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ORIGINAL ARTICLE

Normoxic accumulation of HIF1α is associated with glutaminolysis

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Abstract
Objectives The stabilization of the transcription factor and prognostic tumor marker hypoxia-inducible factor 1α (HIF1α) is considered to be crucial for cellular metabolic adaptations to hypoxia. However, HIF1α has also been shown to accumulate under normoxic conditions, although this phenomenon is poorly understood.
Methods We investigated the conditions for normoxic HIF1α stabilization in different tumor cell lines (e.g., two mammary carcinoma cell lines and three oral squamous cell carcinoma cell lines) via Western blot analysis or immunohistochemical staining. The transcriptional activity of HIF1 was demonstrated by analyzing the messenger RNA (mRNA) expression of the HIF1 target carbonic anhydrase 9 (CA9) via PCR.
Results Our data demonstrate that the combined incubation of tumor cells with glutamine and growth factors (e.g., EGF, insulin, and serum) mediates the normoxic accumulation of HIF1α in vitro. Consequently, the inhibition of glutaminolysis by a glutaminase inhibitor blocked the normoxic accumulation of HIF1α. Additionally, the normoxic HIF1α protein displayed nuclear translocation and transcriptional activity, which was confirmed by the induction of CA9 mRNA expression. Furthermore, the normoxic accumulation of HIF1α was associated with impaired proliferation of tumor cells. Finally, ammonia, the toxic waste product of glutaminolysis, induced a normoxic accumulation of HIF1α to the same extent as glutamine.
Conclusion Our study suggests that HIF1α is involved in the regulation of glutamine metabolism and the cellular levels of the toxic metabolic waste product ammonia under normoxia. Hence, our results, together with data presented in the literature, support the hypothesis that HIF1α and its target genes play a crucial role in metabolic pathways, such as glutaminolysis and glycolysis, under both hypoxic and normoxic conditions.
Clinical relevance Therefore, the inhibition of HIF1α (and/or HIF1α target genes) could emerge as a promising therapeutic approach that would result in the accumulation of toxic metabolic waste products in tumor cells as well as the reduction of their nutrition and energy supply.

Keywords HIF1α · Normoxia · Glutamine · Ammonia · Ammonium · Glutaminolysis · Warburg effect · Metabolism

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Introduction

Tumors are characterized by their ability to reprogram cellular metabolism as a result of their unregulated proliferation.

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Interestingly, the coexistence of both anabolic and catabolic pathways is specific to cancer [1, 2]. One tumor-associated phenomenon is aerobic glycolysis (the Warburg effect). Both the Warburg effect and anaerobic glycolysis (hypoxia-specific metabolism) appear to use similar pathways to bypass oxidative phosphorylation, although oxygen is available in the case of the Warburg effect [3, 4]. The recent interpretation of the Warburg effect (glucose metabolism) indicates that cells adapt their metabolism to incorporate nutrients into the biomass [3, 5]. Nevertheless, it seems that glucose catabolism (glycolysis) is insufficient to sustain a growing and dividing cancer cell population. Moreover, glutamine has been re-discovered as an essential bioenergetic and anabolic substrate for many cancer cell types, although under physiological conditions, glutamine is a nitrogen carrier that transports ammonia to the liver [6–8]. Previous studies have shown that glutamine catabolism (glutaminolysis) is important for the anabolic synthesis of amino acids and membranes in cancer cells [9]. This special metabolic feature in tumor cells seems to be associated with and might be essential for the Warburg effect. Glutaminolysis is performed by cells under normoxic, hypoxic, and anoxic conditions [10–12].

An important transcription factor associated with metabolism is HIF1. The accepted definition states that the α-unit of the transcription factor HIF1 is usually not detectable under normoxic conditions because the α-unit is hydroxylated by prolyl hydroxylases [13] and degraded by an E3 ubiquitin ligase complex containing the VHL tumor suppressor protein [14, 15]. Under hypoxic conditions, HIF1α accumulates and dimerizes with HIF1β (ARNT) to form HIF1, which then is translocated to the nucleus and activates a panel of target genes (including glucose transporter-1 (GLUT-1), glucose transporter-3 (GLUT-3), carbonic anhydrase IX (CAIX), and vascular endothelial growth factor (VEGF)) [15]. In this respect, it is remarkable that almost 20 % (12/65) of the HIF1-regulated genes (e.g., GLUT-1, pyruvate kinase (PKM), phosphoglucomutase1 (PGM1), lactate dehydrogenase (LDHA), hexokinase 1 and 2 (HK1 and HK2), or glucokinase (GCK) http://www.ncbi.nlm.nih.gov/biosystems/138045?Sel=geneid:8553#show=genes) are directly or indirectly involved in glucose metabolism (glycolysis). HIF1α has been detected in different human diseases, such as coronary artery diseases, wound healing, colitis, and tumors [16]. Moreover, HIF1α has been described as an important prognostic tumor factor [15, 17–21]. Therefore, HIF1 appears to be essential for both tumor and non-tumor cells [22] to mount an adaptive metabolic response to hypoxia for survival, and it also acts as a cellular oxygen sensor.

Metabolic intermediates are known to affect HIF1α accumulation. The citric acid cycle intermediates fumarate and succinate cause HIF1α stabilization in fumarate hydratase- and succinate dehydrogenase-deficient tumors [23–25]. In addition, some metabolic substrates affect HIF1 accumulation under hypoxic conditions [26, 27]. However, specific metabolic imbalances, including stress, growth factor application, oncogene activation (such as the phosphatidylinositol 3′-kinase/protein kinase B pathway (PI3K/AKT), rat sarcoma (Ras), serine/threonine-protein kinase (Raf), and avian myelocytomatosis viral oncogene homolog (c-Myc)) signaling, or the cell culture density, are able to induce HIF1α accumulation, even under normoxic conditions. However, the accumulation of HIF1α under normoxic conditions is not well understood [15, 28–34].

Recently, Parks and colleagues discussed the impact of hypoxia-inducible factor 1 (HIF1) on the Warburg effect. These authors presented the key pH-regulating systems and discussed the possibility that rapidly growing tumors exploit acute cell death by metabolic catastrophe [35].

Our previous work tried to explain the phenomenon of normoxic HIF1α/HIF1 accumulation in tumor cells by demonstrating metabolism imbalance, enhanced glutaminolysis, and ammonia release and a change in the intracellular pH value [36, 37], which may link nitrogen and glucose metabolism.

An association between the HIF1 pathway and the metabolic pathway (glycolysis) has already been reported by other authors, supporting our hypothesis that HIF1α is linked to metabolic processes and is regulated in an oxygen-independent manner [27, 38–40]. Because little is known regarding the metabolic function of the HIF1 pathway in terms of glutaminolysis, it has more recently become a subject of growing interest [37, 41–43].

Here, we demonstrate for the first time that glutamine and ammonia, the waste products of glutaminolysis, lead to HIF1α accumulation under normoxic conditions, independent of the activation of the PI3K/AKT pathway. We also discuss the type of mechanism that could be involved and how this mechanism might be used as a potential tumor therapy.

Results

HIF1α accumulation in the MCF-7 cell line under normoxic conditions

In the initial experiments, we noticed a normoxic stabilization of HIF1α. We hypothesized that this stabilization occurs trough an intrinsic mechanism due to the accumulation of metabolic intermediates. First, we analyzed the effect of glucose in combination with pyruvate or tricarboxylic acid cycle intermediates (citrate, succinate, α-ketoglutarate, and malate) but found no effect on the normoxic accumulation of HIF1α in the MCF-7 breast cancer cell line (data not shown). Next, we decided to investigate the effect of amino acids, such as glutamine, and growth factors, such as EGF and insulin, on the normoxic accumulation of HIF1α under serum-free

conditions. We detected a very weak accumulation of HIF1α in MCF-7 cells under normoxic conditions when incubated with glutamine, EGF, and insulin, respectively (Fig. 1a). However, this HIF1α accumulation was strongly enhanced when the cells were treated with a combination of glutamine and either EGF or insulin (Fig. 1a).

Physiological effects of normoxic HIF1α on proliferation

Next, we examined the effects of the glutamine and/or insulin/EGF treatment on the cell cycle distribution and proliferation of MCF-7 cells.

The data revealed that the MCF-7 cells arrested in G1 phase after serum deprivation (G1 cells 73 versus 45 % when cultured with 10 % serum) (Fig. 1b). The application of EGF (G1 cells 40 %) or insulin (G1 cells 28 %) rescued the cells from the G1 cell cycle arrest even when cultivated without serum. The application of the PI3K inhibitor Ly294002 (Ly) as a negative control induced a very strong G1 arrest (G1 cells 92 %) (Fig. 1b).

The proliferation assays (sulforhodamine B (SRB) assay) demonstrate that the addition of EGF or insulin to glutamine-free RPMI medium (containing 11.1 mM glucose) had no effect on the proliferation of MCF-7 cells (Fig. 1c). Compared with the corresponding control (without glutamine and EGF or insulin), the application of glutamine alone increased the proliferation and the number of MCF-7 cells up to 1.7-fold. Remarkably, the combination of glutamine and EGF or insulin increased the number of MCF-7 cells up to 3- and 5-fold, respectively (Fig. 1c). Together, our data suggest that glutamine and a growth factor stimulus (EGF/insulin) are responsible for the normoxic accumulation of HIF1α and the increased proliferation of MCF-7 cells.

The transcriptional activity of HIF1α under normoxic conditions: activation of the mRNA expression of the HIF1 target gene CA9 in different cell lines

Incubation with glutamine and insulin resulted in nuclear staining of HIF1α in normoxic cells at a similar level as the hypoxic MCF-7 cells (Fig. 2a). This nuclear localization is required for the transcriptional activity of the nuclear transcription factor HIF1 (Fig. 2a) and for the messenger RNA (mRNA) expression of its target gene carbonic anhydrase 9 (CA9) (Fig. 2b).

We analyzed the HIF1α protein level and CA9 mRNA expression in five human cell lines (MCF-7, MDA-MB-231, SAOS-2, HT1080, and HEK293) under normoxic conditions. The HIF1α protein level was elevated in MDA-MB-231 cells treated with glutamine alone and in MDA-MB-231 and MCF-7 cells treated with a combination of glutamine and insulin. As expected, an increase in the HIF1α protein level was associated with an increase in the CA9 mRNA levels. The increase in CA9 mRNA expression after the combined treatment with glutamine and insulin was inhibited in the MDA-MB-231 and MCF-7 cells after the small interfering RNA (siRNA)-mediated knockdown of HIF1α. However, normoxic treatment with glutamine and insulin did not enhance the accumulation of the HIF1α protein in the HT1080, HEK-293, and SAOS-2 cells. Nevertheless, in all cell lines, HIF1α was strongly accumulated after a combined treatment with glutamine and serum (includes an undefined mix of different growth factors). This was associated with an elevated CA9 mRNA expression in all cell lines, except for the HEK293 normal embryonic kidney cell line (Fig. 2b).

Next, we asked whether the phenomenon of normoxic HIF1α accumulation after a treatment with both glutamine and insulin could be observed in other cell lines as well.

HIF1α accumulation in head and neck tumor cell lines under normoxic conditions

We analyzed the accumulation of HIF1α under serum-free and normoxic conditions in the head and neck tumor cell lines SAS, XF354, and Cal33 (Fig. 3). The cultivation of the SAS and Cal33 tumor cell lines in glutamine-supplemented media resulted in an increased accumulation of HIF1α. An additional treatment with EGF or insulin had no further effect on the accumulation of HIF1α. Treatment with EGF or insulin alone did not result in distinct HIF1α accumulation. In XF354 cells, the application of glutamine alone induced only a weak accumulation of HIF1α, which was increased after an additional application of EGF or insulin (Fig. 3). Subsequently, we analyzed whether the PI3K/AKT pathway was involved in the normoxic accumulation of HIF1α. To test the activity of AKT, the level of phosphorylated AKT (pAKT) was studied in SAS, XF354, and Cal33 cells under different culture conditions. However, with the exception of the activation of AKT by insulin, no further increase in AKT phosphorylation was observed after the addition of glutamine. Therefore, we suggest that normoxic HIF1α accumulation does not result from the activation of the PI3K/AKT pathway.

Extrinsic ammonia promotes normoxic HIF1α accumulation

We set out to test if ammonia, a waste product of glutaminolysis, would affect the normoxic accumulation of HIF1α in the manner similar to that of glutamine. Thus, we adjusted the pH_e value of the medium using hydrochloric acid or a sodium hydroxide solution. In control experiments under normoxic conditions, we found that changing the pH_e value alone did not affect HIF1α accumulation in MCF-7 cells (pH_e 7.3–7.9), XF 354 cells (pH_e 6.5–7.8), or MDA-MB-231 cells (pH_e 6.7–7.8) (Fig. 4b–d). However, the application of non-toxic ammonium (as NH_4Cl) induced a pH_e value-dependent

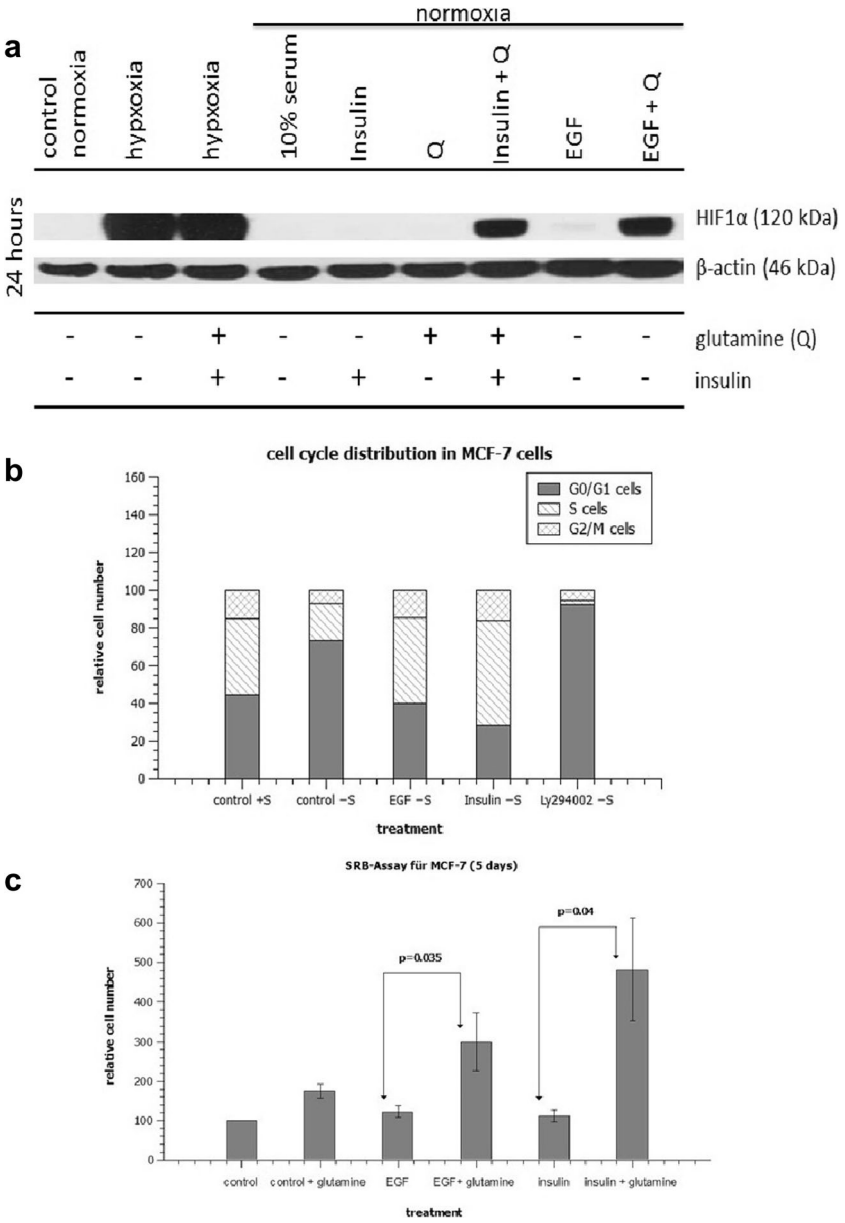

Fig. 1 **a** Western blot analysis of HIF1α and β-actin expression in MCF-7 cells 24 h after the application of 10 % serum, EGF, or insulin with or without 5 mM glutamine under normoxic conditions using glutamine- and serum-free RPMI medium containing 11.1 mM glucose. The expression of HIF1α occurred under hypoxic (*lanes 2* and *3*) and normoxic conditions after treatment with a combination of insulin and glutamine (*lane 7*) or a combination of EGF and glutamine (*lane 9*). **b** Analysis of the cell cycle distribution of MCF-7 cells. The experiment was performed in serum-free RPMI 1640 medium with glutamine (2.2 mM) under normoxic conditions. The distribution of cells in G1 cell changed from 45 % (media containing 10 % fetal bovine serum) to 73 % (media after serum deprivation), 40 % (media with EGF without serum), 28 % (media with insulin without serum), and 93 % (application of the PI3K inhibitor Ly294002 without serum) in the different treatment groups. **c** Cell proliferation in the MCF-7 cells, as determined by the SRB assay. The cells were treated with RPMI medium (without glutamine) containing EGF or insulin under normoxic conditions

increase in the HIF1α protein level, with the maximum level at the highest pH_e value for all three cell lines (Fig. 4b–d). In contrast, the HIF1α protein was not accumulated at lower pH_e values (<7.4), even though NH_4Cl was added, because the concentration of diffusible ammonia is very low at extracellular pH values <7.4. The following formula is used to calculate the concentration of free ammonia inside and outside the cell if one knows the concentration of the applied ammonium, the temperature, and the extracellular pH value [44]: ($[\%NH_3] = 100/1 + 10^{(pKA\text{-}pH\ value)}$) (pKa = 0.0925 + 2728.795 / temperature in °C + 273.15). Using this formula, we calculated that the HIF1α protein starts to accumulate under normoxic conditions due to the influence of ammonia (NH_3) in concentrations ranging from 50 to 150 μM. Stronger HIF1α accumulation is detectable at NH_3 concentrations ranging from 150 to 300 μM (Fig. 4b–d). Moreover, we want to note that in the MDA-MB-231 tumor cell line, the normoxic application of glutamine in concentrations from 80 to 160 and 160–320 μM induced moderate and strong HIF1α accumulation, respectively (Fig. 4e). Thus, the glutamine-induced accumulation of HIF1α (Fig. 4e) was comparable to the pH-dependent accumulation of HIF1α induced by the application of 2 mM NH_4Cl, as shown in Fig. 4d.

Additionally, the treatment of the MDA-MB-231 cells (autocrine-stimulated cell line) with L-glutamine, but not D-glutamine, induces the normoxic accumulation of HIF1α, which agrees with the fact that L-glutamine but not D-glutamine is the substrate for the glutaminase that catalyzes the hydrolysis of the amino group of glutamine (Fig. 4f).

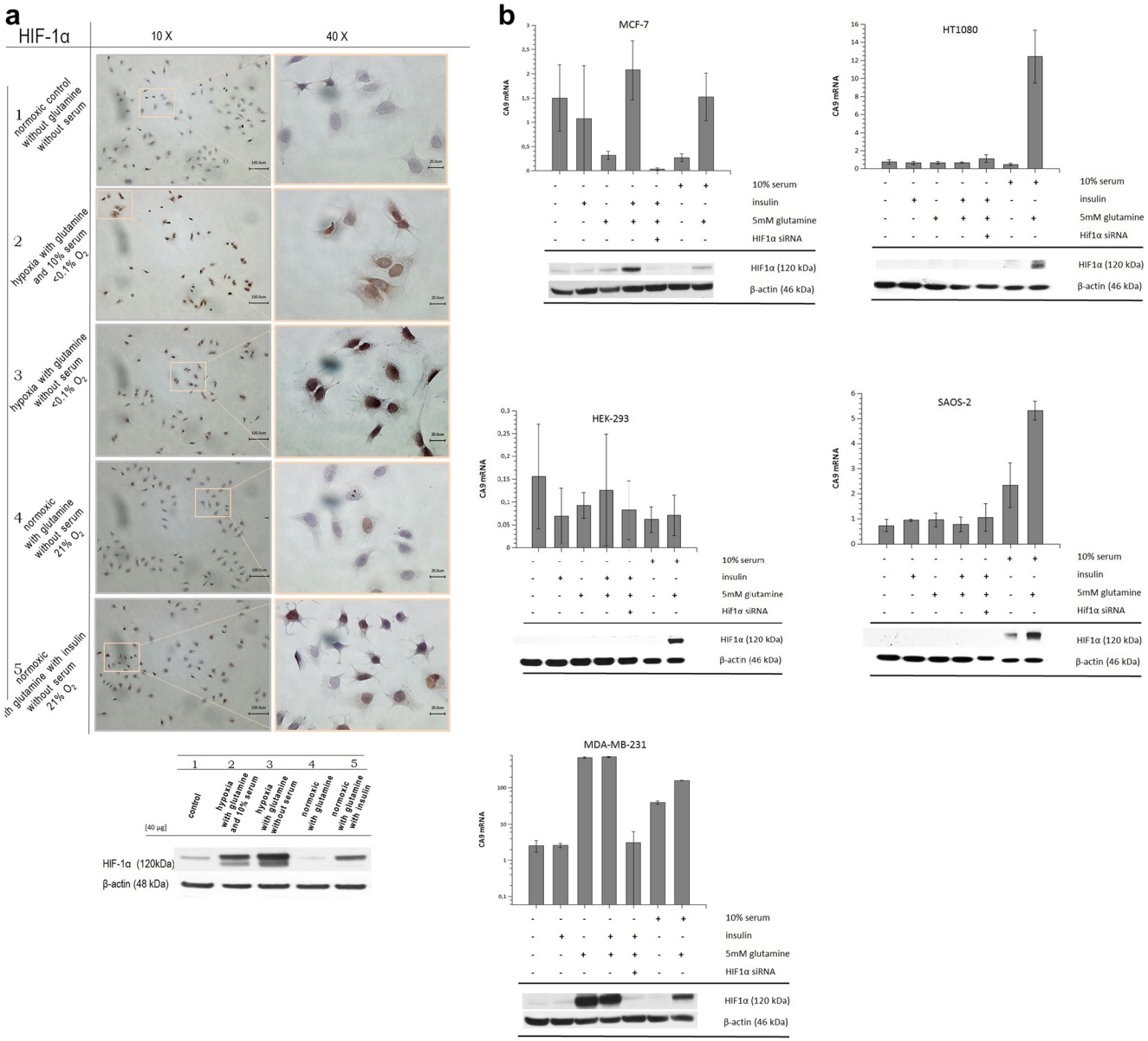

Fig. 2 **a** Immunohistochemical staining of HIF1α in MCF-7 cells indicates the nuclear localization of HIF1α (stained in *brown*) in the hypoxic cells (*2*, *3*) (positive control), in the normoxic cells (*5*) treated with glutamine, or in the cells treated with media without glutamine (negative control). The corresponding cells in each Petri dish were used for Western blot analysis. **b** The MCF-7, MDA-MB-231, SAOS-2, and HT1080 tumor cell lines and the HEK293 embryonic kidney cell line (normal cell line) were stimulated with medium only or with medium supplemented with insulin or glutamine, with insulin and glutamine, insulin and glutamine (these cells were transfected with 5 nM HIF1α-specific siRNA overnight), 10 % serum, or 10 % serum and 5 mM glutamine under normoxic conditions. At 24 h after treatment, the expression of the CA9-mRNA was measured per copy of CA9 mRNA and normalized to 10^5 copies of RPII-mRNA (RNA polymerase II) that were measured from three technical replicates. Strong CA9-mRNA induction was observed in the MDA-MB-231 cells that had been stimulated with (i) only glutamine, (ii) the combination of glutamine and insulin, (iii) only serum, or (iv) serum and glutamine. In MCF-7 cells, CA9 mRNA expression was induced after the cells were stimulated with the combination of glutamine and insulin or with serum. Invariably, the expression of the CA9 mRNA was not induced in cells that were not stimulated or were stimulated with insulin only. The HIF1α siRNA-treated cells that were stimulated with glutamine and insulin showed a lack of CA9 activation or a strong decrease in CA9 mRNA expression (MCF-7 cell line). In the Saos-2 and HT1080 cell lines, we found abundant CA9 mRNA activation following treatment with serum and glutamine. In the Saos-2 and MDA-MB-231 cell lines, we only observed CA9 mRNA activation following the serum treatment. In the normal cell line HEK293, none of the treatments or supplements was able to induce CA9 mRNA expression. A Western blot analysis of HIF1α and β-actin expression was added

To verify the impact of glutaminolysis on normoxic HIF1α accumulation, we treated the MCF-7 cells with a combination of insulin and glutamine, which caused normoxic HIF1α accumulation (Fig. 1a). When these cells were treated with the

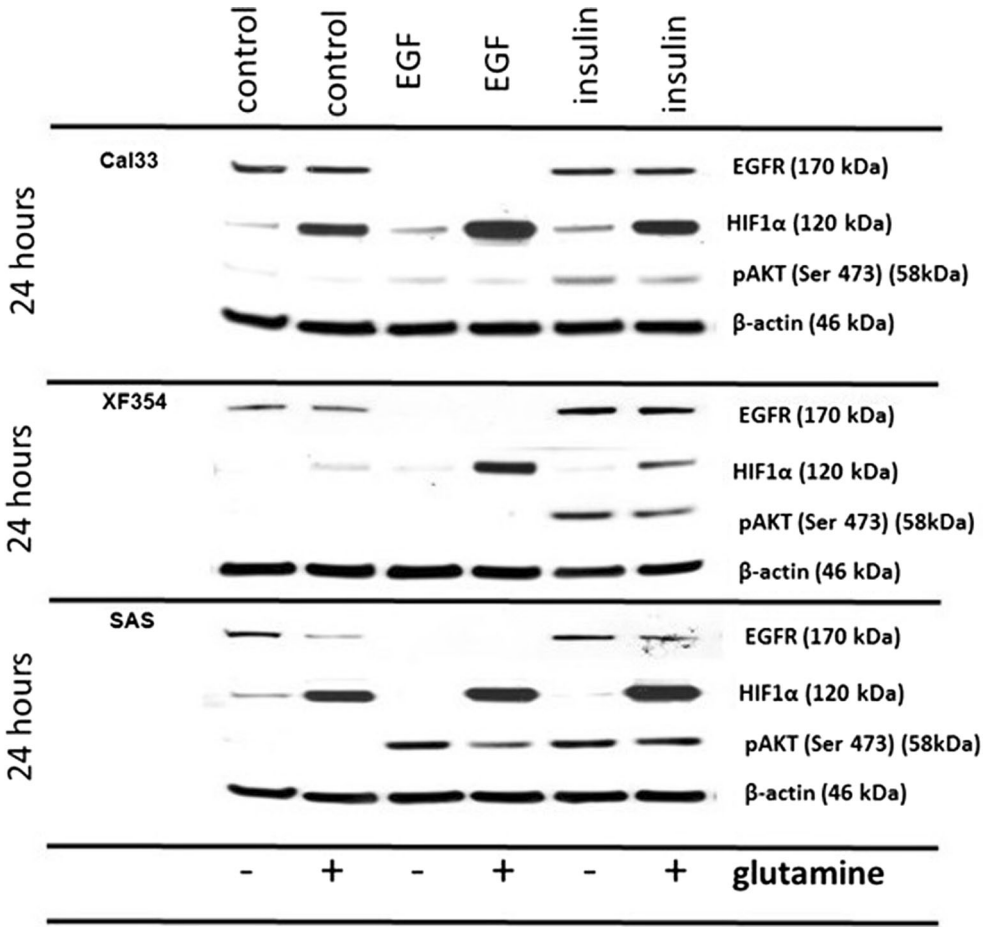

Fig. 3 Western blot analysis of HIF1α, pAKT (ser473), EGFR, and β-actin expression 24 h after the application of 5 mM glutamine, EGF, or insulin under normoxic conditions (without glutamine or serum in RPMI medium in the Cal33, XF354, and SAS cell lines). AKT phosphorylation (pAKT ser473) after the application of EGF or insulin demonstrated the activation of the PI3K/pAKT pathway

glutaminase inhibitor Compound 968, we observed a dose-dependent decrease in detectable normoxic HIF1α accumulation (Fig. 4g).

Together, these results indicate that ammonia, the toxic metabolic waste product of glutaminolysis, mediates the accumulation of the HIF1α protein under normoxic conditions (Fig. 5).

Discussion

We sought to identify the conditions that were responsible for the normoxic accumulation of HIF1α. By combining glutamine and growth factors (EGF, insulin, or serum), we observed an increased accumulation of HIF1α in different cancer cell lines under normoxic conditions. The transcriptional activity of the hypoxia-induced factor HIF1 was supported by its nuclear localization and confirmed by the induction of one of its target genes, CA9. Comparable to glutamine, its degradation (glutaminolysis) product ammonia/ammonium induces HIF1α accumulation under normoxic conditions. Our data promote the speculation that the protein HIF1α is detectable under normoxic conditions if cells consume the amino acid glutamine during growth signal-dependent (EGF, insulin, serum or an intrinsic mechanism) proliferation and that the toxic waste product of glutaminolysis, ammonia, could be the crucial molecule for this detected normoxic effect.

The distinct metabolism observed in cancer tissues compared with normal tissue is one of the most interesting characteristics of cancer. Two questions that still remain unanswered are as follows: why do tumors change their metabolism and what are the consequences of this change? The Warburg effect (aerobic glycolysis) and hypoxic metabolism use similar pathways to bypass oxidative phosphorylation. Reduced mitochondrial function yields an increase in anabolic substrates due to the increased glucose uptake in tumor cells and the decreased consumption for energy generation [45]. Dang et al. concluded that glycolysis is insufficient to sustain growing and dividing cancer cells. The Warburg effect metabolizes glucose; however, cellular proliferation requires glutamine (glutaminolysis) for both redox balance and lipid synthesis [8]. Both glucose and glutamine are consumed in massive quantities during the proliferation of most cell types [46, 47]. Moreover, glucose and glutamine are usually oxidized to lactate, which is a characteristic of the Warburg effect [46]. Other studies have noted the importance of glutaminolysis and suggested that the Warburg

effect may be a secondary metabolic consequence of the nitrogen anabolism that underlies glutaminolysis [48]. Therefore, it seems that enhanced glycolysis (the Warburg effect) and glutaminolysis are combined metabolic pathways that are inextricably linked with each other.

However, under physiological conditions, glutamine is a nitrogen carrier that transports ammonia to the liver (as part of the urea cycle) and to the kidneys [6]. This mechanism enables cells to transport and eliminate the toxic metabolic waste product ammonia out of the cell. Cells can consume the amino acid glutamine, particularly during rapid proliferation [46]. The degradation of glutamine (glutaminolysis) produces waste products, such as lactate [46] and the toxic waste product ammonia (Fig. 4a). Most of the nitrogen originating from glutamine degradation is secreted as ammonia and alanine [46]. Thus, rapid cell proliferation appears to have a downside, i.e., the accumulation of large amounts of toxic waste products, such as ammonia. In that context, Ward and Thompson ascertained that altered metabolism by itself can be oncogenic and may be a hallmark of cancer [49].

The amino acid glutamine is able to induce the mammalian target of rapamycin (mTOR) activation, which is one of the most frequent events in human cancer [50–52]. Our data on different tumor cell lines (e.g., the mammary carcinoma cell lines MCF-7 and MDA-MB-231 and oral squamous cell carcinoma lines CAL-33, XF354, and SAS) confirm that HIF1α might accumulate under normoxic conditions depending on the availability and metabolism of the amino acid glutamine and the induction of cellular proliferation, but not on the activation of the PI3K/AKT pathway, at least in the studied cell lines (Fig. 3). In the case of increased proliferation (increased glycolysis), glutamine is consumed by the cells, which subsequently releases large amounts of the metabolic waste product ammonia at the local cell level, which is toxic, even for tumor cells. Semenza argued that hypoxia induces adaptive changes in the cellular metabolism, which includes a switch from oxidative phosphorylation to glycolysis and a switch from glucose to glutamine, and that metabolic reprogramming is coordinated at the transcriptional level by HIF1, which is a master regulator that balances oxygen supply and demand [41]. However, our findings describe a similar consequence of glutamine consumption under normoxic conditions and focus on the release of ammonia as a reason for that observation.

It seems that the toxic waste product ammonia is responsible for the normoxic accumulation of HIF1α (Fig. 4b–d). Therefore, we suggest that HIF1α is equally responsible for the adjustment process, even for the normoxic metabolism. Hence, the accumulation of the HIF1α protein (which can easily be visualized by the transcriptional activation of CA9) (Fig. 2a) may be a marker for the metabolic changes (e.g., fast proliferation).

Our data suggest a metabolic test for the ability of cells to consume glutamine. The application of glutamine and different growth factors (e.g., insulin, EGF, or even serum) would identify tumor cells that stabilize and increase the amount of HIF1α protein in vitro, even under normoxic conditions (e.g., MCF-7 cells treated with EGF) (Figs. 1a and 2b). Although the induction of HIF1α by insulin or EGF is a well-known phenomenon [29, 53], to our knowledge, the glutamine-induced accumulation of HIF1α has not yet been shown. Thus, a potential individual therapeutic strategy is to identify the functional growth factor pathways in a tissue sample (e.g., EGFR therapy in the case of MCF-7 cells). In the HT1080 or Saos-2 cell lines, an unknown serum-derived growth factor strongly induced CA9 mRNA expression after glutamine supplementation (Fig. 2b). The identification of the responsible growth factor and the specific targeting of its induced signaling pathways would, in theory, provide a selective treatment opportunity for those tumor cells. Moreover, the sole application of glutamine and subsequent activation of the HIF1α/CA9 system could even identify autocrine-stimulated tumor cells or tumor cells with activating mutations in growth factor pathways, as observed in the MDA-MB-231 cells (Fig. 2b). The MDA-MB-231 cell line contains an intrinsic alteration (autocrine EGF stimulation) that results in continuous stimulation, similar to that of a supplemented growth factor (please see: https://www.lgcstandards-atcc.org/products/all/HTB-26.aspx?geo_country=de&slp=1#characteristics or [54]). Thus, we propose that the normoxic transcriptional activation of the CA9 gene by HIF1α after glutamine and/or growth factor stimulation could be used as an indicator test to separate different tumor types with regard to glutamine metabolism and the induction of proliferation. Therefore, this test would identify the appropriate individualized tumor therapy and thus minimize the risk of therapy failure.

The local release of ammonia by glutamine-consuming cells has an additional important consequence. In aqueous solution, ammonia deprotonates water to form ammonium and hydroxide: $NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$. At high pH values, this equilibrium is shifted toward free ammonia, whereas nontoxic ammonium is present at a low extracellular pH value (pH <7.0). Only ammonia, but not ammonium, can diffuse across all membranes and may enter the cell. This can decrease the intracellular pH value (pH_i) [55, 56]. Previous experiments have demonstrated that the metabolically produced ammonium ions of intracellular origin are rapidly excreted into the extracellular space, which causes the intracellular accumulation of one proton (H^+) per NH_3 molecule released [55, 56]. As a result of this change, the intracellular pH value reaches a new, lower, steady-state value which is critical for the cells. This value is characterized by the fact that the former equilibrium between the H^+ and OH^- ions would have been disturbed. Notably, even slight changes in the pH_i could be harmful; specifically, a decrease in the pH_i value of 0.2 is sufficient to completely deactivate phosphofructokinase [57, 58]. Moreover, intracellular ammonia can diffuse across all

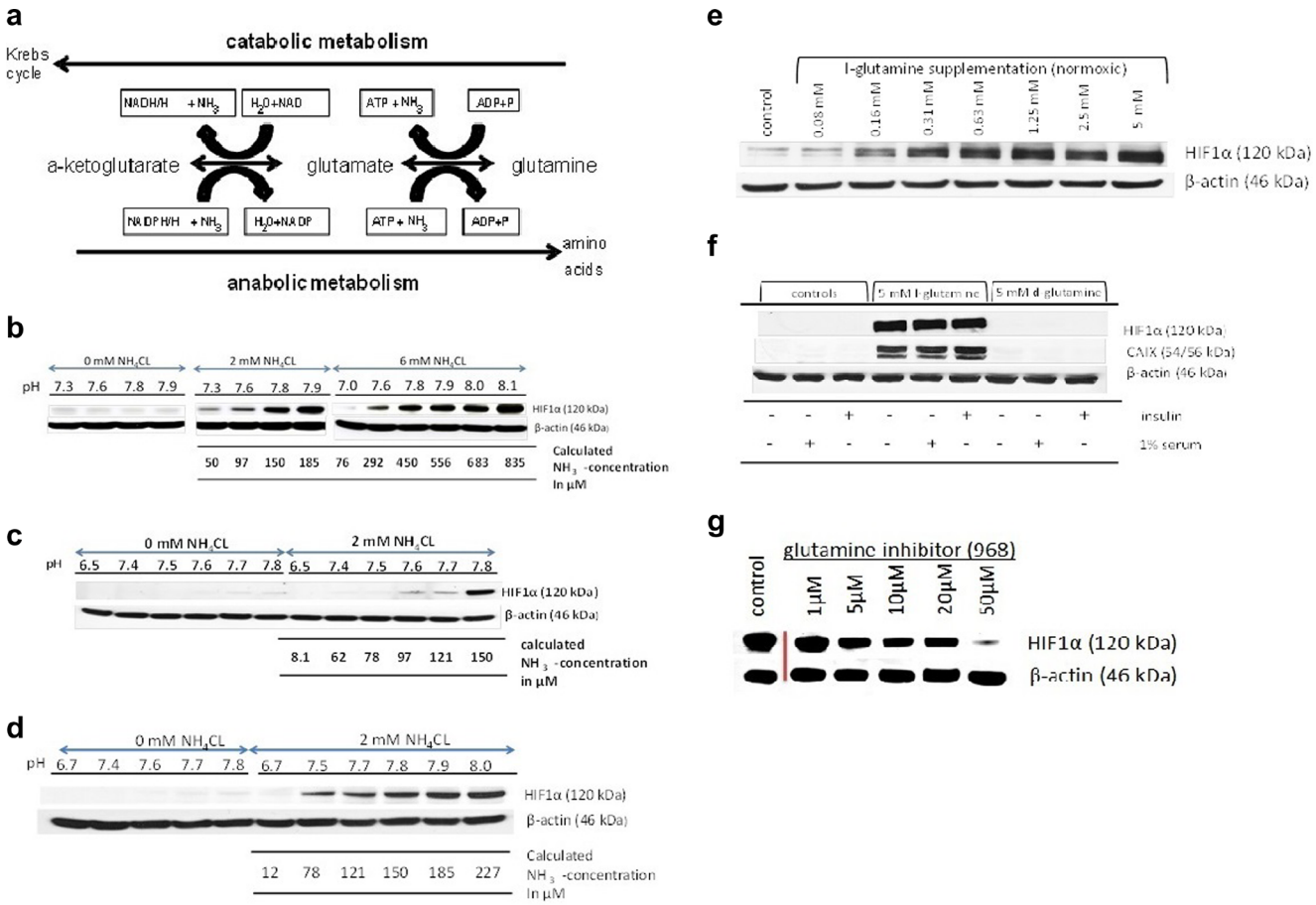

Fig. 4 **a** Part of the glutaminolysis pathway. This illustration shows the release of toxic ammonia during catabolic glutamine/glutamate degradation and the importance of α-ketoglutarate as the ammonia acceptor during anabolic metabolism [Fig. 4a adapted from [37] (Fig. 1)]. **b** Western blot analysis of HIF1α and β-actin expression in MCF-7 cells after the application of 0, 2, or 6 mM NH_4Cl for 24 h. The pH value of the medium was calibrated before the start of the experiment (using NaOH or HCl) and validated after the cells were harvested (the pH value in the figure is the value that was noted at the end of the experiment). The concentrations of ammonium and ammonia are dependent on temperature and the pH_e value and can be calculated using the formula [%NH3] = 100 / 1 + 10pKa-pH (whereas pKa = 0.0925 + (2728.795 / (temperature + 273.154))) and are documented under each blot [44]. The application of ammonia in the form of NH_4Cl is not toxic because this form does not cross the cell membrane. In contrast, highly toxic molecular ammonia can easily cross all membranes. All experiments were performed using serum-free RPMI 1640 medium without glutamine under normoxic conditions. **c** Western blot analysis of HIF1α and β-actin expression in XF354 cells after the application of 0 or 2 mM NH_4Cl for 24 h. The pH value of the medium was calibrated before the start of the experiment (using NaOH or HCl) and validated after the cells were harvested (the pH value in the figure is the value that was noted at the end of the experiment). The concentrations of ammonium and ammonia are dependent on temperature and the pH value. All experiments were performed using serum- and glutamine-free RPMI 1640 medium under normoxic conditions. **d** Western blot analysis of HIF1α and β-actin expression in MDA-MB-231 cells after the application of 0 or 2 mM NH_4Cl for 24 h. The pH value of the medium was calibrated before the start of the experiment (using NaOH or HCl) and validated after the cells were harvested (the pH value in the figure is the value that was noted at the end of the experiment). All experiments were performed using serum- and glutamine-free RPMI 1640 medium under normoxic conditions. **e** Western blot analysis of HIF1α and β-actin expression in MDA-MB-231 cells after the application of 0–5 mM L-glutamine for 24 h. The experiments were performed using serum- and glutamine-free RPMI 1640 medium under normoxic conditions (see also Figs. 2b and 4f) (the MDA-MB-231 cell line generates different growth factors in an autocrine manner, please see "Discussion" section). **f** Western blot analysis of HIF1α and β-actin expression in the cell line MDA-MB-231 cell line after the application of 5 mM L-glutamine (*lanes 4–6*) or 5 mM D-glutamine (*lanes 7–9*) with or without the application of 1 % serum or insulin under normoxic conditions (see also Figs. 2b and 3e). The experiments were performed in glutamine-free RPMI 1640 medium under normoxic conditions. **g** Western blot analysis of HIF1α and β-actin expression in MCF-7 cells that were stimulated with a combination of insulin and 5 mM glutamine for 24 h and treated with glutaminase inhibitor (Compound 968, 1–50 µM) or a control treatment. The experiments were performed in serum- and glutamine-free RPMI 1640 medium under normoxic

membranes but is protonated to ammonium (e.g., in the lysosomes) at a lower pH value. This mechanism is toxic because the cation ammonium cannot diffuse out of the lysosome and induces the functional inhibition of lysosomal processes,

including iron metabolism [59]. Moreover, the extracellular ammonium ions are deprotonated at higher pH values (>7.0) and re-enter the cells repeatedly, because the uncharged NH_3 molecules can passively diffuse across the membrane [55, 56]. To block the re-entry of NH_3, the cells must decrease the extracellular pH value, resulting in the protonation of NH_3 to NH_4^+, which cannot passively diffuse back into the cell. Importantly, the most toxic metabolic waste (ammonia) generated by an overstimulated/altered metabolism must be rapidly neutralized by a molecular mechanism (Fig. 5).

To achieve a decrease in the extracellular pH value, cancer cells initiate a protection program. Initially, CAIX can be activated by HIF1. CAIX decreases the extracellular pH value by hydrating CO_2, which generates an extracellular proton and contributes to further extracellular acidification [15, 57, 60]. A low extracellular pH value is an additional further hallmark of cancer [61, 62]. We demonstrated the normoxic intranuclear location of HIF1α and the transcriptional activity of HIF1, which was demonstrated by the induction of CA9 mRNA expression, in different human cell lines treated with glutamine (Fig. 2a, b). Because hypoxia always decreases the pH_i value of the cell due to alterations in the metabolic response [57, 60, 63], the HIF1/HIF1α system must stabilize the intracellular pH (pH_i) value via the activation of CAIX and the monocarboxylate transporter (MCT4), which decrease the extracellular pH_e [64, 65].Therefore, the HIF1/HIF1α/CA9 system must regulate the extracellular and intracellular pH levels that are changed by the metabolic activities of cancer cells.

In our opinion, HIF1 has two essential functions that are directly connected. First, HIF1 and its target gene CAIX must rapidly regulate the pH_e value resulting from the increased metabolic activity of the glutaminolysis pathway, which produces the toxic product ammonia. Thus, we propose that HIF1α/HIF1 is a major regulator that enables cells to address with high concentrations of ammonia/ammonium. Our interpretation indicated that HIF1 is a sensitive regulator of an oxygen-independent mechanism to stabilize metabolic imbalances (e.g., rapid changes in pH values) (Fig. 5) [36, 37].

Second, HIF1 may stabilize the glutamine catabolism (glutaminolysis) and the release of ammonia/ammonium. To achieve this, HIF1 activates glucose transporters, such as GLUT-1 and GLUT-3, which promote the intracellular accumulation of high levels of glucose to enable glycolysis and the generation of metabolites, such as pyruvate, which is necessary to bind toxic ammonia and produce alanine. In support of this hypothesis, 20 % of the HIF1-regulated genes are associated with glycolysis. The increased consumption of glucose is a characteristic of the Warburg effect [66]. The paradox in which catabolism and anabolism can coexist in the presence of hypoxia [57] can be explained by our interpretation of the function of HIF1 and may explain the suggestion of one previous study that the Warburg effect may be a secondary metabolic consequence of nitrogen anabolism [48]. More precisely, this statement is most likely a consequence of glutaminolysis and ammonia release.

Conclusions

The data presented here demonstrate a link between cellular proliferation and HIF1α accumulation under normoxic conditions. HIF1α seems to accumulate due to glutamine consumption (glutaminolysis) and increased ammonia concentrations. Thus, the normoxic HIF1α/HIF1 system appears to be the unifying element of two pathways that regulate ammonia metabolism. The first stabilizes the pH_i value by decreasing the pH_e value via CAIX. The second pathway neutralizes ammonia, the toxic waste product of glutamine catabolism, of via the enhanced expression of genes involved in glycolysis and, therefore, the subsequent enhancement of glycolysis and the Warburg effect.

The inhibition of HIF1 and/or HIF1 target genes could be a new therapeutic approach, because it results in the generation and accumulation of toxic metabolic waste products (e.g., ammonia) by the tumor-specific metabolism itself (specifically, the metabolic pathways associated with glutaminolysis, the Warburg effect, and hypoxia). This new insight may aid in the development of new therapies for cancer and other metabolic diseases. Finally, we propose that glutaminolysis and glycolysis (Warburg effect) are two metabolic pathways that are inseparably linked to each other in tumor cells.

Together, our results demonstrate that HIF1α can be stabilized under hypoxic and normoxic conditions. Moreover, HIF1α orchestrates the early intracellular adaptations to changes in metabolic conditions that are necessary for further tumor progression.

Materials and methods

Cell culture

In our study, we analyzed the human breast cancer cell lines MCF-7 and MDA-MB-231, the human osteosarcoma cell line SAOS-2, the fibrosarcoma cell line HT1080, and the embryonic kidney cell line HEK293, all of which were

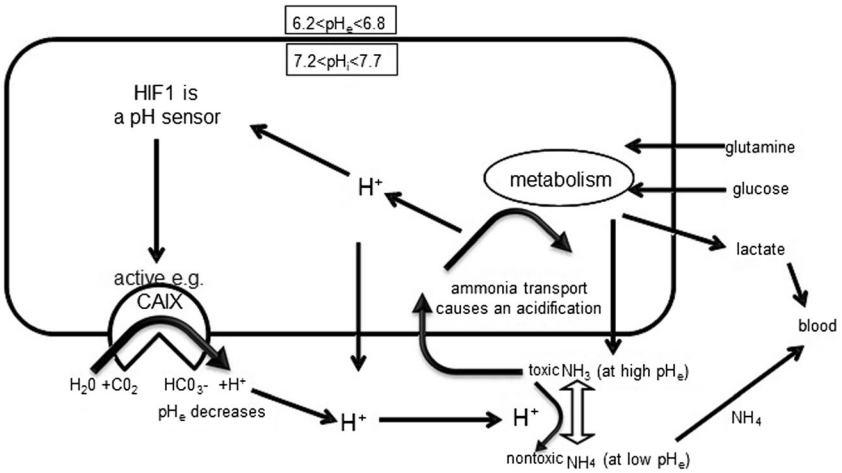

Fig. 5 The elimination of ammonia/ammonium from the cell is facilitated by the HIF1-induced activation of CAIX. CAIX causes extracellular acidification, a milieu in which ammonia can be protonated to ammonium. At a higher extracellular pH value (pH_e), more ammonia can re-enter the cell and cause intracellular acidification. The HIF1-induced GLUT-1 activation leads to enhanced activation of glycolysis, which supplies the citric acid cycle [Fig. 5 adapted from [37] (Fig. 2)]

obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The CAL-33 cell line was derived from a primary tumor of the tongue (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), and the XF354 and SAS cell lines were derived from a primary SCC of the floor of the mouth (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cells were cultured as monolayers in RPMI 1640 medium (Lonza, Walkersville, MD, USA) containing 10 % fetal bovine serum, 185 U/ml penicillin, and 185 μg/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere supplemented with 3 % CO_2.

In preparation for the experimental procedure, the cells were cultured overnight in RPMI medium containing glutamine and 0 or 1 % serum. Then, the medium was replaced with serum- and glutamine-free medium. The cells were treated with 50 ng/ml human EGF (BD Bioscience, Heidelberg, Germany) or 0.35 μg/ml insulin (Humalog, Indianapolis, IN, USA), as well as with L-and D-glutamine, L-glutamate, D-glucose, pyruvate, citrate, succinate, α-ketoglutarate, malate, and glycine (Sigma, Steinheim, Germany). The glutaminase inhibitor Compound 968 was purchased from Merck Millipore (Darmstadt, Germany) and was dissolved in DMSO. All experiments on the effect of glutamine were performed using phenol red-free RPMI 1640 (PAA, Pasching, Austria and Life Technologies, Darmstadt, Germany) without glutamine supplementation. The experiments were usually performed under normoxic conditions (21 % oxygen). Hypoxia (<1 % oxygen) was achieved using a gas generator system, as previously described [67]. Experiments investigating the effects under hypoxic conditions were performed in RPMI medium containing glutamine and 10 % serum.

Western blot analysis

The cells were treated in lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.25 % deoxycholate, and protease and phosphatase inhibitors). The protein concentration was determined using the Bradford method. Western blot analysis was performed as previously described [67].

Detection antibodies against EGFR (Clone V279, dilution 1:2000, host rabbit) and phosphoserine 473 AKT (Clone D9E, dilution 1:2000, host rabbit) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The anti-β-actin antibody (Clone AC-15, dilution 1:3000, host mouse) was obtained from Sigma (Steinheim, Germany), the anti-CAIX antibody (Clone M75, dilution 1:2000, host mouse) was obtained from Bioscience (Bratislava, Slovak Republic), and the anti-HIF1α antibody (dilution 1:2000, host mouse) was obtained from BD Bioscience (Heidelberg, Germany).

Immunohistochemical staining

For immunohistochemical staining (Fig. 2a), the cells were cultivated on sterile slides in dishes. The cells were cultured for 24 h (1) without glutamine and without serum under normoxic conditions (control), (2) with 5 mM glutamine with 10 % serum under hypoxic conditions, (3) with 5 mM glutamine without serum under hypoxic conditions, (4) with 5 mM

glutamine without serum under normoxic conditions, or (5) with 5 mM glutamine and 0.25 ng/ml insulin without serum under normoxic conditions, each in phenol red-free RPMI 1640 (PAA, Pasching, Austria) without glutamine supplementation. The cultures were maintained at 37 °C in a humidified atmosphere supplemented with 3 % CO_2, 185 U/ml penicillin, and 185 μg/ml streptomycin. The cells on the slide surface were fixed in 10 % formalin (Carl Rot GmbH, Karlsruhe, Germany) for 20 min at room temperature and then incubated in 10 mM citrate buffer for 10 min at 95 °C. Immunohistochemical staining was performed as described in [17] using a rabbit/mouse REAL™ Detection System with Peroxidase/DAB+ (Dako, Glostrup, Denmark) according to the manufacturer's instructions. Anti-HIF1α antibody (1:50) was obtained from BD Bioscience (Heidelberg, Germany). For microscopy, we used a Keyence BZ-8000 microscope (Plan Apo 20×/0.75) (Nikon, Japan). The corresponding cells in each dish were used for Western blot analysis (Fig. 2a).

Cell proliferation

The cell proliferation was assayed in the MCF-7 cell line using the SRB dye colorimetric assay. The cells were grown in glutamine-free, serum-free RPMI 1640 medium under normoxic conditions. The cells were seeded in 24- or 96-well microtiter plates at concentrations ranging from 2000 to 16,000 cells per 300 μl or 1 ml of media supplemented with 5 mM glutamine, EGF, or insulin (Fig. 1c) and incubated at 37 °C under 3 % CO_2 for 120 h. Assays were terminated by the addition of 100 μl/well of ice-cold 10 % TCA and incubated for 60 min at 4 °C to fix the cells. The plates were washed with water and air-dried. A SRB solution (100 μl; 0.4 %) in 1 % acetic acid was added, and the plates were incubated for 20 min at room temperature. Then, the plates were washed several times with 1 % acetic acid. Next, the plates were air-dried, and the bound stain was subsequently eluted with 20 mM Tris buffer. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percent growth, which was calculated on a plate-by-plate basis for test wells relative to the control wells, is expressed as the ratio of average absorbance of the test well to the average absorbance of the control well ×100. These experiments were performed in triplicate; the cell viability is presented as the mean ± standard deviation of the three analyses.

Cell cycle analyses

The methods for cell cycle analysis were previously described in detail [68]. Briefly, approximately 5×10^5 cells were harvested, washed in PBS, and stored in 80 % ethanol. The DNA content was measured by flow cytometry using a FACScan (Becton Dickinson, Heidelberg, Germany), and the data were analyzed using CellFit software (Version 2.0 Becton Dickinson, Heidelberg, Germany).

RNA preparation, cDNA synthesis, and transcript analysis by qRT-PCR

The cells used for RNA analysis were harvested 24 h after the start of the experiments. RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) or TRIzol reagent (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA samples were stored at −80 °C. For complementary DNA (cDNA) synthesis, a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) was used according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using a Rotor-Gene RG-6000 (LTF, Wasserburg, Germany) and a QuantiTect SYBR Green PCR Kit (Invitrogen, Karlsruhe, Germany).

Analysis of the transcriptional activity of normoxic HIF1α

The human breast cancer cell lines MCF-7 and MDA-MB-231, the human osteosarcoma cell line SAOS-2, the fibrosarcoma cell line HT1080, and the embryonic kidney cell line HEK293 (normal cell line) were used for these experiments. First, the cells were seeded in serum-free RPMI medium (Lonza, Walkersville, MD, USA) containing glutamine, 185 U/ml penicillin, and 185 μg/ml streptomycin and incubated overnight. Some cells were transfected with 5 nM Silencer Select val HIF1α-specific siRNA (Nr. 4427038) (Life Technologies, Darmstadt, Germany) in parallel overnight using the INTERFERin reagent (Polyplus Transfection, Illkirch, France), according to the manufacturers' instructions. The next day, the cells were incubated in serum-free and glutamine-free RPMI medium (Life Technologies, Darmstadt, Germany) and stimulated with the incubation medium (I); the incubation medium supplemented with 25 ng/ml insulin (II) (Humalog, Indianapolis, IN, USA), 5 mM glutamine (III), and insulin and 5 mM glutamine (IV) and with insulin and glutamine (these cells were transfected with 5 nM Silencer Select val HIF1α-specific siRNA) (V), 10 % serum (VI), or 10 % serum and 5 mM glutamine (VII) under normoxic conditions. At 24 h after treatment, the expression of the HIF1 target gene CA9 was measured per copy of CA9 mRNA and normalized per 10^5 copies of RPII (RNA polymerase II) in the same volume mRNA using a Rotor-Gene RG-6000 (LTF, Wasserburg, Germany) and a QuantiTect SYBR Green PCR Kit (Invitrogen, Karlsruhe, Germany). RNA preparation and cDNA synthesis were performed as described above. The CA9 primers used were as follows: CA9fw, 5′-CATCGTCTTGGACCTCTTTG-3′ and CA9rw,

5′-CCAGAAAGGCGGCCAACACG-3′. RPII (RNA polymerase II) was used as a housekeeping gene (for standardization) and as a marker for integrity of the cDNA. The RPII primers used were as follows: RPII fw 5′-CTTGCCCCGTGCCATGCAGA-3′ and RPII rev, 5′-CTCGCACCCGGCCTTCCTTG-3′.

The experimental results were analyzed using a two-sided paired Student's *t* test. A p value ≤ 0.05 was considered significant.

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Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

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Informed consent For this type of study, formal consent was not required.

References

1. Semenza GL (2009) Regulation of cancer cell metabolism by hypoxia-inducible factor 1. Semin Cancer Biol 19(1):12–16. doi:10.1016/j.semcancer.2008.11.009
2. Semenza GL (2010) HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev 20(1):51–56. doi:10.1016/j.gde.2009.10.009
3. Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324(5930):1029–1033. doi:10.1126/science.1160809
4. Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer 11(5):325–337. doi:10.1038/nrc3038
5. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, et al. (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7(1):11–20. doi:10.1016/j.cmet.2007.10.002
6. Miller AL (1999) Therapeutic considerations of L-glutamine: a review of the literature. Altern Med Rev 4(4):239–248
7. Dang CV (2010) Glutaminolysis: supplying carbon or nitrogen or both for cancer cells? Cell Cycle 9(19):3884–3886. doi:10.4161/cc.9.19.13302
8. Dang CV, Hamaker M, Sun P, et al. (2011) Therapeutic targeting of cancer cell metabolism. J Mol Med 89(3):205–212. doi:10.1007/s00109-011-0730-x
9. Dang CV (2010) Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. Cancer Res 70(3):859–862. doi:10.1158/0008-5472.CAN-09-3556
10. Smolková K, Bellance N, Scandurra F, et al. (2010) Mitochondrial bioenergetic adaptations of breast cancer cells to aglycemia and hypoxia. J Bioenerg Biomembr 42(1):55–67. doi:10.1007/s10863-009-9267-x
11. Smolková K, Plecitá-Hlavatá L, Bellance N, et al. (2011) Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. Int J Biochem Cell Biol 43(7):950–968. doi:10.1016/j.biocel.2010.05.003
12. Fendt S, Bell EL, Keibler MA, et al. (2013) Reductive glutamine metabolism is a function of the α-ketoglutarate to citrate ratio in cells. Nat Commun 4:2236. doi:10.1038/ncomms3236
13. Epstein AC, Gleadle JM, McNeill LA, et al. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation//C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107(1):43–54. doi:10.1016/S0092-8674(01)00507-4
14. Ivan M, Kondo K, Yang H, et al. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292(5516):464–468. doi:10.1126/science.1059817
15. Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3(10):721–732. doi:10.1038/nrc1187
16. Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. Cell 148(3):399–408. doi:10.1016/j.cell.2012.01.021
17. Kappler M, Taubert H, Holzhausen H, et al. (2008) Immunohistochemical detection of HIF-1α and CAIX in advanced head-and-neck cancer. Strahlenther Onkol 184(8):393–399. doi:10.1007/s00066-008-1813-7
18. Bache M, Kappler M, Said HM, et al. (2008) Detection and specific targeting of hypoxic regions within solid tumors: current preclinical and clinical strategies. Curr Med Chem 15(4):322–338. doi:10.2174/092986708783497391#sthash.igXXfwmp.dpuf
19. Eckert AW, Lautner MHW, Schütze A, et al. (2010) Co-expression of Hif1alpha and CAIX is associated with poor prognosis in oral squamous cell carcinoma patients. J Oral Pathol Med 39(4):313–317. doi:10.1111/j.1600-0714.2009.00829.x
20. Eckert AW, Lautner MHW, Schütze A, et al. (2011) Coexpression of hypoxia-inducible factor-1α and glucose transporter-1 is associated with poor prognosis in oral squamous cell carcinoma patients. Histopathology 58(7):1136–1147. doi:10.1111/j.1365-2559.2011.03806.x
21. Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26(2):225–239. doi:10.1007/s10555-007-9055-1
22. Zhang H, Bosch-Marce M, Shimoda LA, et al. (2008) Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem 283(16):10892–10903. doi:10.1074/jbc.M800102200
23. Koivunen P, Hirsilä M, Remes AM, et al. (2007) Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. J Biol Chem 282(7):4524–4532. doi:10.1074/jbc.M610415200
24. King A, Selak MA, Gottlieb E (2006) Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene 25(34):4675–4682. doi:10.1038/sj.onc.1209594
25. Ashrafian H, O'Flaherty L, Adam J, et al. (2010) Expression profiling in progressive stages of fumarate-hydratase deficiency: the contribution of metabolic changes to tumorigenesis. Cancer Res 70(22):9153–9165. doi:10.1158/0008-5472.CAN-10-1949
26. Kwon SJ, Lee YJ (2005) Effect of low glutamine/glucose on hypoxia-induced elevation of hypoxia-inducible factor-1alpha in

human pancreatic cancer MiaPaCa-2 and human prostatic cancer DU-145 cells. Clin Cancer Res 11(13):4694–4700. doi:10.1158/1078-0432.CCR-04-2530
27. Vordermark D, Kraft P, Katzer A, et al. (2005) Glucose requirement for hypoxic accumulation of hypoxia-inducible factor-1alpha (HIF-1alpha). Cancer Lett 230(1):122–133. doi:10.1016/j.canlet.2004.12.040
28. Zundel W, Schindler C, Haas-Kogan D, et al. (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 14(4):391–396
29. Peng X, Karna P, Cao Z, et al. (2006) Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 281(36):25903–25914. doi:10.1074/jbc.M603414200
30. Wang L, Zhou W, Gou S, et al. (2010) Insulin promotes proliferative vitality and invasive capability of pancreatic cancer cells via hypoxia-inducible factor 1alpha pathway. J Huazhong Univ Sci Technol Med Sci 30(3):349–353. doi:10.1007/s11596-010-0355-2
31. Blancher C, Moore JW, Robertson N, et al. (2001) Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia-inducible factor (HIF)-1alpha, HIF-2alpha, and vascular endothelial growth factor expression and their regulation by the phosphatidylinositol 3′-kinase/Akt signaling pathway. Cancer Res 61(19):7349–7355
32. Dayan F, Bilton RL, Laferrière J, et al. (2009) Activation of HIF-1alpha in exponentially growing cells via hypoxic stimulation is independent of the Akt/mTOR pathway. J Cell Physiol 218(1):167–174. doi:10.1002/jcp.21584
33. Mayer A, Höckel M, Vaupel P (2008) Endogenous hypoxia markers: case not proven! Adv Exp Med Biol 614(614):127–136. doi:10.1007/978-0-387-74911-2_15
34. Hernandez VJ, Weng J, Ly P, et al. (2013) Cavin-3 dictates the balance between ERK and Akt signaling. Elife 2:e00905. doi:10.7554/elife.00905
35. Parks SK, Chiche J, Pouysségur J (2013) Disrupting proton dynamics and energy metabolism for cancer therapy. Nat Rev Cancer 13(9):611–623. doi:10.1038/nrc3579
36. Kappler M, Taubert H, Eckert AW (2011) Oxygen sensing, homeostasis, and disease. N Engl J Med (365):1845–1846. doi:10.1056/NEJMc1110602#SA2
37. Kappler M, Taubert H, Schubert J, et al. (2012) The real face of HIF1α in the tumor process. Cell Cycle 11(21):3932–3936. doi:10.4161/cc.21854
38. Griffiths JR, McSheehy PMJ, Robinson SP, et al. (2002) Metabolic changes detected by in vivo magnetic resonance studies of HEPA-1 wild-type tumors and tumors deficient in hypoxia-inducible factor-1beta (HIF-1beta): evidence of an anabolic role for the HIF-1 pathway. Cancer Res 62(3):688–695
39. Lu H, Forbes RA, Verma A (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem 277(26):23111–23115. doi:10.1074/jbc.M202487200
40. Lu H, Dalgard CL, Mohyeldin A, et al. (2005) Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem 280(51):41928–41939. doi:10.1074/jbc.M508718200
41. Semenza GL (2013) HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. J Clin Invest 123(9):3664–3671. doi:10.1172/JCI67230
42. Sun RC, Denko NC (2014) Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. Cell Metab 19(2):285–292. doi:10.1016/j.cmet.2013.11.022
43. Tallis S, Caltana LR, Souto PA, et al. (2014) Changes in CNS cells in hyperammonemic portal hypertensive rats. J Neurochem 128(3):431–444. doi:10.1111/jnc.12458
44. Hobiger G (1996) Ammoniak in Wasser: Ableitung einer Formel zur Berechnung von Ammoniak in wäßrigen Lösungen. Berichte/Umweltbundesamt, Umweltbundesamt, Wien
45. Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8(9):705–713. doi:10.1038/nrc2468
46. DeBerardinis RJ, Mancuso A, Daikhin E, 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 U S A 104(49):19345–19350. doi:10.1073/pnas.0709747104
47. Feron O (2009) Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol 92(3):329–333. doi:10.1016/j.radonc.2009.06.025
48. Meng M, Chen S, Lao T, et al. (2010) Nitrogen anabolism underlies the importance of glutaminolysis in proliferating cells. Cell Cycle 9(19):3921–3932. doi:10.4161/cc.9.19.13139
49. Ward PS, Thompson CB (2012) Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. Cancer Cell 21(3):297–308. doi:10.1016/j.ccr.2012.02.014
50. Demidenko ZN, Blagosklonny MV (2014) The purpose of the HIF-1/PHD feedback loop: to limit mTOR-induced HIF-1α. Cell Cycle 10(10):1557–1562. doi:10.4161/cc.10.10.15789
51. Wise DR, Thompson CB (2010) Glutamine addiction: a new therapeutic target in cancer. Trends Biochem. Sci. 35(8):427–433. doi:10.1016/j.tibs.2010.05.003
52. Shanware NP, Bray K, Eng CH, et al. (2014) Glutamine deprivation stimulates mTOR-JNK-dependent chemokine secretion. Nat Commun 5:4900. doi:10.1038/ncomms5900
53. Stiehl DP, Jelkmann W, Wenger RH, et al. (2002) Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS Lett 512(1–3):157–162
54. Hernández-Bedolla MA, Carretero-Ortega J, Valadez-Sánchez M, et al. (2015) Chemotactic and proangiogenic role of calcium sensing receptor is linked to secretion of multiple cytokines and growth factors in breast cancer MDA-MB-231 cells. Biochim Biophys Acta 1853(1):166–182. doi:10.1016/j.bbamcr.2014.10.011
55. McQueen A, Bailey JE (1990) Effect of ammonium ion and extracellular pH on hybridoma cell metabolism and antibody production. Biotechnol Bioeng 35(11):1067–1077. doi:10.1002/bit.260351102
56. Ferreira TB, Carrondo MJT, Alves PM (2007) Effect of ammonia production on intracellular pH: consequent effect on adenovirus vector production. J Biotechnol 129(3):433–438. doi:10.1016/j.jbiotec.2007.01.010
57. Chiche J, Brahimi-Horn MC, Pouysségur J (2010) Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. J Cell Mol Med 14(4):771–794. doi:10.1111/j.1582-4934.2009.00994.x
58. Trivedi B, Danforth WH (1966) Effect of pH on the kinetics of frog muscle phosphofructokinase. J Biol Chem 241(17):4110–4112
59. Yu Z, Persson H, Eaton JW, et al. (2003) Intralysosomal iron: a major determinant of oxidant-induced cell death. Free Radic Biol Med 34(10):1243–1252. doi:10.1016/S0891-5849(03)00109-6
60. Brahimi-Horn MC, Bellot G, Pouysségur J (2011) Hypoxia and energetic tumour metabolism. Curr Opin Genet Dev 21(1):67–72. doi:10.1016/j.gde.2010.10.006
61. Parks SK, Chiche J, Pouyssegur J (2011) pH control mechanisms of tumor survival and growth. J Cell Physiol 226(2):299–308. doi:10.1002/jcp.22400
62. Parks SK, Mazure NM, Counillon L, et al. (2013) Hypoxia promotes tumor cell survival in acidic conditions by preserving ATP levels. J Cell Physiol 228(9):1854–1862. doi:10.1002/jcp.24346
63. Chiche J, Le Fur Y, Vilmen C, et al. (2012) In vivo pH in metabolic-defective ras-transformed fibroblast tumors: key role of the monocarboxylate transporter, MCT4, for inducing an alkaline intracellular pH. Int J Cancer 130(7):1511–1520. doi:10.1002/ijc.26125

64. Fang JS, Gillies RD, Gatenby RA (2008) Adaptation to hypoxia and acidosis in carcinogenesis and tumor progression. Semin Cancer Biol 18(5):330–337. doi:10.1016/j.semcancer.2008.03.011
65. Kroemer G, Pouyssegur J (2008) Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 13(6):472–482. doi:10.1016/j.ccr.2008.05.005
66. Warburg O (1956) On respiratory impairment in cancer cells. Science 124(3215):269–270
67. Kappler M, Rot S, Taubert H, et al. (2007) The effects of knockdown of wild-type survivin, survivin-2B or survivin-3 on the radiosensitization in a soft tissue sarcoma cells in vitro under different oxygen conditions. Cancer Gene Ther 994–1001. doi:10.1038/sj.cgt.7701090
68. Kappler M, Bache M, Bartel F, et al. (2004) Knockdown of survivin expression by small interfering RNA reduces the clonogenic survival of human sarcoma cell lines independently of p53. Cancer Gene Ther 11(3):186–193. doi:10.1038/sj.cgt.7700677