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REVIEW

Rare insights into cancer biology

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Cancer-associated mutations have been identified in the metabolic genes succinate dehydrogenase (*SDH*), fumarate hydratase (*FH*) and isocitrate dehydrogenase (*IDH*), advancing and challenging our understanding of cellular function and disease mechanisms and providing direct links between dysregulated metabolism and cancer. Some striking parallels exist in the cellular consequences of the genetic mutations within this triad of cancer syndromes, including accumulation of oncometabolites and competitive inhibition of 2-oxoglutarate-dependent dioxygenases, particularly, hypoxia-inducible factor (HIF) prolyl hydroxylases, JmjC domain-containing histone demethylases (part of the JMJD family) and the ten-eleven translocation (TET) family of 5methyl cytosine (5mC) DNA hydroxylases. These lead to activation of HIF-dependent oncogenic pathways and inhibition of histone and DNA demethylation. Mutations in *FH*, resulting in loss of enzyme activity, predispose affected individuals to a rare cancer, hereditary leiomyomatosis and renal cell cancer (HLRCC), characterised by benign smooth muscle cutaneous and uterine tumours (leiomyomata) and an aggressive form of collecting duct and type 2 papillary renal cancer. Interestingly, loss of FH activity results in the accumulation of high levels of fumarate that can lead to the non-enzymatic modification of cysteine residues in multiple proteins (succination) and in some cases to their disrupted function. Here we consider that the study of rare diseases such as HLRCC, combining analyses of human tumours and cell lines with *in vitro* and *in vivo* murine models has provided novel insights into cancer biology associated with dysregulated metabolism and represents a useful paradigm for cancer research.

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DYSREGULATED METABOLISM: AEROBIC GLYCOLYSIS

Almost a century ago Otto Warburg reported that cancer cells exhibited dysregulated metabolism compared with normal cells and hypothesised that respiration defects in cells, and a slow adaptation to enhanced aerobic glycolysis, constituted the metabolic switch that caused cancer.[1,2] Until recently, research interest in this so-called 'Warburg effect' waned in favour of the identification and investigation of the crucial role of 'oncogenes' in cancer. That said, the enhanced aerobic glycolysis exhibited by some cancer cells provides them with a characteristic signature and results in increased dependence on glucose.[3] This phenotype has been exploited to image solid tumours through the use of ^{18}fluoro-2-deoxy-glucose positron emission tomography, as increased glucose uptake by tumour cells leads to accumulation of labelled derivative.[4] Cancer cell lines are routinely cultured in medium containing very high levels of glucose (4.5 g/l; 25 mM), approaching blood glucose levels observed in diabetic individuals.[5] Also, some cancer cells and tissues convert glucose to lactate in normoxia (normal oxygen), a process that only occurs in normal cells under conditions of hypoxia (reduced oxygen).[6] Increased glycolysis is also associated with the hypoxia observed in most solid tumours, or the pseudo-hypoxia characteristic of cells deficient in fumarate hydratase (FH), succinate dehydrogenase (SDH) and von-Hippel-Lindau protein (VHL).[7,8]

There are important caveats to associating cancer cells exclusively with the 'Warburg effect'; it is not specific to cancer cells and many cancer cells do not exhibit it, retaining instead mitochondrial respiration. In addition, glucose metabolism *via* glycolysis cannot provide all the building blocks (for example, nitrogen) required by a dividing cell such as for production of biomass and DNA division.[3] Hence, logically such cells require other sources of fuel and need to adapt their use of other metabolic pathways adding to a profile of dysregulated metabolism. Therein may lie a key to understanding the multiple steps in oncogenesis; cells are exquisitely adept at adapting their metabolism as a stress response and such altered metabolism may represent both a driving force in oncogenesis and also an Achilles' heel for therapeutic targeting.

DYSREGULATED METABOLISM: A 'HALLMARK' OF CANCER

Within the past decade there has been a major resurgence of interest and excitement in the links between cancer and altered metabolism, now identified as a 'hallmark' of malignancy.[9] We have entered a 'golden era' in metabolism studies increasing our understanding of normal cell metabolism and an appreciation of the extent and details of dysregulated metabolism associated with cancer (Figure 1).[10] New insights have come from multiple sources; made possible as a result of the identification of mutated metabolic enzymes leading to hereditary cancer syndromes,[11,12] and the use of exquisitely sensitive technologies such as mass spectrometry and nuclear magnetic resonance, combined with labelling of cellular metabolites.[13] These technologies also offer the capacity to analyse altered metabolite levels in a clinical setting (Figures 2 and 3).[14–16] The development of *in vivo* murine models and assorted cell lines has

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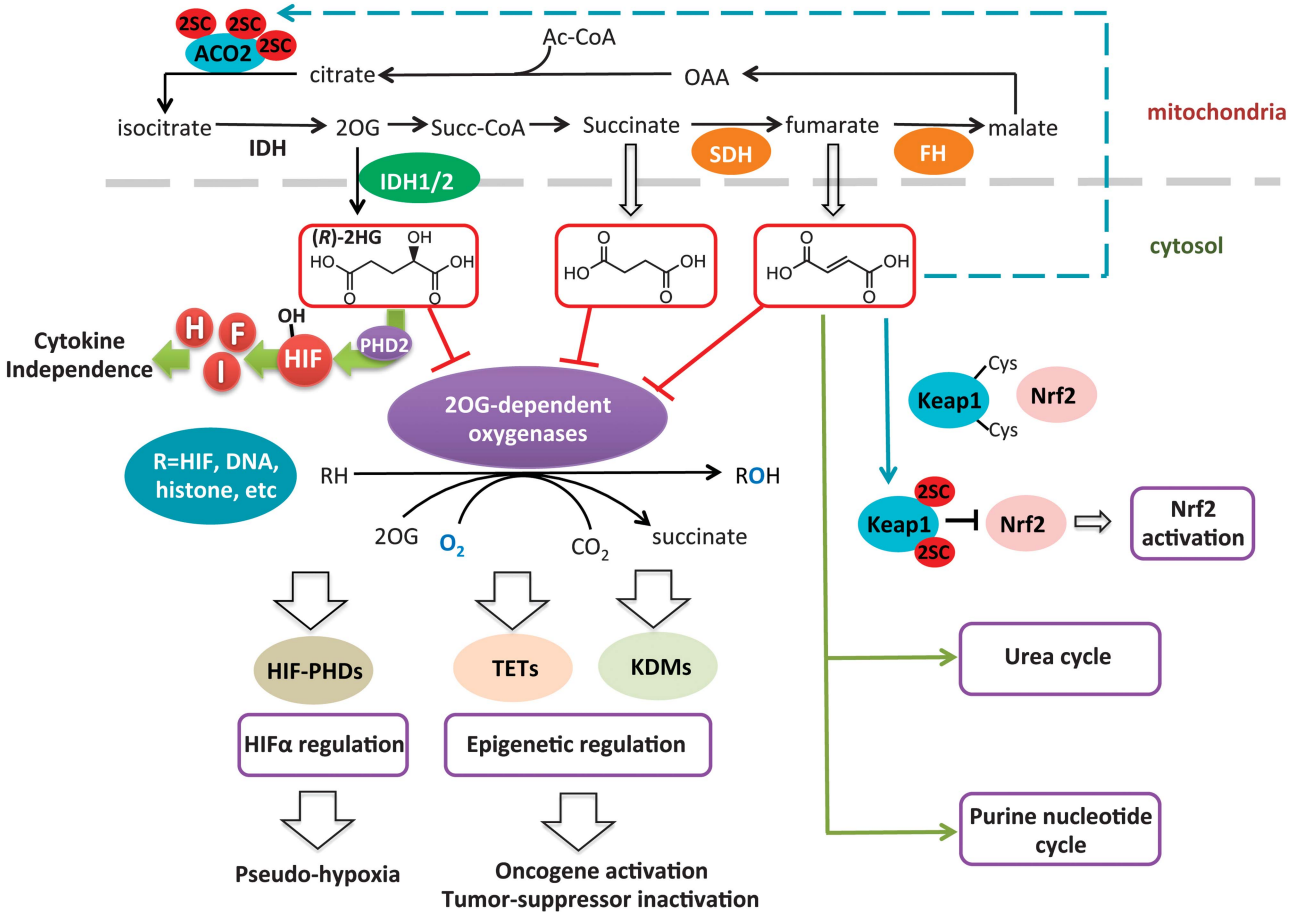

Figure 1. Candidate mechanisms for the oncogenic roles of (*R*)-2-hydroxyglutarate ((*R*)-2HG), succinate and fumarate. (*R*)-2HG is the product of gain-of-function mutations in the cytosolic and mitochondrial isoforms of isocitrate dehydrogenase (*IDH*). Succinate and fumarate are intermediates of the Krebs cycle. Loss-of-function mutations in the tumour-suppressor genes succinate dehydrogenase (*SDH*) and fumarate hydratase (*FH*) cause intracellular accumulation of succinate and fumarate, respectively. These three oncometabolites (*R*)-2HG, succinate and fumarate are sufficiently similar in structure to 2-oxogluratate (2OG) to inhibit a range of 2OG-dependent dioxygenases, including hypoxia-inducible factor (HIF) prolyl hydroxylases (PHDs), histone lysine demethylases (KDMs) and the *ten-eleven* translocation (TET) family of 5-methylcytosine (5mC) hydroxylases. In turn, this leads to modulations of HIF-mediated hypoxia responses and alterations in gene expression through global epigenetic remodelling that may contribute to malignant transformation. Separately, (R)-2HG has been shown in some settings to act as a co-substrate for PHD2 in the prolyl hydroxylation of HIF1α, leading to cellular transformation as a result of reduced HIF expression. In addition, fumarate can irreversibly modify cysteine residues in proteins *via* succination. The succination of Kelch-like ECH-associated protein 1 (KEAP 1) on two cysteine residues in FH-deficient cells results in the constitutive activation of nuclear factor erythroid 2-related factor 2 (NRF2), leading to the transcription of genes involved in antioxidant response. Succination of the Krebs cycle enzyme aconitase 2 (ACO2) on three iron/sulphur-binding cysteine residues leads to impaired aconitase activity in FH-deficient cells. Fumarate accumulation may also impact on cytosolic pathways potentially hampering the urea and purine nucleotide cycles. Ac-CoA, acetyl coenzyme A; Cys, cysteine; OAA, oxaloacetate; Succ-CoA, succinyl coenzyme A; 2SC, succination of cysteine residues.

also allowed for the analysis of early events following loss of enzyme activity in these syndromes.[17–25]

In our own studies,[26,27] we have used a variety of technologies including capillary electrophoresis time-of–flight mass spectrometry,[28–30] which although it cannot analyse neutral compounds and lipids, is a powerful tool for the simultaneous analysis of most charged metabolites in central metabolic pathways including the glycolytic, Krebs cycle and pentose phosphate pathways. Metabolite labelling allows us to follow metabolic pathways in an unbiased and non-disruptive way and, especially, when linked to powerful computational analyses, offers the promise to provide unique metabolic profiles of normal and diseased cells and tissues.[13] Although our knowledge base using these technologies will continue to increase as more cells are analysed under different conditions, the next challenge will be to dovetail the results from these metabolite profiles with microarray data from the same cells, or tissues, and ultimately genome-wide mutational and epigenetic analyses. The requisite genomics data sets are already available and will continue to accumulate.[31]

INSIGHTS FROM CANCER SYNDROMES

The discovery that tumor-associated mutations in *SDH, IDH-1* and *-2*, and *FH* has given us extraordinary insights in cancer biology with the expectation that further studies may similarly implicate other members of metabolic pathways (Figure 1).[32] Two of these genes, *SDH* and *FH*, are classified as tumour suppressors as affected individuals inherit one mutated copy of the relevant gene, whereas the tumours exhibit loss of the wild-type allele following a somatic 'second event' in keeping with Knudson's classic two-hit hypothesis.[33,34] In contrast, isocitrate dehydrogenase (IDH-1 and -2) mutations are somatic and retain the wild-type allele; essentially they are gain-of-function mutations.[35] These cancers are rare; but provide direct

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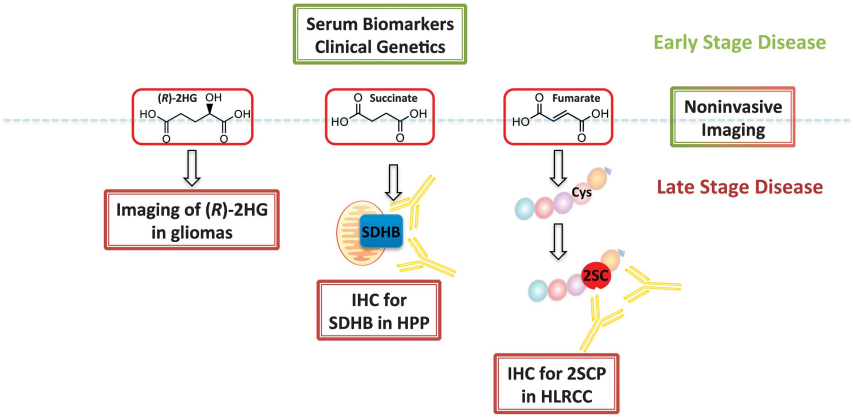

Figure 2. Oncometabolites as clinical biomarkers. Cancer syndromes associated with mutations in the metabolic genes, isocitrate dehydrogenase (*IDH*)–gliomas, succinate dehydrogenase (*SDH*)–hereditary paraganglioma and pheochromocytomas (HPP) and fumarate hydratase (*FH*)–hereditary leiomyomatosis and renal cell cancer (HLRCC), have a number of clear characteristic signatures that might allow for both diagnosis and monitoring of patients. For example, the noninvasive imaging of tumours to detect high levels of (*R*)-2-hydroxyglutarate ((*R*)-2HG) or sensitive immunohistochemistry (IHC) assays. In the case of HLRCC, an antibody raised against S-(2-succino) cysteine (2SCP) can identify FH-deficient cells sensitively and specifically, whereas IHC for SDHA and SDHB can distinguish between identify pheochromocytomas and paragangliomas that are SDH-related. The dysregulated metabolism associated with these diseases offers realistic opportunities for diagnosis and screening of patients *via* imaging such as ^{18}fluoro-2-deoxy-glucose, glutamine and glutamate positron emission tomography, magnetic resonance spectroscopy and magnetic resonance imaging. There is no reason, other than cost, that the use of sensitive research technologies, such as mass spectrometry and nuclear magnetic resonance, cannot be extended into the clinic for screening purposes. A more significant challenge is to identify patients and families in the early stages of disease progression, perhaps by means of serum biomarkers or the like, in order that at-risk individuals have appropriate genetic testing and screening, are correctly diagnosed and are provided with appropriate advice and care. Cys, cysteine; 2SC, succination of cysteine residues.

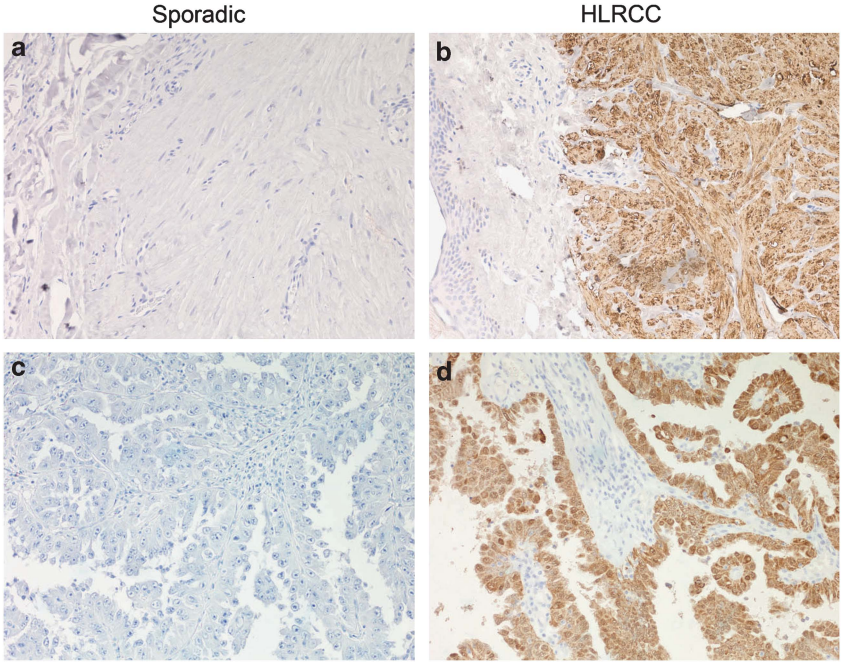

Figure 3. Immunohistochemistry for succination of cysteine residues (2SC) as a clinical biomarker for HLRCC. Immunohistochemistry for 2SC in skin leiomyomata (**a**, **b**) and papillary renal cell cancer (**c**, **d**). Brown staining represents the presence of 2SC. Note the absence of staining in sporadic tumours (**a**, **c**) and the presence of strong staining in HLRCC (FH-mutant) tumours (**b**, **d**). In a prospective study, the 2SC bioassay outperformed conventional sequencing methods in identification of previously undiagnosed HLRCC cases.[134]

evidence of a link between altered metabolism and cancer[12] and afford a unique opportunity to understand the consequences for a cell of dysregulated metabolism.

SDH

SDH has two roles, to couple the oxidation of succinate to fumarate in the Krebs cycle accompanied by the reduction of ubiquinone to ubiquitinol, and as part of the mitochondrial electron transport chain, designated complex II, participating in the production of reduced flavin nucleotides, which support the electron flow used for ATP synthesis.[36] The enzyme complex is comprised of four subunits encoded by four genes: *SDHA, SDHB, SDHC* and *SDHD*, and is activated by SDH assembly factor (*SDHAF2, SDH5*) encoding a protein involved in the incorporation of flavin dinucleotide cofactor.[37] SDH was the first mitochondrial enzyme

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to be associated with an inherited cancer syndrome and identified as a tumour suppressor.[38] Initially, mutations of *SDHD* were found to be associated with familial paraganglioma, neural crest-derived tumours that arise from parasympathetic ganglia of the head and neck or sympathetic ganglia of the chest and abdomen, and pheochromocytomas, tumours of the adrenal medulla.[39] Subsequently, reduced SDH activity was observed in renal cell carcinoma and papillary thyroid cancer associated with mutations in *SDHB* and *SDHC*. Latterly, mutations in *SDHA* and *SDHAF2* have been found in familial and sporadic paraganglioma, pheochromocytomas and in stromal tumours of the gastrointestine.[40–46] Prognosis for patients harbouring *SDHB* germline mutations is particularly poor, carrying a higher risk of metastatic cancer than patients without the mutation.[47–49]

Although loss-of-function mutations have been identified in all five SDH genes predisposing to cancer,[32] little is known about the mechanism for oncogenesis.[50] The loss of SDH activity results in accumulation of intracellular succinate.[51] This leads to the stabilisation of hypoxia inducible factor-1alpha (HIF1α) as a consequence of competitive inhibition of prolyl hydroxylase domain (PHD) 1, 2 and 3 enzymes, the 2-oxoglutarate (2OG)-dependent dioxygenases that regulate HIF.[52,53] Stabilisation of HIF1α is a potential mechanism for oncogenesis as the transcription factor can activate other pathways resulting in an angiogenic and glycolytic response in SDH-mutated tumours.[54–56] Elevated succinate is also associated with enhanced production of reactive oxygen species (ROS) that can induce DNA damage and genome instability[57] and increased apoptosis arising from mitochondrial dysfunction.[58–60]

It has been demonstrated that both succinate and fumarate can inhibit a number of 2OG-dependent dioxygenases in addition to the PHDs. These include the JmjC domain-containing histone lysine demethylases (KDMs)[61] and the ten-eleven translocation (TET) family of 5methyl cytosine (5mC) DNA hydroxylases,[62–64] resulting in altered histone and DNA demethylation patterns (Figure 1).[65] TET has well-defined tumour-suppressor functions and therefore inhibition of these enzymes might contribute to SDH or FH-associated oncogenesis.[66] Equally, alterations in histone methylation might be expected to lead to epigenetic changes that could promote oncogenesis.[67–70] Furthermore, identification and classification of epigenetic changes gives opportunities for use as biomarkers as performed in other cancers.[71–73] Such biomarkers would be a valuable tool, in addition to the use of immunohistochemistry for SDHA and SDHB, to identify effectively the subset of pheochromocytomas and paragangliomas that are SDH-related (Figure 2).[74,75]

IDH

IDH-1 and -2 located in the cytoplasm and mitochondria, respectively, catalyse the reversible oxidative decarboxylation of isocitrate to 2OG with the concomitant reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH.[76] *IDH* mutations are somatic, do not exhibit loss-of-heterozygosity and are linked largely to hotspot arginine residues, Arg132 for IDH-1 and the corresponding Arg172, or Arg140 in IDH-2 resulting in a single amino-acid substitution in the enzyme active site. Sequencing of gliomas (75% of grade 2–3 gliomas and secondary glioblastomas) and acute myeloid leukaemia (20%) identified gain-of-function mutations in one allele, in *IDH-1*, and less often in the *IDH-2* homologue.[77–80] IDH-1 and IDH-2 mutations in these and other residues have now been identified in a bewildering number of tumours including thyroid cancer, chondrosarcoma, enchondroma (Ollier disease and Maffucci syndrome, where mosaic constitutional mutations of IDH-1 and IDH-2 have been reported), melanoma, paraganglioma, prostate cancer, β-acute lymphoblastic leukaemia, angioimmunoblastic T-cell lymphoma and intrahepatic cholaniocarcinoma.[81–91] Metabolomic analyses have linked these mutations in some cell lines and tumour tissues with neomorphic enzymatic activity resulting in the production and accumulation of the oncometabolite enantiomer (*R*)-2-hydroxyglutarate ((*R*)-2HG), which is essentially non-existent in normal cells and cancer cells lacking IDH-1/IDH-2 mutations.[92] (*R*)-2HG is generated efficiently by the NADPH-dependent reduction of 2OG when both mutant and wild-type alleles are present. It has been proposed that (*R*)-2HG competitively inhibits multiple 2OG-dependent dioxygenases, including the PHDs, KDMs and the TET family of 5mC hydroxylases (Figure 1).[93–96] Inhibition of KDMs and TETs linked to DNA methylation alterations have been suggested as a possible explanation for the hypermethylation observed in gliomas bearing IDH-1 mutations[97] and the pattern of mutations in acute myeloid leukaemia.[98,99]

At variance with this hypothesis it has been shown in human astrocytes in culture that (*R*)-2HG, but not (*S*)-2HG, stimulates PHD activity with consequent reduction in levels of HIF expression. This is linked with the evidence of tumorigenesis, exhibited by increased proliferation and an ability to grow in semi-solid agar.[100] Also, it has been demonstrated using a human erythroleukaemic cell line that a stably infected IDH-1 R123H mutation results in increased (*R*)-2HG and promotes growth factor independence, enhanced proliferation and impaired differentiation; all characteristics of leukaemogenesis. These features can be mimicked by addition of (*R*)-2HG to the parental cell line, but not (*S*)-2HG, despite the fact that (*S*)-2HG is a potent inhibitor of TET2. This striking anomaly has been attributed to the fact that in some settings, such as those described above in human astrocytes, (R)-2HG acts as a PHD2 agonist, whereas (S)-2HG acts an antagonist. The implication is that promoting PHD2 activity results in cellular transformation, as inhibition of PHD2 by (S)-2HG prevents such transformation.[101] Surprisingly, the alterations in growth factor independence mediated by the action of (*R*)-2HG can be rapidly reversed. These data were generated *in vitro* and highlight again contradictory; but not mutually exclusive results, obtained from different models and the difficulties of integrating these with analyses of tumours. That said, further investigation and corroboration of the results in other settings such as a mouse model would offer the promise of an exciting therapeutic strategy.

FH

Although generally considered a mitochondrial enzyme, functioning within the Krebs cycle to catalyse the conversion of fumarate to malate, FH is also expressed in the cytoplasm, where it acts to metabolise fumarate that participates in the urea cycle, nucleotide and amino-acid metabolic pathways,[102,103] and in the nucleus where it is thought to be involved in the cellular response to DNA damage.[104,105] Germline loss-of-function mutations in *FH* predispose affected individuals to hereditary leiomyomatosis and renal cell cancer (HLRCC), an under-diagnosed syndrome, characterised by benign but painful smooth muscle cutaneous and uterine tumours (leiomyomata) and an aggressive form of collecting duct and type 2 papillary renal cancer.[106–108] The renal tumours carry a poor prognosis as they metastasise rapidly both nodally and systemically, even if the primary tumour is small.[109,110]

COMPETITIVE INHIBITION OF 2OG OXYGENASES BY ELEVATED FUMARATE

Loss of FH in cells and tumours results in the accumulation of high levels of fumarate.[23,51,111] Initially, mitochondrial dysfunction and in particular the stabilisation of HIF-1α leading to activation of HIF-dependent oncogenic pathways provided the accepted, but unsubstantiated, hypotheses for the neoplasia associated with HLRCC.[112] It had been known for some time that FH-associated tumours exhibit stabilisation of HIF[23,51,113] and as

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with SDH-deficient cells two possible mechanisms were proposed by which this could occur. Either enhanced reactive oxygen species (ROS) production could result in HIF stabilisation in FH-deficient cells[114] and/or fumarate could competitively inhibit 2OG-dependent dioxygenases that control levels of HIF.[115–117] In normal cells prolyl hydroxylation occurs at two sites in a degradation domain within HIFα, thus promoting binding to the VHL E3 ligase complex and subsequent proteolysis by the ubiquitin-proteasome pathway; asparaginyl hydroxylation blocks co-activator recruitment and thus reduces transcriptional activity.[7,118] HIF prolyl hydroxylation is catalysed by three related enzymes PHD- 1, -2 and -3; whereas asparaginyl hydroxylation is catalysed by factor inhibiting HIF (HIF1AN/FIH).[119,120] To address these questions, we generated a panel of four immortalised mouse embryonic fibroblast (MEF) cell lines from a conditional FH1 knockout (KO) mouse model:[17] FH1WT, FH1KO and isogenic FH1KO MEFs, reconstituted with either full-length FH (FH1KO + FH) or cytosolic-restricted FH by deleting the mitochondrial targeting sequence (FH1KO + FHcyt).[121] Our studies demonstrated that FH1KO MEFs exhibited impairment of HIF prolyl hydroxylation; but not asparaginyl hydroxylation that could be ameliorated by the addition of 2OG. Furthermore, re-expression of cytoplasmic FH in FH1KO MEFs (FH1KO + FHcyt) was sufficient to reduce intracellular fumarate levels and to restore the normal pathway of HIF degradation, despite continuing to exhibit defective mitochondrial oxidative metabolism.[121] Thus, as for succinate and (*R*)-2HG, HIF1α stabilisation occurred as a consequence of competitive inhibition of 2OG-dependent dioxygenases by fumarate in addition to KDMs and TET proteins as described above.[117] This was an important finding as it supported the hypothesis that fumarate may act as an 'oncometabolite' (Figure 1).[122] Significantly, it also implied that cytoplasmic FH may have an important role in dysregulated metabolism associated with cancer.

DOES HIF INITIATE ONCOGENESIS?

HIF stabilisation, leading to pseudohypoxia and activation of HIF-dependent pathways, is a characteristic feature of loss of FH-human tumours, human and murine cells and the hyperplastic renal cysts in the FH1KO mouse model[17,27,51,111] and represented a real candidate to drive oncogenesis.[23,34] Renal cysts are considered an early stage in carcinogenesis of hereditary renal cancer syndromes including HLRCC and VHL disease as the cystic epithelium often exhibits dysplastic changes and/or the development of tumours.[123,124] To determine whether HIF was important in initiating renal tumour formation in FH deficiency, we generated multiple murine models with combined inactivation of FH1 and either HIF1α or HIF2α, or HIF1α and HIF2α and as a control, mice in which PHD1, 2 and 3 were inactivated. PHD triple knockout mice did not develop cysts while inactivation of HIF1α, but not HIF2α actually exacerbated renal cyst formation.[27] Thus, we concluded that the formation of renal cysts is both HIF- and PHD-independent and that HIF is not the initiating driver of tumorigenesis. However, the continued stabilisation of HIF and activation of HIF-dependent pathways, including increased expression of glucose transporters and glycolytic enzymes, cannot be discounted from a later role in cancer progression.[125] These findings implied that other players and mechanisms must have a role(s) in FH-associated oncogenesis and that the disease progression could be considered in a stepwise manner. Also, this suggests other potential parallels between cancer syndromes associated with SDH, IDH and FH.[8]

ELEVATED FUMARATE LEADS TO SUCCINATION

An important and novel mechanism linked to FH loss stems from the ability of fumarate to act as an endogenous electrophile, reacting with free sulphydryl groups to make a thioether linkage with cysteine residues in multiple proteins, *via* a Michael addition reaction. This process, termed succination, results in the formation of S-(2-succino) cysteine (2SC).[126–128] Succination, first identified in diabetic models, was postulated to occur as a consequence of mitochondrial stress in adipocytes cultured in high glucose (30 mM, compared with physiological levels of 5 mM), in the skeletal muscle of rats treated with streptozotocin to induce type I diabetes and in adipose tissue of ob/ob type 2 diabetic mice.[129–131] It is hypothesised that the increased glucose results in elevated ATP/ADP, NADH/NAD^+ and mitochondrial membrane potential and that the increased NADH/NAD^+ inhibits oxidative phosphorylation leading to fumarate accumulation and succination.[132]

As outlined earlier, fumarate accumulation in HLRCC tumours is a key feature of loss of FH activity and it has been shown convincingly that immunohistochemistry for 2SC can provide sufficient sensitivity and specificity for its use as a reliable biomarker of HLRCC in research and clinical settings (Figures 2 and 3).[133,134]

Crucially, post-translational modification of cysteine residues in proteins can lead to disruption or loss of function, as demonstrated in the inactivation of glyceraldeyde-3-phosphate dehydrogenase in diabetic models.[130,131] Such modifications have important consequences for the physiology and pathology of FH-deficient cells, renal cysts and tumours. In normal cells, Kelch-like ECH-associated protein 1 (KEAP1), part of an E3 ubiquitin ligase complex, targets the transcription factor, nuclear factor erythroid 2-related factor 2 (NRF2), for degradation.[135] It has been shown that in FH-deficient cells and tumours, succination of key cysteine residues (Cys151 and Cys288) in KEAP1 leads to abrogation of its interaction with NRF2 allowing nuclear accumulation of NRF2, enhanced binding to antioxidant response elements[136,137] and activation of the potentially oncogenic NRF2-mediated anti-oxidant defence pathway[27,138] (Figure 1). Furthermore, this pathway has been shown to be activated in sporadic papillary renal cell carcinoma as a consequence of somatic mutations in *NRF2*, *CUL3* and *SIRT1* strengthening the argument for a role for NRF2 in tumorigenesis.[139] NRF2 activation has also been shown to modulate cell metabolism under the control of P13K-Akt signalling, possibly augmenting the cellular stress response, by directing both glucose and glutamine into pathways that enhance purine synthesis and contributing to cell proliferation through its action on the pentose phosphate pathway.[140] As yet we have no data to explain why activation of NRF2 is important for the proliferation of FH-deficient cells; but this is an active area of research. It would be interesting to combine inactivation of NRF2 and FH1 *in vivo* to determine whether the cystic phenotype is ameliorated and to analyse alterations in the metabolic profile of tissue and cells lacking the function of both proteins. Elucidation of the functional consequences of KEAP1 succination prompted us to search for other 2SC targets that may contribute to the pathogenesis of FH-associated disease and dysregulated metabolism.[122] This has revealed loss of mitochondrial aconitase (ACO2) activity in FH-deficient cells as a consequence of succination of three cysteine residues required for iron-sulphur cluster binding,[26] thus potentially contributing to their dysregulated metabolism (Figure 1).

MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction results in loss of ability to trigger apoptosis and increased levels of reactive oxygen species (ROS) that can cause mutagenic damage to DNA. This has been linked to FH associated cancer.[34] Normally, ROS levels are tightly controlled by antioxidant pathways that are acutely regulated by NRF2 in response to cellular stress.[141,142] In contrast, it seems that NRF2 is permanently activated in some cancers, leading to increased detoxification of ROS.[143] Studies interrogating this mechanism

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through expression of oncogenic alleles, including *Kras* and *Myc*, have shown that these oncogenes increase the activity of NRF2 and the antioxidant programme and lower cellular ROS.[144] These results may be significant in light of the observation that the NRF2 antioxidant pathway is activated in cells lacking FH.[125] Also, haem oxygenase-1, involved in haem degradation, is a target for NRF2. The pathway of haem synthesis from glutamine is upregulated in FH-deficient cells and it has been shown that inhibition of this pathway, and haem oxygenase-1 in particular, leads to synthetic lethality with FH-deficiency.[145] Clearly, further studies are required to investigate the role of NRF2 in HLRCC. There are also multiple other unresolved questions about the role of the mitochondria in FH-deficient cells, such as whether the mitochondrial membrane potential and permeability is altered, and whether autophagy is increased in this environment.

A ROLE FOR CYTOPLASMIC FH

FH metabolises fumarate generated from arginine synthesis and the purine nucleotide cycle in the cytoplasm.[36,146] Using the panel of MEFs, we have shown that re-expression of cytosolic FH reduces fumarate levels in part and ameliorates constitutive activation of both the hypoxia and antioxidant response pathways in FH1-null cells, despite a persistent defect in oxidative metabolism.[27,121] Recently, through metabolomic analyses we have been able to demonstrate that FH1KO cells and tissues exhibit defects in the urea cycle/arginine metabolism and that acute arginine depletion reduced significantly the viability of FH1-deficient cells in comparison to controls. Also, re-expression of cytosolic FH *in vivo* ameliorated both renal cyst development and urea cycle defects associated with renal-specific FH1 deletion in mice. Our findings highlight the importance of extra-mitochondrial metabolic pathways in FH-associated oncogenesis.[147]

ALTERED CELLULAR METABOLISM IN FH-DEFICIENT CELLS

Loss of a functioning Krebs cycle poses real metabolic challenges to FH-deficient cells. Contradictory results based on different cellular models (MEFs, murine renal cells and the human cell line UOK262) have highlighted various different mechanisms by which these cells respond to this metabolic challenge. Human and murine FH-deficient cells exhibit upregulation of aerobic glycolysis and impaired respiration.[114,121] Elevated glutaminolysis has been observed in FH1-deficient murine renal cells, suggesting glutamine is an important source of carbon for the Krebs cycle.[145] Metabolomic analyses of UOK262 cells have demonstrated a partial reversal of the Krebs cycle (glutamine-dependent reductive carboxylation) by which 2OG is reductively carboxylated by IDH to generate isocitrate. This is in turn metabolised to citrate, which is then cleaved to produce oxaloacetate and acetyl coenzyme A (acetyl CoA).[148–150] This acetyl CoA reservoir is necessary for fatty acid synthesis and protein acetylation, whereas oxaloacetate is converted to malate and can thus compensate, in part, for blocks within the Krebs cycle. Recently, we conducted labelling experiments with deuterium-labelled glutamine that indicated the oxidative flux of the Krebs cycle in FH1KO MEFs. At variance with the results obtained with UOK262 cells, our data suggest that in FH1KO MEFs, 2OG can be converted to isocitrate by reversal of the IDH catalysed reaction, but isocitrate cannot be further metabolised to citrate, possibly due to impaired aconitase activity as a result of succination.[26] This suggests that succination of ACO2 may prevent FH1KO MEFs from utilising the reductive carboxylation pathway for citrate synthesis.[26] Clearly, much more metabolomic analyses of various cell lines and tumours need to be conducted before we have a clear picture of dysregulated metabolism associated with FH deficiency.

EXPERIMENTAL MODELS FOR LOSS OF FH LEADING TO HLRCC

None of these studies would have been possible without the development of a variety of models, particularly the conditional mouse model of FH-associated disease in which inactivation of FH1 in kidney tubules causes the formation of hyperplastic cysts similar to the human disease; but not cancer.[17] This has facilitated *in vivo* and *in vitro* studies, the latter using a panel of MEFs[121] and renal cells derived from it.[145] Our own studies have always integrated analyses of murine models with human tumours and UOK262 cells (two human FH-deficient cell lines exist from HLRCC patients: UOK262 derived from a metastasis and UOK268, the first established renal cell line[18,21]). Although many similarities exist between FH1-deficient human and murine cells, there are also a number of clear differences. Both display increased lactate production and stabilisation of HIF1α.[18,21,114,121] The growth rate is reduced/relatively slow in all cell lines lacking FH compared with controls. UOK262 cells use reductive carboxylation, whereas this is not the case for the FH1-deficient renal cells[145] or MEFs.[26] We propose that the murine and human cell lines might be models for different stages in the HLRCC disease process. Hence, the FH1 mouse model is particularly valid and informative for the early stages of FH loss and initiation of oncogenesis that lead as far as cyst formation *in vivo*, although we have successfully extrapolated findings from the murine models to identify pathways in FH-deficient human tumours, such as succination[134] and activation of the NRF2 antioxidant pathway.[27,138] UOK262 cells perhaps better reflect the later stages of renal neoplasia and metastasis, possibly having acquired additional mutations subsequent to loss of FH activity. There is a need to generate more and better human cell lines to extend research, particularly for epigenetic analyses, ideally with normal *versus* FH-deficient cells either from patients or for example, by the use of transcription activator-like effector nucleases.

FUTURE RESEARCH: SYNTHETIC LETHALITY SCREENS

Targeting metabolism offers a realistic promise for controlling renal cancer and the deployment of synthetic lethality screens are one tool with which to identify and interrogate metabolic pathways that are critical for both the survival and neoplastic potential of FH-deficient cells.[151] There are multiple strategies for such screens including the use of short interfering RNA and/or small-molecule libraries to identify compounds or pathways that induce lethality in FH-deficient, but not wild-type cells. Clearly, a variety of cellular models could be used–murine (MEFs and renal cell lines) versus human cell lines; the key is to exploit the fact that FH-deficient cells exhibit dysregulated metabolism and rely on alternative metabolic pathways to cells with functioning FH.

FUTURE RESEARCH: FORWARD SCREEN USING 'SLEEPING BEAUTY' TRANSPOSON-MEDIATED MUTAGENESIS

The failure of the FH1KO mouse model to recapitulate fully a renal cancer phenotype implies a requirement for secondary somatic hits.[17,152,153] The identification of somatic mutations in renal cancer by genome-wide sequencing and the distinction between initiating and non-functional mutations will be expensive in both time and money.[68,69,154] Forward genetic screens in mice such as the *Sleeping Beauty* transposon-mediated mutagenesis[155,156] offer a complement to analyses of human tumours and an alternative and unbiased strategy to identify driver from passenger mutations in genes and pathways that promote renal carcinogenesis and progression in FH deficiency.

CONCLUSIONS

We have discussed the consequences of mutations in a triad of metabolic genes *FH*, *SDH* and *IDH* and highlighted some common

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elements in the capacity of affected cells to adapt when 'metabolically stressed/ challenged' that go beyond the 'Warburg effect'. Fumarate, succinate, 2OG and (*R*)-2HG have similar chemical structures fitting well with the observation that they can competitively inhibit 2OG-dependent dioxygenases. A number of these enzymes are inhibited including PHDs leading to stabilisation of HIF and activation of HIF-dependent oncogenic pathways, KDMs, and the TET family of 5mC hydroxylases leading to global epigenetic changes. Thus, altered metabolism seems to be capable of altering transcription mediated by small molecules that can be considered 'oncometabolites' and represents an important candidate in oncogenesis and consideration for future studies. We propose that succination as a consequence of elevated fumarate is a significant mechanism in oncogenesis associated with loss of FH and may provide a link with increased cancer risks. Although there is neither a time frame nor a clear idea of all the steps in the oncogenic process associated with mutations in these genes, we do know more about some of the players and pathways that are activated and are building a complex, but exciting perspective on these protagonists. Altered metabolic states in disease offer additional opportunities for diagnosis *via* imaging[4,157,158] and measurement of altered levels of metabolites.[14–16] Our ongoing studies of the consequences of FH deficiency have highlighted multiple candidate pathways that are not mutually exclusive and come from an integrated and unbiased research approach encompassing *in vivo* murine models, cellular models and analyses of human tumour material. We suggest that the study of rare diseases such as HLRCC has already given new and exciting insights into links between dysregulated metabolism and cancer and represents a real paradigm for cancer research with the promise of potential novel therapeutic strategies for patients.

CONFLICT OF INTEREST

Professor Soga is a founder of Human Metabolome Technologies. The remaining authors declare no conflict of interest.

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