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Redox stress is not essential for the pseudo-hypoxic phenotype of succinate dehydrogenase deficient cells

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Abstract

HIFα prolyl hydroxylases (PHDs) are a family of enzymes that regulate protein levels of the α subunit of the hypoxia inducible transcription factor (HIF) under different oxygen levels. PHDs catalyse the conversion of a prolyl residue, molecular oxygen and α-ketoglutarate to hydroxy-prolyl, carbon dioxide and succinate in a reaction dependent on ferrous iron and ascorbate as cofactors. Recently it was shown that pseudo-hypoxia, HIF induction under normoxic conditions, is an important feature of tumours generated as a consequence of inactivation of the mitochondrial tumour suppressor 'succinate dehydrogenase' (SDH). Two models have been proposed to describe the link between SDH inhibition and HIF activation. Both models suggest that a mitochondrial-generated signal leads to the inhibition of PHDs in the cytosol, however, the models differ in the nature of the proposed messenger. The first model postulates that mitochondrial-generated hydrogen peroxide mediates signal transduction while the second model implicates succinate as the molecular messenger which leaves the mitochondrion and inhibits PHDs in the cytosol. Here we show that pseudo-hypoxia can be observed in SDH-suppressed cells in the absence of oxidative stress and in the presence of effective antioxidant treatment.
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1. Introduction

Succinate dehydrogenase (SDH) couples the oxidative dehydrogenation of succinate to the reduction of ubiquinone and thereby functions as a member of both the Krebs (tricarboxylic acid; TCA) cycle and the electron transport chain (ETC-complex II). SDH is comprised of four protein subunits (SDHA-D) which utilize FAD, iron–sulphur centres, haem$_b$ and ubiquinone as prosthetic groups (for more details, see [1,2]). Interestingly, despite its crucial role in energy metabolism, SDH inactivation has been shown to be associated with the formation of several types of tumours, primarily paraganglioma (including functional paraganglioma or phaeochromocytoma) [3–5]. Initially, mutations in SDHB, SDHC and SDHD were discovered in families with hereditary paraganglioma syndrome (HPGL) but very soon thereafter it was shown that somatic mutations in these genes (particularly SDHB and SDHD) also play a role in a significant number of sporadic paragangliomas [6,7]. Recently, a link between SDHB mutations and renal cell and thyroid carcinomas has also been described [8]. Despite extensive genetic evidence demonstrating the importance of SDH inactivation in the formation of these tumours, very little biochemical evidence has been provided which clarifies the relationship between SDH inactivation and tumorigenesis. One important biochemical link was elucidated from studies in the nematode C. elegans [9,10]. In this work, it was shown that a mutation in the C. elegans SDHC gene (Mev-1) leads to the accumulation of reactive oxygen species (ROS). More recently, it has been shown that the relevant murine SDHC mutant can also induce ROS production when transfected into mouse fibroblasts, leading to accelerated mutagenesis of nuclear DNA in these cells [11].

One hallmark of tumours generated due to SDH inactivation is pseudo-hypoxia, a condition in which cells

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activate an hypoxic response in the presence of oxygen. This is principally characterised by the activation of the hypoxia inducible transcription factor (HIF) and its target genes, leading to accelerated glycolysis and blood vessel growth [12–16]. The best-characterised route of regulating HIF activity is by a group of three HIFα prolyl hydroxylases (PHDs) which hydroxylate and destabilize the HIFα subunit of the HIF α/β heterodimer [17]. PHDs are members of a larger family of α-ketoglutarate-dependent dioxygenases which utilize molecular oxygen to hydroxylate their protein targets on specific prolyl residues and are therefore important oxygen sensors in cells [18]. Once hydroxylated, HIFα binds to pVHL which is part of an E3 ubiquitin ligase complex that ubiquitylates and targets HIFα for proteasomal-mediated degradation. Importantly, PHDs require ferrous iron and ascorbate for activity and hydrogen peroxide was shown to oxidise these cofactors, thereby inhibiting PHD and leading to HIFα stabilization [19]. Furthermore, mitochondrial-generated ROS were also shown to play a role in oxygen sensing (under hypoxia) leading to HIF activation [20–22].

Overall, these observations suggest that ROS generated by mutated SDH could play an active role in mutagenesis and pseudo-hypoxia leading to tumorigenesis. However, experimental studies have failed to confirm the involvement of ROS in SDH-mutated tumours [14,16]. Recently, an alternative model based on a metabolic signalling pathway was shown to link SDH inactivation to pseudo-hypoxia [23]. This pathway, in which succinate functions as a messenger between mitochondrial and cytosolic compartments, has been supported by metabolomic studies of primary tumours and additional biochemical studies [16,24,25]. All in all, there is an ongoing debate regarding the biochemical cause for HIF activation in SDH-mutated tumours. It is important to elucidate this cause as it may indicate ways of treating these tumours. If ROS are the primary cause, then it is likely that antioxidants can be used either as a preventive treatment (in families carrying SDH mutations) or as a potential treatment. However, if succinate is the relevant player in pseudo-hypoxia, increasing intracellular levels of α-ketoglutarate may overcome this effect [26].

2. Materials and methods

2.1. Materials

Dihydroethidium (DHE) and 2′7′dichlorodihydrofluorescein diacetate (DCFDA) were purchased from Molecular Probes; antimycin A, ascorbate and N-acetyl cysteine were purchased from Sigma; ThioGlo-1 was purchased from Calbiochem.

2.2. Plasmids

The scrambled and the SDHD-targeting siRNA short hairpins, Sc and Di3, respectively, which were previously described [23], were now cloned into pSUPER.neo+GFP (OligoEngine).

2.3. Cell culture and transfection

HEK293 cells were incubated routinely at 37 °C, 5% CO_2 in DMEM containing 25 mM glucose and supplemented with 10% foetal calf serum (FCS) and 50 μM β-mercaptoethanol. CCL16-B9 and CCL16-B30 Chinese Hamster lung fibroblasts were grown in DMEM containing 25 mM glucose supplemented with 10% FCS, 50 μM β-mercaptoethanol, 1× non-essential amino acids (Gibco), 2 mM glutamine and 2 mM pyruvate. Cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

2.4. Glutathione detection

Cells were treated as indicated and lysed using a lysis buffer (Cell Signaling) freshly supplemented with protein inhibitor cocktail for mammalian tissues (Sigma). Immediately following centrifugation to clear the lysates, aliquots of the supernatant were assayed for GSH in 25 mM KH_2PO_4 (pH 7.2) containing 20 μM ThioGlo-1 (Calbiochem) and for total glutathione after adding 100 μM NADPH and 3.5 U/ml glutathione reductase. Florescence intensities were monitored using an excitation wavelength of 405 nm and emission wavelength of 485 nm and were converted to GSH concentrations by comparison with standards.

2.5. Analysis of SDH activity

Succinate-DCIP oxidoreductase activity was carried out as was previously described[23].

2.6. Protein analysis

Cells were extracted in Laemmli sample buffer. Following SDS-PAGE, proteins were blotted onto nitrocellulose and probed with the following antibodies: anti-human HIF1α (clone 54; BD Biosciences) or anti-multi-species HIF1α (clone H1alpha67; Abcam) or anti-actin (clone AC-40; Sigma).

3. Results

Two models have emerged to explain HIFα stabilization resulting from SDH mutations. The first concerns the effects of ROS arising from an impaired complex II of the ETC in which ROS are proposed to inhibit PHDs as a result of cofactor oxidation. The second points to signalling mediated by succinate originating from a blocked TCA cycle which inhibits PHD activity by product inhibition. Although ROS can arise in some SDH-mutated mitochondria, no evidence supports their role in mediating HIFα stabilization in SDH-deficient tumours. To test this possibility, a small interfering RNA (siRNA), termed Di3 which has previously shown to effectively knockdown SDH activity in human embryonic kidney HEK293 cells [23], was cloned into pSuper.neo+GFP vector. This vector, once transfected into cells, co-expresses green fluorescent protein (GFP) and the Di3 small hairpin RNA (shRNA) that targets the SDHD subunit, hence GFP-positive cells are effectively deficient in SDH activity. Following transient transfection with this vector, ROS levels were monitored using the probe dihydroethidium (DHE) which undergoes oxidation by superoxide to form a red fluorescent product. Superoxide formed by one electron reduction of molecular oxygen can serve as progenitor for other ROS such as hydrogen peroxide, hydroxyl radical and peroxynitrite and was thus selected for measurement. The combination of dihydroethidium and GFP allow ROS generation (red) in cells deficient in SDH activity (green) to be assessed. The presence of simultaneous green and red

fluorescence in cells indicates a specific induction of superoxide by SDH inhibition. As can be seen from Fig. 1A, whilst some scattered cells are positive for DHE oxidation, these are not necessarily the SDH-deficient (green) cells. Moreover, there is no change in the degree or pattern of fluorescence of control cells transfected with a scrambled shRNA-encoding pSuper.neo + GFP vector (Sc) compared to that of the SDHD-specific Di3-transfected cells (Fig. 1A). These results indicate that superoxide formation is not evident in SDH-deficient cells relative to control cells.

Reduced glutathione (GSH) is the most abundant non-enzymatic antioxidant in cells and is crucial for scavenging ROS. GSH removes hydrogen peroxide in a reaction catalyzed by glutathione peroxidase which generates GSSG; GSSG is then reduced to GSH by glutathione reductase. In normal cells, ~90–95% of the glutathione pool is GSH and ~5–10% GSSG. A decrease in [GSH], increase in [GSSG] and decrease in GSH/GSSG ratio are indicative of ROS production and oxidative stress. The fluorescent thiol reagent ThioGlo-1 was used to measure GSH levels in cell extracts before and after the addition of glutathione reductase which was used to convert GSSG to GSH in the presence of NADPH. Using this methodology, the GSH/GSSG ratios of untreated, Sc- or Di3-transfected cells were calculated. Antimycin A was used as a positive control to induce superoxide production resulting from inhibition of complex III of the electron transport chain. While the inhibition of complex III with antimycin A has a marked effect on the GSH/GSSG ratio compared to untreated and control Sc-transfected cells, no effect was observed following the inhibition of complex II (SDH) with the Di3 shRNA (Fig. 1B). This observation corroborates and extends the data from the DHE experiments (Fig. 1A) and strongly suggests that no detectable alteration in the redox state of cells is observed following the inhibition of SDH.

To more convincingly determine whether ROS are responsible for HIFα induction following SDH inhibition, cells were pre-treated with the potent antioxidants ascorbate and N–acetyl cysteine prior to either hydrogen peroxide treatment or transfection with the shRNAs. To confirm the effectiveness of the antioxidant regimen, cells were pre-incubated with or without antioxidants prior to treatment with hydrogen peroxide. The hydrogen peroxide detector, $2'7'$ dichlorodihydrofluorescein diacetate (DCFDA) was added before extracting the cells and DCFDA oxidation was fluorescently assessed. As can be seen in Fig. 2A, the
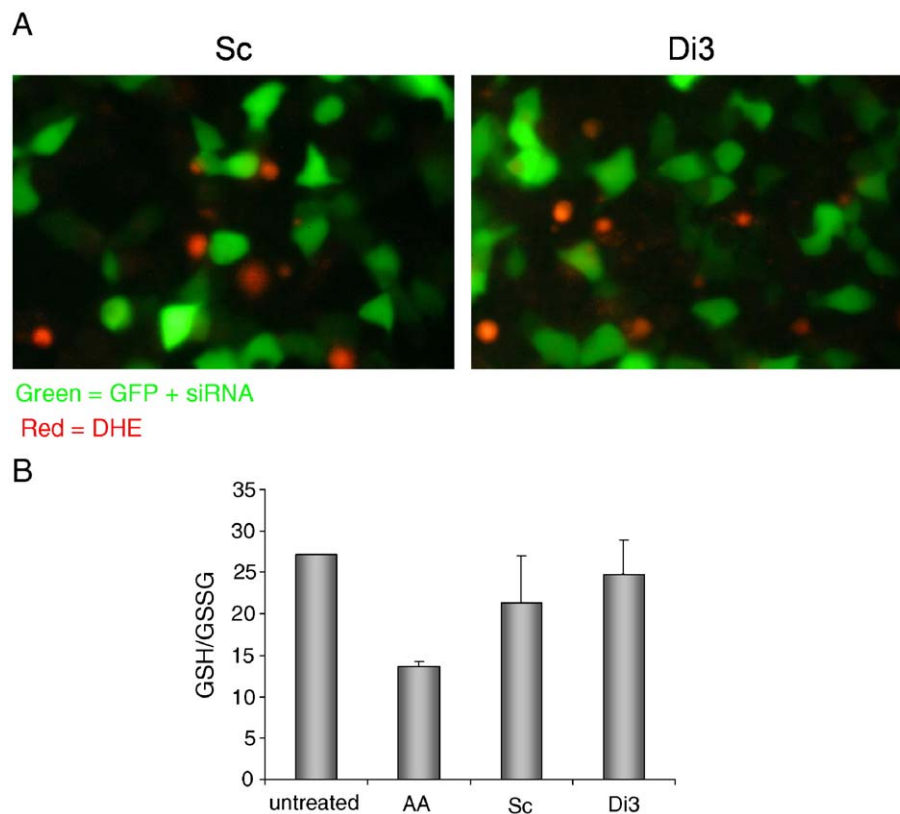

Fig. 1. (A) ROS were not detected in SDH-deficient cells. HEK293 cells were transfected with pSUPER.neo + GFP encoding either control scrambled shRNA (Sc) or the SDHD-targeting shRNA (Di3). Forty eight hours after transfection cells were loaded with 10 μM dihydroethidium (DHE) for 30 min. Superoxide levels (determined by dihydroethidium oxidation to red-fluorescent ethidium) was analysed microscopically. Green fluorescence (GFP-expressing) denotes cells which were efficiently transfected with the indicated shRNA. (B) SDH inactivation had no apparent effect on the redox state of cells. HEK293 cells were either left untreated, treated with 2 μM antimycin A (AA) for 12 h or transfected with the control scrambled shRNA-expressing plasmid (Sc) or with the SDHD-targeting shRNA (Di3) for 48 h. The ratio of reduced (GSH) to oxidized (GSSG) glutathione was used as an indicator of the redox state of the cells. While antimycin A efficiently shifted cells to a more oxidized state, SDH inactivation has no effect on the redox state.

antioxidants effectively removed the excess hydrogen peroxide added to the cells. Moreover, as was previously described [27,28], hydrogen peroxide led to the accumulation of HIF1α protein (a HIFα prototype) under normoxic conditions and treatment with the antioxidants effectively prevented HIF1α induction in hydrogen peroxide-treated cells (Fig. 2B left panel). By contrast, antioxidants did not block HIF1α accumulation in cells treated with an SDHD-targeting shRNA. (Fig. 2B right panel). These results indicate that while antioxidant treatment has a strong protective effect against PHD inhibition mediated by hydrogen peroxide, it has no effect on PHD inhibition following SDH inactivation.

Although SDH-knockdown does not lead to increased ROS production in cells, the possibility remained that aberrant SDH activity in tumours with a mutated form of SDH may lead to ROS generation. One technical difficulty to testing this hypothesis is the lack of tumour-derived cell lines. We therefore used *SDHC*-mutated hamster fibroblast cells, CCL16-B9 (B9), which have been previously characterised [29]. These cells contain a point mutation in the *SDHC* gene

resulting in premature termination of translation of the protein. We first compared SDH activity in B9 cells to its activity in cells reconstituted with an SDHC-GFP fusion protein (B30) [30] and confirmed lack of SDH activity in B9 cells and a significant recovery of SDH activity in B30 cells (Fig. 3A). Next, we compared ROS production and established that, despite a marked reduction in SDH activity due to the mutation in SDHC, no increase in DHE oxidation could be detected in B9 cells compared to B30 cells (Fig. 3B). On the other hand, HIF1α protein level under normoxic conditions was higher in B9 cells compared to B30 cells (Fig. 3C). These results reinforce the previous conclusion that SDH inactivation does not necessarily lead to ROS production and that pseudo hypoxia in SDH-deficient cells can be observed in the absence of redox stress. It is however important to mention that not much is known about the hamster HIFα protein(s), and the protein observed in Fig. 3C is of ~65 kDa, a considerably lower molecular weight than that of HIF1α of other species. However, the level of this detected protein was significantly elevated under hypoxic conditions and when
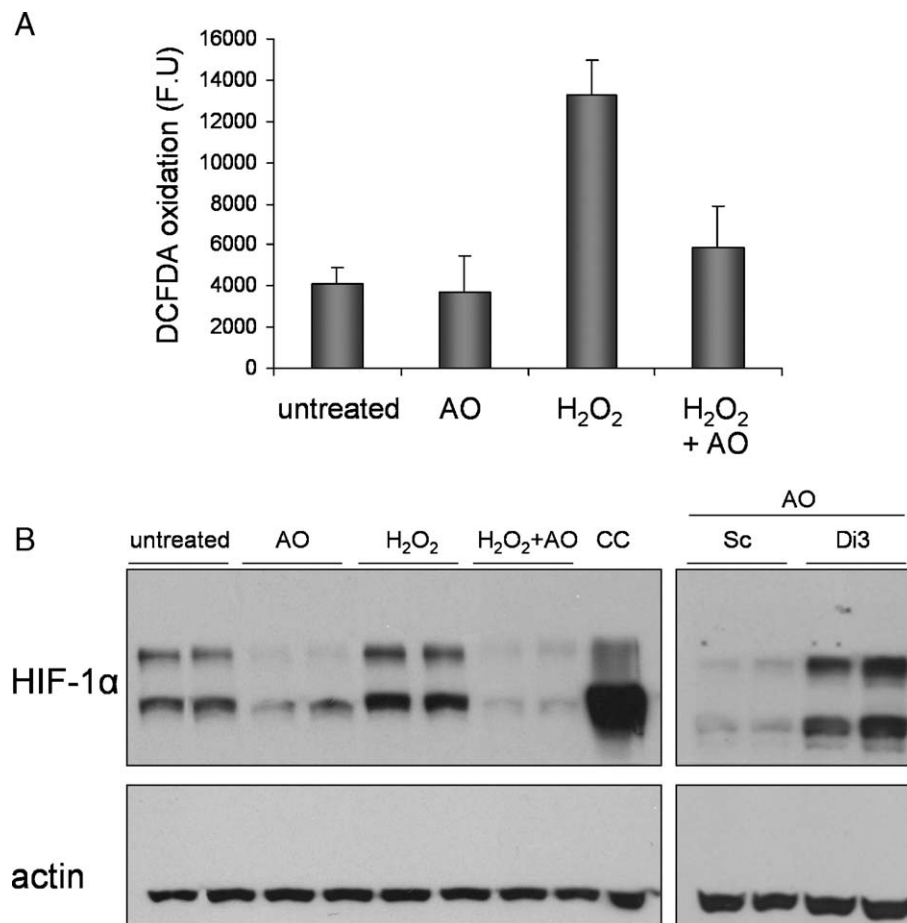

Fig. 2. (A) Antioxidants efficiently remove exogenously added hydrogen peroxide. HEK293 cells either left untreated or pre-treated with antioxidants (AO) [2 mM N-acetyl cysteine and 2 mM ascorbate] for 2 h. Cells were incubated for 30 min with 20 μM DCFDA (Molecular Probes) before further treating with or without 500 μM hydrogen peroxide. Cells were extracted in RIPA buffer 3 h later and DCFDA oxidation was analyzed in the lysate using a spectrofluometer. (B) Antioxidants prevented HIF1α induction in hydrogen peroxide-treated cells but not in SDH-deficient cells. HEK293 cells were treated with antioxidants (AO) with or without 500 μM hydrogen peroxide or transfected with control scrambled shRNA (Sc) or with shRNA targeting SDHD (Di3). HIF1α protein level was detected by western blot. CoCl2-treated cells (CC) were used as a positive control for HIF1α induction and actin level was used as a loading control.
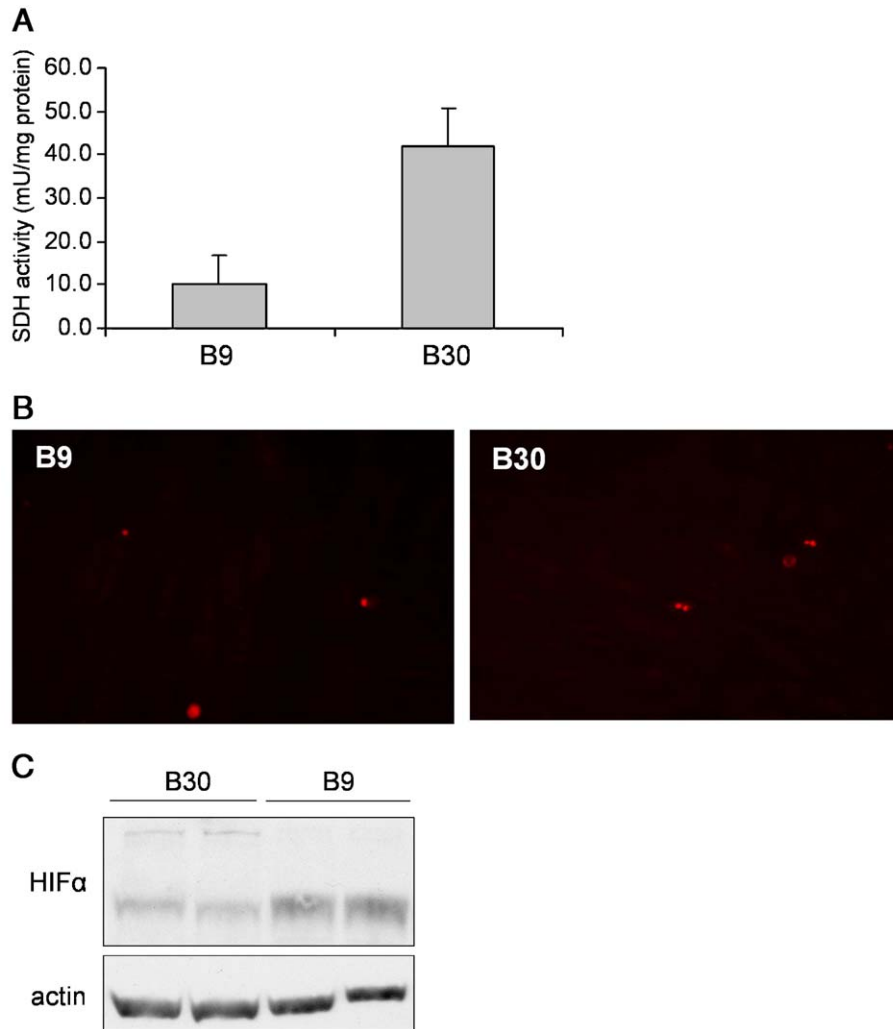
Fig. 3. (A) SDH activity was analyzed as was previously described [23] in the *SDHC*-mutated B9 cells and in the *SDHC*-reconstituted B30 cells. (B) ROS level was not increased in B9 cells compared to B30 cells. Cells were incubated with DHE and analyzed microscopically as described in Fig. 1. (C) HIFα protein level is elevated in the SDH-deficient B9 cells compared to B30 cells. Actin was used as a loading control.

CoCl$_2$ was used to inhibit PHD activity, suggesting that it is indeed a HIFα protein (data not shown).

4. Discussion

Although mutations in the B, C or D subunits of SDH have been predicted (and in one case of SDHC has been observed) to increase ROS production by restricting electron flow through complex II, there is currently no evidence which supports the contention that ROS mediates HIF1α stabilization in SDH-deficient cells. Indeed, to the contrary, the data presented here show that siRNA suppression of SDH activity stabilized HIF1α by a mechanism independent of ROS production. By using an RNA interference technique to transiently target SDH in cells and by using cells which are carrying an endogenous *SDHC* mutant, we have demonstrated both that SDH-inhibited cells do not generate ROS and that antioxidant treatment does not block HIF1α induction in transfected cells. These considerations, and the fact that tumours with SDH mutations fail to exhibit signs of oxidative stress, indicate that an alternate mechanism, namely metabolic signalling mediated by succinate, is likely to account for HIF stabilization under the pseudo-hypoxic conditions observed in SDH deficiency.

The present observations have important clinical implications as they indicate that antioxidants will not be effective in blocking HIF activation seen in SDH-deficient tumours. By contrast, PHD activity and thus normal low HIF1α levels in these tumours may be restored by any treatment which decreases or diverts succinate away from PHD and/or increases the concentration of α-ketoglutarate. Because HIF accumulation may promote tumour progression, drugs that inhibit HIF and its downstream targets may be useful in treating carcinomas. We suggest that manipulation of intracellular concentrations of key metabolic intermediates such as succinate and α-ketoglutarate with the capability to modulate PHD activity may provide the basis for a metabolic approach to cancer chemotherapy.

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