Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-α prolyl hydroxylase

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Summary

Several mitochondrial proteins are tumor suppressors. These include *succinate dehydrogenase* (*SDH*) and *fumarate hydratase*, both enzymes of the tricarboxylic acid (TCA) cycle. However, to date, the mechanisms by which defects in the TCA cycle contribute to tumor formation have not been elucidated. Here we describe a mitochondrion-to-cytosol signaling pathway that links mitochondrial dysfunction to oncogenic events: succinate, which accumulates as a result of SDH inhibition, inhibits HIF-α prolyl hydroxylases in the cytosol, leading to stabilization and activation of HIF-1α. These results suggest a mechanistic link between *SDH* mutations and HIF-1α induction, providing an explanation for the highly vascular tumors that develop in the absence of *VHL* mutations.

Introduction

Inherited or somatic mutations in subunits *B*, *C*, or *D* of the *SDH* genes are associated with the development of pheochromocytoma and paraganglioma (Baysal, 2003; Eng et al., 2003; Pollard et al., 2003; Rustin and Rotig, 2002). More recently, *SDHB* mutations have also been associated with renal cell carcinoma and papillary thyroid cancer (Neumann et al., 2004), and downregulation of *SDHD* was reported in gastric and colon carcinoma (Habano et al., 2003). Furthermore, mutations in *SDH* were shown to induce the hypoxia-response pathway in tumors (Baysal, 2003; Pollard et al., 2003). In particular, elevated levels of hypoxia inducible factor-1α (HIF-1α) in nuclei and the induction of hypoxia-inducible genes were observed in paraganglioma carrying an *SDHD* mutation (Gimenez-Roqueplo et al., 2001). HIF-α subunit is the oxygen-inducible component of the HIF transcription factor, a heterodimer comprised of HIF-α and HIF-β subunits (Pugh and Ratcliffe, 2003; Semenza, 2002). The physiological function of HIF is to promote adaptation of cells to low oxygen by inducing neovascularization and glycolysis (Pugh and Ratcliffe, 2003; Semenza, 2002). In addition, it was recently demonstrated that HIF activation resulting from *VHL* mutations promotes metastasis by inducing the expression of *met* and *CXCR4* (Pennacchietti et al., 2003; Staller et al., 2003). The *VHL* gene product (pVHL) is part of an E3 ubiquitin ligase complex that binds to the oxygen-dependent degradation (ODD) domain of HIF-α in an oxygen-dependent manner and targets it for degradation (Pugh and Ratcliffe, 2003; Semenza, 2002). Therefore, in tumors carrying *VHL* mutations that can no longer bind to and destabilize HIF-1α, HIF-1α is stabilized even under normoxic conditions (Kaelin, 2002).

The binding of pVHL to HIF-α is regulated by the hydroxylation of two specific prolyl residues in the ODD domain (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). Hydroxylation at the 4-position of Pro-402 and Pro-564 of HIF-α (numbers refer to human HIF-1α) enables formation of two hydrogen bonds to pVHL and increases the binding of pVHL to HIF-α by several orders of magnitude (Hon et al., 2002; Min et al., 2002). This posttranslational modification is catalyzed by the HIF-α-prolyl hydroxylases (HPH1-3 or PHD1-3) (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2002). PHD activity is dependent on molecular oxygen and is considered to be an important oxygen sensing mechanism in animal cells (Safran and Kaelin, 2003; Schofield and Ratcliffe, 2004). In addition to oxygen, the

SIGNIFICANCE

Succinate dehydrogenase (SDH) is a mitochondrial TCA cycle enzyme and a tumor suppressor. Inherited or somatic mutations in subunits B, C, or D of *SDH* lead to pheochromocytoma, paraganglioma, or renal cell carcinoma, and *SDH* downregulation is observed in gastric and colon carcinoma. No mechanism that links these mutations to tumor formation has been reported previously. In this study we show that succinate, a TCA cycle metabolite, is accumulated due to *SDH* downregulation and transmits an "oncogenic" signal from mitochondria to the cytosol. Once in the cytosol, succinate inhibits HIF-α prolyl hydroxylase (PHD) leading to HIF-1α stabilization under normoxic conditions. Thus, succinate can increase expression of genes that facilitate angiogenesis, metastasis, and glycolysis, ultimately leading to tumor progression.

PHDs utilize α-ketoglutarate as a cosubstrate and require ferrous iron (Fe^{2+}) and ascorbate as cofactors (Kaelin, 2002; Schofield and Zhang, 1999). The PHD isozymes belong to the Fe^{2+} and α-ketoglutarate-dependent family of dioxygenases that split molecular oxygen to hydroxylate their substrates and, in parallel, oxidize and decarboxylate α-ketoglutarate to succinate (Schofield and Zhang, 1999). The transcriptional activity of HIF-α is also regulated by hydroxylation of an asparaginyl residue in its C-terminal transactivation domain in a reaction catalyzed by factor inhibiting HIF (FIH) (Hewitson et al., 2002; Lando et al., 2002a, 2002b). Significantly, both α-ketoglutarate and succinate are TCA cycle intermediates; in the TCA cycle, succinate is converted to fumarate by SDH. Furthermore, succinate has been shown to inhibit some α-ketoglutarate-dependent oxygenases, including procollagen prolyl hydroxylase and thymine-7-hydroxylase (Holme, 1975; Myllyla et al., 1977). Succinate moves freely between the mitochondria and the cytosol via the dicarboxylic acid translocator in the mitochondrial inner membrane and the voltage-dependent anion channel (VDAC/porin) in the mitochondrial outer membrane. In this paper, we show that downregulation of SDH activity results in the accumulation of succinate in cells, which inhibits HIF-α hydroxylases (product inhibition), leading to the dissociation of pVHL from HIF-1α and consequently leading to HIF-1α stabilization and overall HIF activation. We suggest that succinate can act as an intracellular messenger, linking TCA cycle dysfunction to HIF regulation.

Results

Inhibition of SDH in cells using siRNA leads to HIF-1α accumulation and the induction of HIF transcription activity

To study the mechanism that links SDH inhibition to HIF-1α induction, the RNA interference (RNAi) technique was used to transiently target *SDH* in cells. Vectors encoding small interference RNA (siRNA) that target the *SDHD* subunit (Di3 or Di4) were constructed, and their function was analyzed by transfection into human embryonic kidney cells (HEK293). Transfection efficiency was analyzed by cotransfecting the cell population with a GFP-encoding vector and was ~50% in all experiments described below (data not shown). Endogenous *SDHD* mRNA levels were analyzed by RT-PCR and found to be lower in cells transfected with either of the two SDHD siRNA constructs (Di3 or Di4) as compared to scrambled siRNA (scRNAi) control-transfected cells (Figure 1A), demonstrating the effectiveness of Di3 and Di4. To analyze the efficacy of siRNA at inhibiting endogenous SDH activity, succinate and 2,6-dichloroindophenol (DCIP) were used as electron donor and acceptor, respectively, to spectrophotometrically measure SDH activity (Trounce et al., 1996). When cells were transfected with either Di3 or Di4, SDH activity was decreased by ~50% in the total cell population, as compared to scRNAi-transfected cells (Figure 1B). This suggests that SDH activity in transfected cells is severely diminished at the time of analysis. Transfection of any of the siRNAs had no effect on citrate synthase activity, an unrelated nuclear-encoded TCA cycle enzyme (data not shown). Since elevated levels of HIF-1α protein are observed in tumors harboring *SDH* mutations, the effect of SDH inhibition on HIF-1α protein levels was analyzed by Western blot. An induction of HIF-1α protein under normoxic conditions was observed in Di3- and Di4-transfected cells as compared to the control-transfected cells (Figure 1C).

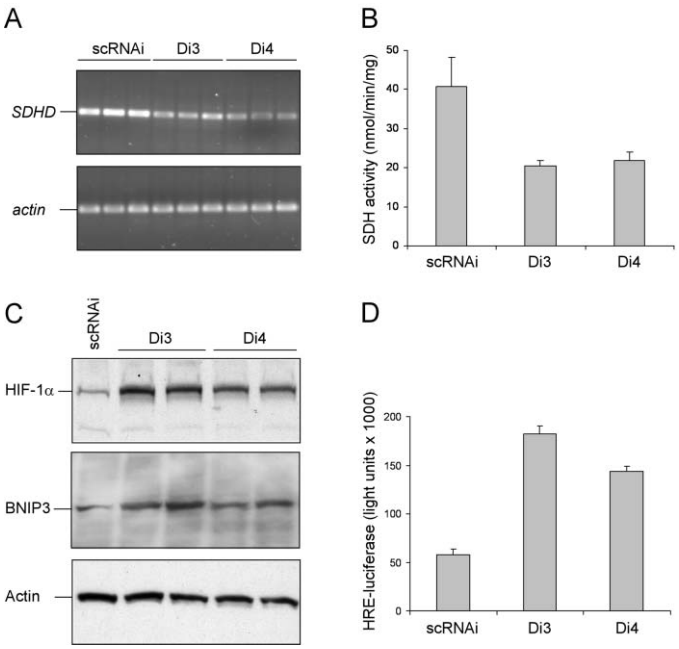

Figure 1. Inhibition of SDH activity increases HIF-1α levels and HIF activity

A: Cells were transfected (in triplicate) with either scrambled siRNA (scRNAi) or siRNA directed at *SDHD* subunit (Di3 or Di4). Following transfections, mRNA levels of endogenous *SDHD* and *actin* were quantified by RT-PCR.

B: Succinate-DCIP oxidoreductase activity was analyzed in cells transfected as in **A** to confirm inhibition of SDH activity.

C: Endogenous HIF-1α and BNIP3 protein levels, under normoxic conditions, were detected by Western blot analysis following transfection with scRNAi, Di3, or Di4. Actin was used as loading control.

D: HIF transcriptional activity following SDH inhibition (as in **C**) was assessed by the dual luciferase reporter assay system (Promega) using pGL2/HRE-luciferase as a reporter for HIF activity.

Moreover, when HIF activity was analyzed by cotransfection of a vector containing luciferase reporter gene downstream to a minimal promoter with a hypoxia responsive element (HRE), a 3-fold induction in HIF activity was observed in the SDH-inhibited cell population (Figure 1D). BNIP3 is a proapoptotic Bcl-2 family member that was shown to be induced in a HIF-dependent manner in hypoxic zones of tumors (Sowter et al., 2001). Analysis of the endogenous level of BNIP3 showed a marked induction of BNIP3 protein in SDH inhibited cells as compared to control-transfected cells (Figure 1C).

Next, we compared HIF-1α protein levels in SDH-inhibited cells under normoxic conditions to HIF-1α physiological levels under hypoxic conditions. Cells were transfected with either scRNAi or Di3 under normoxic conditions, or incubated at 3% oxygen or treated with $CoCl_2$, an inhibitor of PHD and a hypoxic mimetic compound (Safran and Kaelin, 2003), for 3 hr. Hypoxia or hypoxic mimetic treatment induced higher levels of HIF-1α than SDH inhibition (Figure 2A, upper panel). However, it is important to note that while all cells experienced hypoxic conditions, only the transfected cells have reduced SDH activity. When the expression of an endogenous HIF target gene, *VEGF*, was analyzed, a clear induction in *VEGF* mRNA levels was observed in the population of Di3-transfected cells (Figure 2B). Allowing for the transfection efficiency of the culture, the level

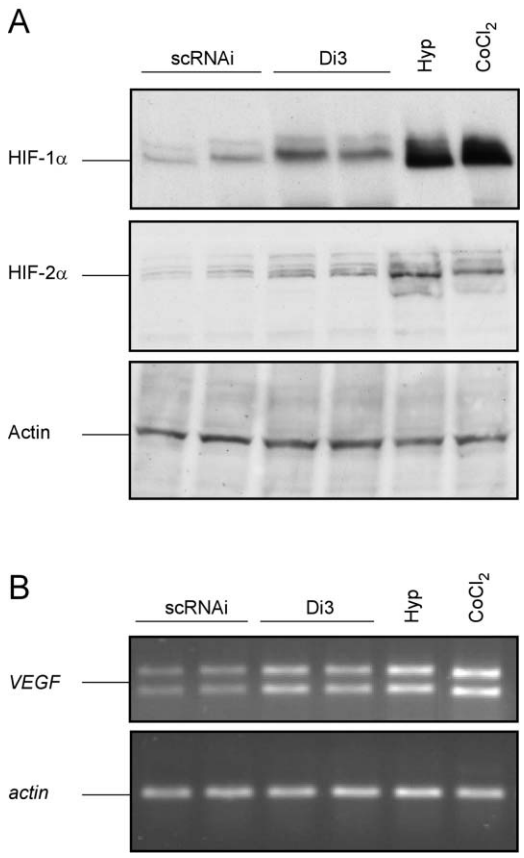

Figure 2. HIF-1α and HIF-2α induction in SDH inhibited or hypoxic cells

A: Protein levels of HIF-1α and HIF-2α in cells transfected with either control scRNAi or siRNA directed at *SDHD* subunit (Di3), or in cells exposed to hypoxic conditions (3% oxygen; 3 hr = Hyp) or to the hypoxic mimetic ($CoCl_2$). Actin protein level was used as loading control.

B: mRNA levels (analyzed by RT-PCR) of *VEGF* or *actin* in cells treated as in **A**. The two distinct products of *VEGF* cDNA are most likely due to alternatively spliced forms of the *VEGF* mRNA.

of *VEGF* mRNA in Di3-transfected cells is comparable to that in cells exposed to hypoxic conditions or $CoCl_2$ (Figure 2B).

HIF transcriptional activity is regulated by the levels of both HIF-1α and HIF-2α, which heterodimerize with the HIF-β subunit and induce the transcription of genes containing HREs in their promoter/enhancer region. However, the regulation of HIF-1α and HIF-2α expression is not necessarily similar in tumors (Beasley et al., 2002) or during physiological response to hypoxia or hypoglycemia (Brusselmans et al., 2001). For this reason, HIF-2α induction was also analyzed in SDH-inhibited cells. Although there was a noticeable increase in HIF-2α protein in Di3-transfected cells, this increase was not as pronounced as that of HIF-1α (Figure 2A). Moreover, when cells were exposed to hypoxia or hypoxic mimetic conditions, it was evident that HIF-1α is more subject to induction than HIF-2α (Figure 2A). This observation may be explained by the fact that different PHD enzymes, i.e., PHD2 and PHD3, differentially regulate the stability of HIF-1α and HIF-2α, respectively (Appelhoff et al., 2004), and these enzymes may exhibit differences in their response to succinate and/or hypoxia. Alternatively, other mechanisms may facilitate HIF-2α degradation in these cells, despite the fact that PHD activity is inhibited.

Reactive oxygen species do not play a role in HIF-1α induction after SDH inhibition

One possible explanation for the stabilization of HIF-1α in SDH-inhibited cells is the generation of reactive oxygen species (ROS) (Baysal, 2003; Eng et al., 2003; Pollard et al., 2003; Rustin and Rotig, 2002). To test this hypothesis, we analyzed ROS generation in siRNA-transfected cells using dihydroethidium (DHE), a fluorescent probe for superoxide. The tumor suppressor p53, which is known to elevate ROS production (Polyak et al., 1997), was used as a positive control. While there was a pronounced increase in ROS level following transfection with a p53-encoding plasmid as compared to control-transfected cells (Figures 3A and 3B), there was no detectable difference in DHE oxidation in Di3 and Di4-transfected cells relative to the control (compare Figures 3C and 3D to 3A). In addition, we examined siRNA-transfected cells for evidence of ROS formation and oxidative stress by measuring the levels of reduced glutathione (GSH) and total glutathione (GSH + GSSG). In agreement with the DHE results, no significant difference was observed in the redox state of SDH-inhibited cells as compared to the control scRNAi-transfected cells (Table 1).

Succinate is accumulated in SDH-inhibited cells

An alternative explanation for the induction of HIF-1α in SDH-deficient cells is the accumulation of succinate, the substrate for SDH in mitochondria and a product of PHD in the cytosol. Succinate can move freely between mitochondria and the cytosol via the dicarboxylic acid translocator in the mitochondrial inner membrane and VDAC in the mitochondrial outer membrane. Once in the cytosol, succinate may impede PHD activity by inhibiting the decarboxylation of α-ketoglutarate to succinate, a required coreaction for PHD while hydroxylating its target.

To determine if SDH inhibition led to an accumulation of succinate, gas chromatography-mass spectrometry (GCMS) was used to measure succinate levels in cells following transfection with the indicated siRNA. The amount of succinic acid in cell extracts was calculated by comparison to a known amount of deuterated (D_4)-succinic acid used as a reference in the lysis solution (Figure 4). When cells were transfected with either of the *SDHD*-targeting siRNAs, an increase in succinic acid level was observed as compared to control-transfected cells (see Figure 4 for Di3). The concentration of succinate in the transfected cells was then estimated based on 50% transfection efficiency as judged by cotransfection with GFP and the mean cell volume measured by Coulter counter prior to extraction. A significant increase in succinate levels was observed in SDH-inhibited cells (Table 1). It is noteworthy that in contrast to succinate, the basal level of α-ketoglutarate was estimated to be ~50 μM, with no significant change after SDH inhibition (data not shown).

Succinate inhibits PHD activity in vitro

To test the hypothesis that the elevated succinate level may inhibit PHD by product inhibition, PHD activity was analyzed in vitro in the presence of increasing amounts of succinate (Figure 5A). In vitro translated HA-tagged ODD domain was used as a substrate, and cell extracts were used as a source of PHD activity. When the HA-ODD is incubated with cell extracts in the presence of physiological levels of α-ketoglutarate, Fe^{2+}, and ascorbate, it undergoes hydroxylation and migrates faster

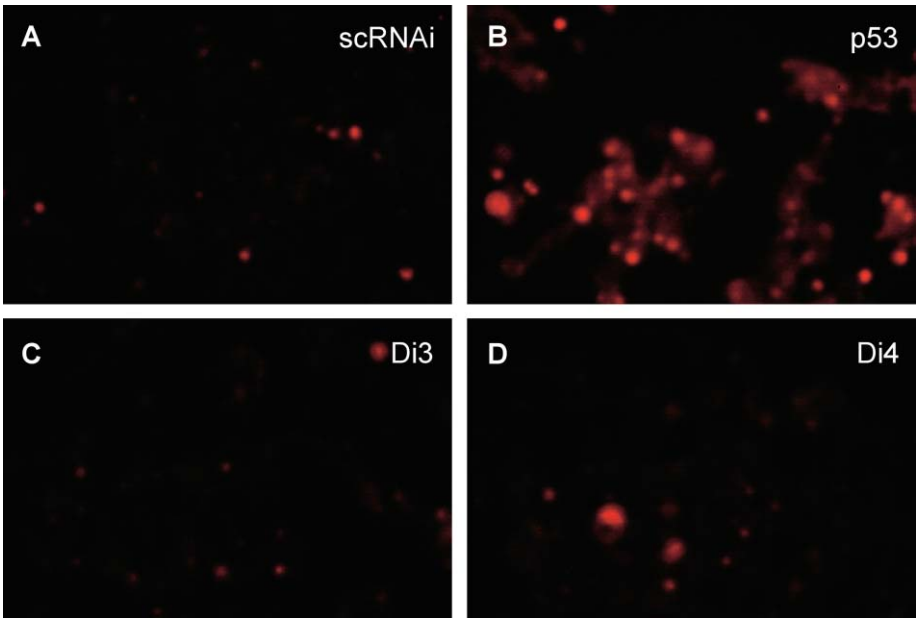

Figure 3. Inhibition of SDH activity has no effect on ROS production

A–D: Microscopic analysis of DHE oxidation in cells transfected with either scRNAi control (**A**), p53, as a positive control (**B**), or siRNA directed at *SDHD* subunit Di3 (**C**) or Di4 (**D**).

on SDS-PAGE (Huang et al., 2002; Ivan et al., 2001). Deferoxamine (DFO) is an iron chelator that inhibits PHD activity and can therefore be used as a hypoxia mimetic agent to stabilize HIF-α levels in certain cells (Safran and Kaelin, 2003). DFO inhibited PHD activity in vitro and retarded HA-ODD mobility on SDS-PAGE (Figure 5A, lane 6). Importantly, increasing amounts of succinate in the hydroxylation reaction led to a dose-dependent decrease in the production of the faster migrating, hydroxylated HA-ODD (Figure 5A, lanes 1–5). The IC_{50} was ~0.5 mM for succinate under these reaction conditions, a level that is attainable in SDH-inhibited cells (Table 1).

Elevation of succinate level following downregulation of SDH leads to PHD inhibition and HIF-1α stabilization in cells

To determine if high levels of succinate are sufficient to elevate HIF-1α levels in cells, we incubated cells with the membrane-permeable dimethyl-ester succinic acid derivative (DMS) and analyzed for HIF-1α levels. Although the rates of DMS uptake, its conversion into succinate, and its metabolism are unknown, cells incubated with 20 mM DMS for 48 hr showed elevated levels of HIF-1α under normoxic conditions (Figure 5B). Moreover, a clear accumulation of HIF-1α in the nuclei of cells treated with DMS was observed (Figure 5C), resembling the HIF-1α pattern of expression in SDH-deficient tumors (Gimenez-Roqueplo et al., 2001).

Next, it was determined whether elevated succinate levels following SDH inhibition decreased PHD activity in cells. A GFP-ODD domain fusion protein was used, the degradation of which is enhanced by pVHL overexpression (Figure 6Aiii and 6Aiv) and

Table 1. Effects of SDH inhibition on cellular redox state and succinate concentration

	scRNAi	Di3	Di4
GSH (nmol/mg)	44.0 ± 5.03	42.3 ± 9.76	46.3 ± 1.05
GSH + GSSG	45.9 ± 5.13	44.2 ± 9.80	48.7 ± 0.82
Succinate (μM)	120.6 ± 32.0	446.8 ± 86.1	440.9 ± 36.0

The levels of reduced (GSH) and total (GSH + GSSG) glutathione presented as nmol/mg protein and the level of succinate (μM) in cells transfected with the indicated siRNA. Results are average ± SD of three or six independent transfections for glutathione or succinate, respectively.

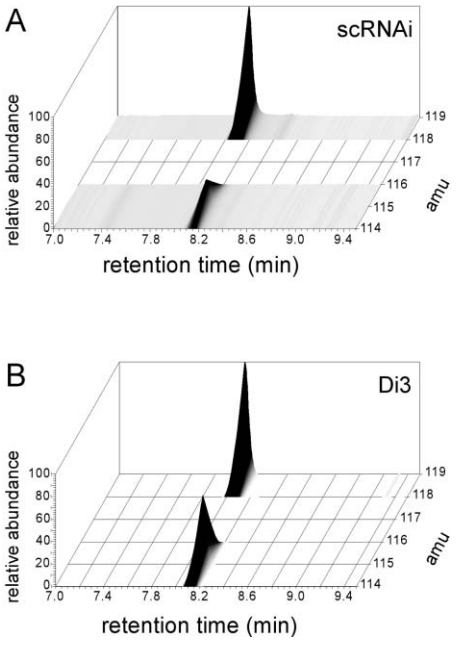

Figure 4. SDH inhibition leads to increased levels of succinate

GCMS profiles of selected ionized fragments in extracts from cells transfected with either scRNAi (**A**) or Di3 (**B**). Deuterated (D_4)-succinic acid was used as a reference and was identified by its major ion component of 119 amu at retention time of 8.05 min. Succinic acid was identified by its major ion component of 115 amu at retention time of 8.15 min.

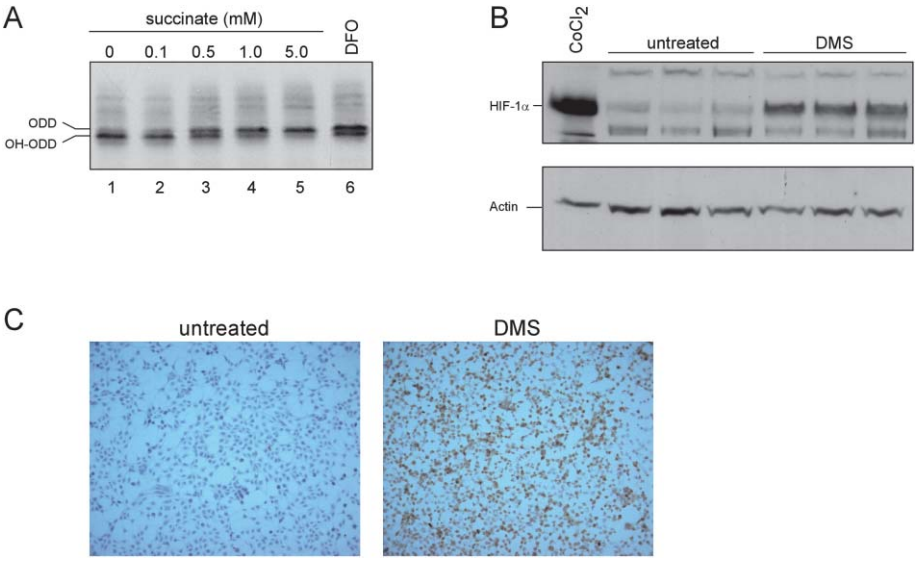

Figure 5. Succinate inhibits PHD activity in vitro and induces HIF-1α levels in cells

A: Cell extracts were mixed with in vitro translated HA-ODD in the presence of Fe^{2+}, ascorbate, and α-ketoglutarate. Where indicated, succinate was added to the reaction. Deferoxamine (DFO), an iron chelator, was added to inhibit PHD activity. The hydroxylated polypeptide migrates faster on SDS-PAGE.

B: HIF-1α and actin levels were assessed by Western blot of extracts from either untreated cells or from cells treated with DMS or $CoCl_2$ for 48 hr under normoxic conditions.

C: Immunostaining was performed to analyze the subcellular localization of HIF-1α in DMS-treated and untreated control cells.

therefore depends on hydroxylation of the ODD domain for degradation, in contrast to GFP (Figures 6Ai and 6Aii). When cells were cotransfected with GFP-ODD, HA-tagged pVHL, and either of the siRNA constructs, higher GFP levels were observed in Di3- and Di4-transfected cells compared to scRNAi-transfected cells (Figures 6Aiv–6Avi). To quantify the differences in GFP-ODD levels, cells were lysed, and the amounts of GFP-ODD in the extracts were determined by Western blot using an anti-GFP antibody (Figure 6B, upper panel). The differences in GFP-ODD levels could not be attributed to differences in pVHL levels in the transfected cells, since HA-pVHL was expressed equally in each of the transfections (Figure 6B, lower panel). Therefore, it is likely that in SDH-inhibited cells, the HIF-1α ODD domain is hydroxylated less efficiently, and consequently the binding of pVHL to GFP-ODD is reduced.

To test this possibility, cells were transfected with HA-pVHL, either scRNAi or Di3, and GFP-ODD or GFP (the latter used as negative control). GFP fluorescence was analyzed in extracts prior to or after immunoprecipitation with an anti-HA antibody, to measure both total and pVHL-bound GFP. Following stringent

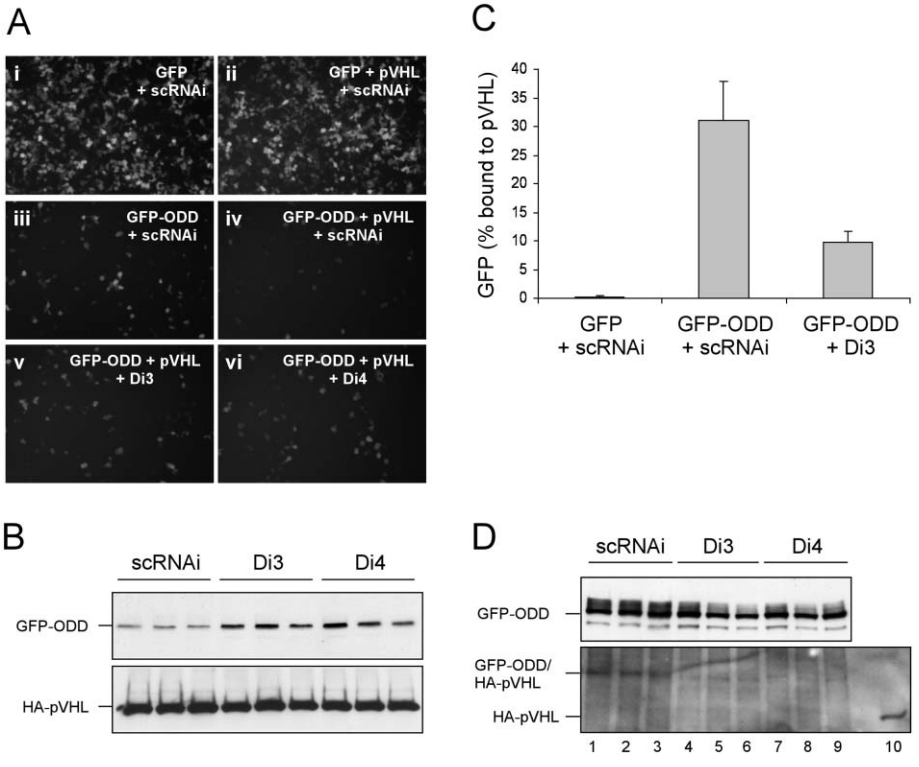

Figure 6. Inhibition of SDH decreases PHD activity

A: Cells were transfected with plasmids encoding either GFP (i and ii) or GFP-ODD (iii–vi) without pVHL (i, iii, v) or with pVHL (ii, iv, vi) together with one of the indicated siRNA constructs: scRNAi (i–iv), Di3 (v), or Di4 (vi). GFP fluorescence was detected microscopically.

B: GFP-ODD and HA-pVHL protein levels in cells transfected (in triplicate) as in **Aiv–vi**.

C: Cells were transfected with plasmids encoding GFP or GFP-ODD together with HA-pVHL and with the indicated siRNA. GFP fluorescence was analyzed in cell extracts before and after immunoprecipitation with an anti-HA antibody. The results are presented as the percent of GFP fluorescence bound to HA-pVHL and are the average and standard deviation of three independent transfections.

D: Direct detection of ODD domain hydroxylation by far Western blot analysis. Cells were transfected (in triplicate) with plasmids encoding GFP-ODD and the indicated siRNA (but without HA-pVHL). Protein extracts were blotted onto nitrocellulose membrane and the binding of immunopurified HA-pVHL to the blotted GFP-ODD protein was detected using an anti-HA antibody. 10 ng of the immunopurified HA-pVHL protein was loaded on lane 10.

washes, immunoprecipitated proteins were eluted using HA peptide, and GFP fluorescence was analyzed (pVHL-bound GFP) and compared to that of extracts prior to immunoprecipitation (total GFP). Results are presented as percent pVHL-bound GFP of total GFP (Figure 6C). Although GFP-transfected cells have much higher total fluorescence than GFP-ODD-transfected cells (Figures 6Aii and 6Aiv), pVHL-associated GFP fluorescence was hardly detectable, demonstrating that in the GFP-ODD transfected cells, pVHL-associated GFP fluorescence is dependent on the ODD domain. In Di3-transfected cells, even though the total GFP-ODD level was higher than in scRNAi-transfected cells (Figures 6Aiv and 6Av), the levels of pVHL-associated fluorescence were significantly lower (Figure 6C). Since pVHL binding to the ODD domain is dependent on PHD activity (Kaelin, 2002; Safran and Kaelin, 2003), these results indicate that inhibiting SDH activity in cells results in decreased PHD activity.

Finally, cells were transfected with GFP-ODD and either scRNAi, Di3, or Di4 (but not with pVHL). The hydroxylation status of GFP-ODD was determined by examining the binding of purified HA-pVHL protein to GFP-ODD expressed in these cells by far Western blot. The nitrocellulose membrane was probed with purified HA-pVHL protein and GFP-ODD/HA-pVHL complexes were detected by an anti-HA antibody (Figure 6D). HA-pVHL binding to the GFP-ODD extracted from scRNAi-transfected cells was observed (Figure 6D, lanes 1–3), but a large decrease in HA-pVHL binding to GFP-ODD was evident in Di3- and Di4-transfected cells (Figure 6D, lanes 4–9). These results suggest that in SDH-inhibited cells, PHD activity is reduced.

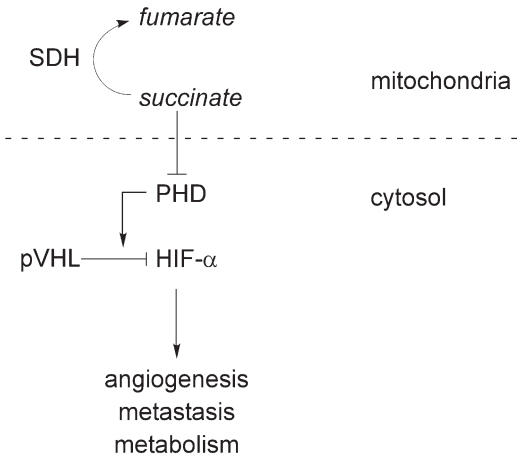

Figure 7. A schematic model that summarizes the role of succinate in the mitochondrion-to-cytosol signaling pathway

Succinate accumulated in the mitochondria due to SDH inhibition is transported to the cytosol. Elevated cytosolic succinate inhibits PHD and thereby HIF-α hydroxylation. Consequently, pVHL binding to HIF-α is decreased, and elevated HIF activity induces expression of genes that facilitate angiogenesis, metastasis, and metabolism, leading to more aggressive tumors.

Discussion

Our results provide evidence for a previously unidentified mechanism for regulating PHD activity and consequently HIF-1α levels. We describe a direct signaling pathway that links mitochondrial dysfunction to tumorigenesis, providing a mechanistic link between mutations in *SDH* and the highly vascularized tumors which develop as a consequence of HIF-1α induction in the absence of *VHL* mutations. These results show that succinate may function as an intracellular messenger between mitochondria and the cytosol and has a profound effect on cytosolic enzymes (PHD) and consequently on nuclear events (gene expression by HIF) (Figure 7). It is noteworthy that, in addition to the described *SDH* mutations in pheochromocytoma and paraganglioma, significant reduction in *SDH* expression has also been described in gastric and colorectal carcinoma (Habano et al., 2003), in particular during transition to the more aggressive Dukes' stage C colorectal cancer, as compared to the confined Dukes' stage B tumors (Frederiksen et al., 2003). Therefore, it is plausible that the succinate signaling pathway elucidated here plays a more general role in tumorigenesis, in particular in the transition to a more infiltrating form.

It is frequently hypothesized in the literature that ROS generation due to *SDH* mutations may mediate HIF-1α induction (Baysal, 2003; Eng et al., 2003; Pollard et al., 2003; Rustin and Rotig, 2002). However, to our knowledge, there are no published data that actually show ROS generation by the mutated or inhibited SDH in mammalian cells or human tumors. Indeed, although we found that hydrogen peroxide can induce HIF-1α levels as was previously described (Chandel et al., 2000) (data not shown), we observed neither ROS generation nor a shift to a more oxidized state following SDH inhibition (Figure 3 and Table 1). Structural and functional analysis of bacterial SDH complex suggested a mechanism for ROS production at the FAD site (Yankovskaya et al., 2003). The study of Yankovskaya et al. further indicated that the bacterial homolog fumarate reductase, which catalyzes the reverse reaction of SDH, is more likely to produce ROS than SDH. SDH itself can work in reverse and generate ROS under hypoxic conditions (Paddenberg et al., 2003), but such a reaction (and ROS generation) does not occur under normoxic conditions and is unlikely to be catalyzed by mutated SDH (Paddenberg et al., 2003). ROS production following SDH inhibition has been reported previously to occur in *C. elegans* carrying a *SDHC* mutation, *mev-1* (Senoo-Matsuda et al., 2001). Interestingly, this mutation has no effect on succinate levels in the nematode, indicating that succinate was removed by oxidation to fumarate and that oxygen may have been used as the final electron acceptor, hence generating superoxide ions (Senoo-Matsuda et al., 2001). This observation is not consistent with the data reported in the present study, and may suggest either that human and nematode SDH differ in their ability to generate ROS, or that different mutations have different outcomes. The observation that succinate level is elevated in tumors with SDH mutations (P. Rustin; personal communication) suggests that our model is more relevant for human tumors.

Succinate has been recently shown to be a ligand for a G protein-coupled receptor, suggesting that succinate can also be released from cells under some physiological/pathological circumstances (He et al., 2004). It is likely that succinate may function as a physiological as well as a pathological intracellular messenger. For instance, physiological levels of nitric oxide (NO) can block the induction of HIF-1α under hypoxic conditions by preventing oxygen consumption at cytochrome c oxidase (complex IV), hence allocating more oxygen for PHD activity in the cytosol (Hagen et al., 2003). Under these conditions, SDH

inhibition and succinate accumulation reverse this effect (data not shown). This suggests a more complex mechanism for HIF induction under hypoxic conditions involving oxygen, NO, and succinate levels. Hence, different cells may have different thresholds and mechanisms of HIF activation. Moreover, unlike pVHL, which regulates HIF-1α levels, succinate may additionally regulate HIF activity by modulating another α-ketoglutarate dependent dioxygenase, FIH, thereby altering the ability of HIF-1α to bind the coactivator p300 (Hewitson et al., 2002; Lando et al., 2002a, 2002b). Such a regulatory mechanism may dictate the outcome (the pattern of gene expression) of HIF activity and add another level of complexity to this signaling pathway.

Experimental procedures

Plasmids

The *SDHD* siRNA hairpins were cloned into pBABE-Puro ΔLTR together with the U6 promoter, as previously described (Fox et al., 2003). A PCR-based strategy was employed using pEF6-hU6 as a template and primers containing *SDHD* DNA hairpin sequences (Di3 = sense 5′-GGTCAGACCT GCTCATATCTCAGCA-3′; Di4 = sense 5′-GGTGTGGAGTGCAGCACATA CAC-3′). scRNAi was cloned into pSuperRetro (OligoEngine) with the hairpin sequence, sense, 5′-GATACGGTAGGGCGACAA-3′.

pGL2/HRE-luciferase was generated by inserting 3 copies in tandem of the 24-mer oligonucleotide (5′-tgtcacgtcctgcacgactctagt) in front of a minimal thymidine kinase promoter into pGL2-basic (Promega). The oligonucleotide contains 18 bp from PGK promoter including the HRE.

pRK5/HA-ODD was generated by cloning the human HIF-1α ODD domain (amino acids 530–652) in frame into a vector containing HA-tag and Gal4 DNA binding domain. Then the entire cDNA was cloned into pRK5 vector.

pEGFP/ODD, the plasmid encoding the GFP-ODD domain fusion protein, was generated as follows: A PCR fragment of *hHIF-1α* ODD domain was generated using PRK5/HA-ODD as a template and ligated in frame into pEGFP-C1 (Clontech).

RT-PCR analysis

Cells were treated as indicated in the text and total RNA was isolated using RNA-B solution (Biogenesis) according to the manufacturer's instructions. Semiquantitative reverse transcription was performed using a GeneAmp RNA PCR kit (Applied Biosystems). RNA (1 μg) was reverse-transcribed using random hexamers and PCR was carried out with the following conditions: 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 57°C, 1 min at 72°C, then 15 min at 72°C. Primers used for amplification were: *SDHD* forward 5′-ATGGCGGTTCTCTGGAGGCTG-3′; reverse 5′-GAGCTTCCAC AGCATGGCAAC-3′, *VEGF* forward 5′-ATGAACTTTCTGCTGTCTTGGGT-3′; reverse 5′-TCACCGCCTCGGCTTGTCACA-3′, *actin* forward 5′-TCCATCA TGAAGTGTGACGT-3′; reverse 5′-TACTCCTGCTTGCTGATCCAC-3′.

In vitro PHD activity

HA-ODD was in vitro translated (IVT) using wheat germ extract (Promega), and 4 μl aliquots of the IVT reaction were incubated with 50 μl of HEK293 cellular extracts (20 mM Tris [pH 7.4], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, supplemented with "complete" protease inhibitor cocktail [Roche] and 100 μM ALLN). The reaction was carried out for 15 min at 37°C in the presence of 5 mM ascorbate and 100 μM $FeCl_2$ with either 5 mM DFO or the indicated amounts of succinate. Reactions were terminated by adding Laemmli sample buffer and immediate boiling. Following SDS-PAGE, samples were analyzed by Western blot using an anti-HA antibody.

SDH activity (complex II; succinate-DCIP oxidoreductase)

Cells were lysed with 0.1% v/v Triton X100 in an assay buffer composed of 25 mM $KHPO_4$ (pH 7.4), 20 mM succinate, 50 μM decylubiquinone, 5 μM rotenone, 2 μM antimycin A, and 10 mM NaN_3. Following a 15 min incubation at room temperature, the baseline absorbance at 600 nm was recorded and the reaction initiated by adding 50 μM DCIP ($\epsilon = 21\ mM^{-1}\ cm^{-1}$). The change in absorbance was monitored for 2–3 min before and after addition of 50 μM 2-thenoyltrifluoroacetone (TTFA), a complex II inhibitor used to confirm reaction specificity. The specific activity of TTFA-sensitive succinate-DCIP oxidoreductase is reported as nmol/min/mg cell protein.

Redox state analysis

For microscopic analysis of ROS generation, cells were transfected as indicated in the text and 30 hr later incubated with 10 μM DHE (Molecular Probes) for 30 min followed by fluorescent microscopic analysis.

For studying reduced and total glutathione levels, cell lysates were prepared using a lysis buffer (Cell Signaling) freshly supplemented with protease 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. Fluorescence 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.

Succinic acid quantification by GCMS

Cell extracts were prepared in 90% methanol, 10% acetic acid containing 1 μg/ml (D_4)-succinic acid. Extracts were evaporated to dryness under nitrogen at 60°C and methylated by redissolving in 1% HCl in methanol for 10 min at 60°C. The extracts were redried and dissolved in hexane and 1 μl was injected into an Automass Multi GCMS system. The instrument was fitted with a ZB-1 column (30 meters × 0.32 mm id × 1 μm film), the oven was programmed as follows: 80°C (5 min) then 10°C/min to 170°C, the head pressure was 60 kPa. The mass spectrometer was operated in EI mode at 70 eV and selected ion monitoring was carried out for ions at 119 and 91 amu (methyl ester of [D_4]-succinic acid) and 115 and 87 amu (methyl ester of succinic acid).

Immunostaining

HEK293 cells plated on fibronectin-coated chamber slides were incubated in DMEM or DMEM supplemented with 20 mM DMS for 24 hr. The cells were fixed in formalin and permeabilized in a 0.05% saponin solution followed by incubation in 0.3% hydrogen peroxide. Subcellular localization of HIF-1α was monitored by staining with a monoclonal anti-HIF-1α antibody (BD Biosciences Pharmingen) followed by peroxidase-conjugated secondary antibody (Immpress reagent, Vector Laboratories) and diaminobenzidine (DAB) substrate to detect the signal. Cells were counterstained with hematoxylin.

Protein analyses

For HIF-1α immunoblot, cells were extracted in Laemmli sample buffer and for all other analyses in Tris lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.5% NP40, supplemented with "complete" protease inhibitor cocktail [Roche] and 100 μM ALLN). Following SDS-PAGE, proteins were blotted onto nitrocellulose and analyzed with the following antibodies: anti-HIF-1α (BD Biosciences), anti-HA (Roche), anti-GFP (BD Biosciences), or anti-actin (Sigma). Immunoprecipitation for HA-pVHL was carried out using an anti-HA antibody (Roche) and protein G Sepharose beads (Phramacia) in Tris lysis buffer. Proteins were eluted by incubating the beads with 1 mg/ml HA-peptide in TBS for 15 min at 37°C. Eluates were immediately subjected to fluorometric analysis in a 96-well plate fluorometer (Molecular Devices) to determine GFP or GFP-ODD levels.

Preparation of HA-pVHL protein as a probe for far Western blot analysis was carried out as follows: pRc-CMV/HA-pVHL was transfected into HEK293 cells, and 30 hr later, HA-pVHL protein was immunopurified from the cell extract on an anti-HA matrix (Roche) according to manufacturer's instructions. Following elution of the protein with HA peptide, the eluate was dialyzed overnight in TBS and HA-pVHL levels were assessed by SDS-PAGE followed by Coomassie blue staining and Western blot analysis.

For far Western blot analysis, protein extracts from GFP-ODD-expressing cells were blotted onto nitrocellulose membrane and blocked in 5% nonfat dry milk in TBST for 2 hr. Following several washes with TBST, HA-pVHL protein in TBST/milk (1 μg/ml) was added to the nitrocellulose membrane and incubated over night at 4°C, followed by several washes with TBST and detection with an anti-HA antibody.

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