain-of-function mutations. Nonetheless, 2HG elevation resulting from hypoxia in cells with wild-type IDH1/2 may hold promise as a cellular or serum biomarker for tissues undergoing chronic hypoxia and/or excessive glutamine metabolism.

The IDH2-dependent reductive carboxylation pathway that we propose in this report allows for continued citrate production from glutamine carbon when hypoxia and/or HIF1 activation prevents glucose carbon from contributing to citrate synthesis. Moreover, as opposed to continued oxidative TCA cycle functioning in hypoxia which can increase reactive oxygen species (ROS), reductive carboxylation of α-ketoglutarate in the mitochondria may serve as an electron sink that decreases the generation of ROS. HIF1 activity is not limited to the setting of hypoxia, as a common feature of several cancers is the normoxic stabilization of HIF1α through loss of the VHL tumor suppressor or other mechanisms. We demonstrate here that altered glutamine metabolism through a mitochondrial reductive pathway is a central aspect of hypoxic proliferating cell metabolism and HIF1-induced metabolic reprogramming. These findings are relevant for the understanding of numerous constitutive HIF1-expressing malignancies, as well as for populations, such as stem progenitor cells, which frequently proliferate in hypoxic conditions.

Materials and Methods

Cell Culture and Media. SF188 cells (University of California, San Francisco, Brain Tumor Research Center) and RCC4 cells (10) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen 11965), 10% FBS (Gemini Biosystems), 100 units/mL Penicillin, 100 μg/mL Streptomycin, 25 mM glucose, and 6 mM L-glutamine. For glutamine starvation experiments, DMEM without glutamine (Invitrogen 11960) was supplemented with 10% dialyzed FBS (Gemini Biosystems). For metabolic tracing experiments, DMEM without glutamine and with 10% dialyzed FBS was supplemented with [U-^{13}C]-L-glutamine (Isotec 605-166). DMEM without glucose (Sigma) was supplemented with [U-^{13}C]-D-glucose (Cambridge Isotope Laboratories CLM-1396). Viability was determined by trypan blue exclusion. Hypoxia was generated using an InVivo$_2$ 400 hypoxic workstation (Biotrace).

Metabolite Extraction and Derivatization and GC-MS Analysis. For extraction of cellular organic acids, culture medium was gently aspirated and the cells were rapidly quenched with 80% methanol chilled to −80 °C. After a 15-min incubation at −80 °C, the extract was transferred and spun down at 10,000 rpm for 15 min at 4 °C. The cellular organic acid pool in the supernatant was recovered and dried under nitrogen gas. Organic acids were purified by redissolving the dried extract in 1 mL of deionized water and then applying to an AG-1 X8 100-200 anion exchange resin (Bio-Rad 140-1441). After washing with five column volumes, organic acids were eluted with 3 N HCl and dried under nitrogen. Extracts were redissolved in a 1:1 mixture of acetonitrile (Regis 270010) and *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA; Regis 270243) and derivatized for 1 h at 95 °C. GC-MS analysis was conducted as described (16). Isotopic enrichment was assessed by monitoring the abundance of the following ions: *m/z* 459–465 for citrate, *m/z* 418–422 for aspartate, *m/z* 419–423 for malate, *m/z* 287–291 for fumarate, and *m/z* 433–438 for 2HG. An explanation for these ions is provided in *SI Materials and Methods*. Isotopomer distributions were simultaneously corrected for naturally occurring heavy isotopes of all elements in each mass fragment using a correction matrix as detailed in *SI Materials and Methods*.

Medium Metabolite Analysis. Medium glucose, glutamine, and lactate were analyzed using the Nova Biomedical Flex Metabolite Analyzer. Medium metabolite levels were compared with the measured levels in control medium not exposed to cells and then normalized to cell number to arrive at metabolite consumption/production values.

ACKNOWLEDGMENTS. We thank Anthony Mancuso and members of the C.B.T. laboratory for helpful discussion, and Niklas Thompson for help with isotopomer correction algorithms. This work was supported in part by grants from the National Cancer Institute and National Institutes of Health. D.R.W., P.S.W., J.E.S.S., J.J.G., U.M.S., and J.M.P. were also supported by the University of Pennsylvania Medical Scientist Training Program.

1. Hatzivassiliou G, et al. (2005) ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* 8:311–321.
2. Wellen KE, et al. (2009) ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* 324:1076–1080.
3. Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324:1029–1033.
4. DeBerardinis RJ, et al. (2007) Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA* 104:19345–19350.
5. Gao P, et al. (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 458:762–765.
6. Bertout JA, Patel SA, Simon MC (2008) The impact of O2 availability on human cancer. *Nat Rev Cancer* 8:967–975.
7. Zhou Y, et al. (2011) Metabolic alterations in highly tumorigenic glioblastoma cells: Preference for hypoxia and high dependency on glycolysis. *J Biol Chem* 286: 32843–32853.
8. Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* 3:177–185.
9. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC (2006) HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab* 3:187–197.
10. Lum JJ, et al. (2007) The transcription factor HIF-1alpha plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev* 21:1037–1049.
11. Maxwell PH, et al. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399:271–275.
12. Schroedl C, McClintock DS, Budinger GR, Chandel NS (2002) Hypoxic but not anoxic stabilization of HIF-1alpha requires mitochondrial reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* 283:L922–L931.
13. Wheaton WW, Chandel NS (2011) Hypoxia. 2. Hypoxia regulates cellular metabolism. *Am J Physiol Cell Physiol* 300:C385–C393.
14. Wise DR, et al. (2008) Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci USA* 105:18782–18787.
15. Dang L, et al. (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* 462:739–744.
16. Ward PS, et al. (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 17:225–234.
17. Ward PS, et al. (2011) Identification of additional IDH mutations associated with oncometabolite *R*(-)-2-hydroxyglutarate production. *Oncogene Epub*, 26: 10.1038/onc.2011.416.
18. Struys EA, Verhoeven NM, Brunengraber H, Jakobs C (2004) Investigations by mass isotopomer analysis of the formation of D-2-hydroxyglutarate by cultured lymphoblasts from two patients with D-2-hydroxyglutaric aciduria. *FEBS Lett* 557:115–120.
19. Holleran AL, Briscoe DA, Fiskum G, Kelleher JK (1995) Glutamine metabolism in AS-30D hepatoma cells. Evidence for its conversion into lipids via reductive carboxylation. *Mol Cell Biochem* 152:95–101.
20. Metallo CM, Walther JL, Stephanopoulos G (2009) Evaluation of 13C isotopic tracers for metabolic flux analysis in mammalian cells. *J Biotechnol* 144:167–174.
21. Chance B, Thorell B (1959) Localization and kinetics of reduced pyridine nucleotide in living cells by microfluorometry. *J Biol Chem* 234:3044–3050.
22. Frezza C, et al. (2011) Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival. *PLoS ONE* 6:e24411.
23. Rydström J (2006) Mitochondrial NADPH, transhydrogenase and disease. *Biochim Biophys Acta* 1757:721–726.
24. Lemons JM, et al. (2010) Quiescent fibroblasts exhibit high metabolic activity. *PLoS Biol* 8:e1000514.
25. Struys EA, et al. (2005) Mutations in the D-2-hydroxyglutarate dehydrogenase gene cause D-2-hydroxyglutaric aciduria. *Am J Hum Genet* 76:358–360.