A Role for Cytosolic Fumarate Hydratase in Urea Cycle Metabolism and Renal Neoplasia

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SUMMARY

The identification of mutated metabolic enzymes in hereditary cancer syndromes has established a direct link between metabolic dysregulation and cancer. Mutations in the Krebs cycle enzyme, fumarate hydratase (FH), predispose individuals to hereditary leiomyomatosis and renal cell cancer (HLRCC) (Tomlinson et al., 2002). Affected individuals also develop renal cysts, a phenotype that is recapitulated in FH1 (murine FH)-deficient mice (Pollard et al., 2007). Loss of FH activity results in accumulation of intracellular fumarate, which, in turn, affects multiple signaling pathways, including inhibition of 2-oxoglutarate (2OG)-dependent dioxygenase enzymes (Isaacs et al., 2005; Loenarz and Schofield, 2008; O’Flaherty et al., 2010; Xiao et al., 2012) and posttranslational modification (succination) of cysteine residues (Adam et al., 2011; Alderson et al., 2006; Bardella et al., 2011; Yang et al., 2012). However, the mechanism(s) of tumorigenesis and particularly the role of defective mitochondrial and cytosolic FH isoforms remain undefined. On the basis of comprehensive metabolomic analyses, we demonstrate that FH1-deficient cells and tissues exhibit defects in the urea cycle/arginine metabolism. Remarkably, transgenic re-expression of cytosolic FH ameliorated both renal cyst development and urea cycle defects associated with renal-specific FH1 deletion in mice. Furthermore, acute arginine depletion significantly reduced the viability of FH1-deficient cells in comparison to controls. Our findings highlight the importance of extramitochondrial metabolic pathways in FH-associated oncogenesis and the urea cycle/arginine metabolism as a potential therapeutic target.

INTRODUCTION

Since first highlighted in the last century, altered metabolism has been a consistent observation in cancer cells (Warburg, 1956). Recently, the identification of mutated Krebs cycle enzymes in familial cancer syndromes has linked altered metabolism and cancer directly (reviewed in Bayley and Devilee, 2010; Frezza et al., 2011). Mutations in one of these enzymes, fumarate hydratase (FH), predispose individuals to hereditary leiomyomatosis and renal cell cancer (HLRCC) (Tomlinson et al., 2002). Affected individuals also develop renal cysts, a phenotype that is recapitulated in FH1 (murine FH)-deficient mice (Pollard et al., 2007). Loss of FH activity results in accumulation of intracellular fumarate, which, in turn, affects multiple signaling pathways, including inhibition of 2-oxoglutarate (2OG)-dependent dioxygenase enzymes (Isaacs et al., 2005; Loenarz and Schofield, 2008; O’Flaherty et al., 2010; Pollard et al., 2005; Xiao et al., 2012) and posttranslational modification (succination) of cysteine residues (Adam et al., 2011; Alderson et al., 2006; Bardella et al., 2011; Yang et al., 2012). However, the mechanism(s) of tumorigenesis and particularly the role of defective mitochondrial metabolism in FH-associated disease remain undetermined. Though considered a Krebs cycle enzyme, FH is also expressed in the cytosol and the nucleus (Yoge et al., 2010, 2011). Moreover, re-expression of cytosolic FH ameliorates constitutive activation of both the hypoxia and antioxidant response pathways in FH1-null cells, despite a persistent defect in oxidative metabolism (Adam et al., 2011; O’Flaherty et al., 2010). To investigate the role of extramitochondrial FH in renal cyst development, we have undertaken high-resolution mass-spectrometry-based metabolomic analyses of FH-deficient cells, renal cysts, and tumors. To corroborate our findings in vivo, we generated two transgenic murine models where either FH or extramitochondrial FH (FH cyt) is stably expressed from the
Rosa26 locus (Zambrowicz et al., 1997). We demonstrate that re-expression of cytosolic FH in FH1-deficient mice is critical for the suppression of renal cyst development and restoration of defects in the arginine biosynthesis pathway. Furthermore, FH-deficient cells exhibit a greater dependence on exogenous arginine than wild-type counterparts. Taken together, our data support a role for extramitochondrial metabolic pathways in renal neoplasia and arginine deprivation as a candidate target for therapy.

RESULTS

Urea Cycle Metabolites Accumulate in FH1KO Kidneys

Previously, we demonstrated that mice with deletion of FH1 in renal tubular epithelial cells (Shao et al., 2002) (FH1lox/lox Ksp-Cre\(^{+/−}\); FH1KO) develop hyperplastic renal cysts (Pollard et al., 2007). This model has been characterized further by genetic crosses and subsequent gene expression analyses (Adam et al., 2011; Ashrafian et al., 2010), but without comprehensive analysis of metabolism. Therefore, we determined metabolite levels in control and FH1KO kidneys using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS; Soga et al., 2009). Levels of fumarate, argininosuccinate, and citrulline were increased significantly in FH1KO kidneys compared to controls, whereas aspartate was depleted (Figures 1A–1D; Table S1). Metabolic pathway analyses using IPA (Ingenuity Pathway Analysis, Ingenuity Systems) showed significant changes in the urea cycle/arginine biosynthesis pathway (Table S1).

FH1KO Mouse Embryonic Fibroblasts Exhibit Multiple Defects in the Krebs Cycle and Utilize the Urea Cycle, but Not Reductive Carboxylation

There were at least two hypotheses to test: whether the urea cycle is dysregulated in the FH1KO mouse embryonic fibroblasts (MEFs) as predicted above, and whether they use the reductive carboxylation pathway in FH-deficient cells has been reported for other FH-deficient cells (Mullen et al., 2012). Hence, we cultured wild-type (FH1WT) and FH1KO MEFs in medium containing the stable isotope tracer glutamine-2,3,3,4,4-d\(_5\) ([D\(_5\)]-glutamine) for 3 and 9 hr and determined the incorporation of deuterium label in Krebs cycle and urea cycle metabolites by CE-TOFMS analyses (Figure 1E; Table S1). Use of [D\(_5\)]-glutamine by the canonical oxidative Krebs cycle would result in m+4 for 2OG and succinate, m+2 for fumarate and malate, and m+1 for oxaloacetate and aspartate and thus provides a means of differentiating whether argininosuccinate is generated by arginine and fumarate, or alternatively by condensation of citrulline and aspartate (Figure 1E). Significantly, we detected argininosuccinate m+2, and, in addition, the isotopic distribution pattern of argininosuccinate matched that of fumarate, but not of aspartate (Figure 1E). Therefore, we concluded that argininosuccinate is synthesized directly from fumarate. The glutamine-dependent reductive carboxylation pathway metabolizes 2OG to citrate for lipid synthesis, forcing partial reversal of the Krebs cycle (Metallo et al., 2012; Mullen et al., 2012; Wise et al., 2011). We did not observe evidence for a reversal of the Krebs cycle in our MEF model, and furthermore, levels of citrate, cis-aconitate, and isocitrate were significantly lower in FH1KO compared to FH1WT MEFs (Figure 1E; Table S1). We have proposed previously that, in FH1KO MEFs, 2OG can be converted to isocitrate by isocitrate dehydrogenase (IDH), but isocitrate cannot be further metabolized to citrate, probably as a result of impaired Aconitase 2 activity due to succination. We did not observe any label in citrate and have suggested that this may be the consequence of inactive aconitase in both the mitochondria and cytoplasm of FH1KO MEFs (Ternette et al., 2013).

Cytosolic FH Suppresses Renal Cyst Development In Vivo

Given that the part of the urea cycle affected by fumarate accumulation functions in the cytosol (Shambaugh, 1977), we hypothesized that cytosolic FH may be important in the pathogenesis of HLRCC. Previously, we demonstrated that expression of cytosolic FH in FH1KO MEFs reduced fumarate levels significantly with concomitant loss of nuclear factor (erythroid-derived 2)-like 2 (NFE2L2/NRF2) and hypoxia-inducible factor (HIF) expression, but did not restore defects in oxidative metabolism (Adam et al., 2011; O’Flaherty et al., 2010). To investigate the in vivo role of cytosolic FH, we constructed two transgenic mouse lines stably expressing either FH or FH\(^{F372}\) (excluded from the mitochondria) with a C-terminal V5 affinity tag and under the control of the CAG promoter (Niwa et al., 1991). Equivalent expression between both lines was ensured by targeting the FH transgenes to the Rosa26 locus (Zambrowicz et al., 1997) using integrase-mediated cassette exchange (Chen et al., 2011) (Figures 2A and S1). Targeting fidelity was assessed using PCR (Figure S1), and FH protein localization was confirmed in embryonic stem (ES) cells by immunofluorescence (Figures 2B and 2C). Transgenic expression of FH-V5 was analyzed by immunoblotting and immunofluorescence (Figure S1). Similar to HLRCC patients with renal cancer, mice with kidney-specific FH1 deletion develop hyperplastic renal cysts (Pollard et al., 2007). We intercrossed FH1KO mice with both transgenic lines (FH1KO+FH and FH1KO+FH\(^{F372}\)). Macroscopic analyses of kidneys from 30-week-old mice (Figure 2D) indicated that expression of either transgene was sufficient to ameliorate the increased renal mass in FH1KO mice, and microscopic analysis at three time points (13, 20, and 30 weeks) confirmed that transgenic expression of cytosolic FH was sufficient to suppress cyst development (Figures 2E–2H).

Cytosolic Expression of FH in FH1KO Mice Restores Urea Cycle Metabolism

Since re-expression of cytosolic FH “rescued” the cystic phenotype associated with FH1 deletion, we hypothesized that this might be mediated in part through alterations in metabolism. Hence, we determined the metabolic consequences of restoring cytosolic FH in FH1KO kidneys from 15-week-old mice by CE-TOFMS and IPA analysis (Table S1). This time point was chosen to avoid severe pathological changes in the kidneys and to match previous analyses (Adam et al., 2011). Metabolites showing the most significant changes are indicated using a heat-map (Figure 3A). Notably, transgenic rescue of FH1 deficiency with either FH or FH\(^{F372}\) restored levels of fumarate and urea cycle...
metabolites comparable to controls (Figures 3B–3E). Interestingly, levels of citrulline (which exists in both the mitochondria and cytosol) were not fully rescued in FH1KO+FHcyt animals (Figure 3E).

**Fumarate and Argininosuccinate Levels Are Increased in HLRCC Tumors**

To determine whether the increase in urea cycle metabolites observed in FH1KO kidneys was recapitulated in FH mutant HLRCC tumors,](cellreports/issue3/4) we performed metabolomic analyses using CE-TOFMS. We observed a significant increase in fumarate and argininosuccinate levels in FH mutant HLRCC tumors compared to control HLRCC tumors. These findings suggest a potential role for FH deficiency in the regulation of urea cycle metabolism in HLRCC tumors.
Figure 2. Generation and Analyses of FH-Expressing Transgenic Mice to Investigate the Role of Cytosolic FH/Fumarate in Renal Cyst Development

(A) The FH and FH\textsuperscript{cyt} transgenes were cloned into the CB92 vector and targeted to the Rosa26 locus, using phage-mediated recombination (Chen et al., 2011).

(B and C) Localization of FH and FH\textsuperscript{cyt} was confirmed in ES cells by immunocytochemistry. Colocalization (yellow) of FH (green) and mitochondria (red) is evident (B), whereas FH\textsuperscript{cyt} is absent from the mitochondria (C). Nuclei (blue) are visualized with DAPI.

(D) Kidneys harvested from 30-week-old control, transgene-only (FH, FH\textsuperscript{cyt}), and genetically “rescued” mice (FH1KO+FH and FH1KO+FH\textsuperscript{cyt}) appeared macroscopically normal compared to FH1KO kidneys, which appeared enlarged and cystic.

(E) Hematoxylin and eosin (H&E) staining of kidneys harvested from mice at 13, 20, and 30 weeks reveal that transgenic expression of either FH or FH\textsuperscript{cyt} is sufficient to ameliorate cyst development.

(F–H) Numbers and frequency of macrocysts (>0.5 mm) and microcysts (>0.1 mm) were determined in each group (n = 6). Error bars represent the SEM. Scale bars, 5 \textmu m (B and C), 10 mm (D), and 100 \textmu m (E). Error bars indicate SEM. See also Figure S1.
tumors, we used CE-TOFMS and IPA to analyze the metabolome of normal kidney (n = 2) and type 2 papillary tumors (n = 3) from two HLRCC patients (Table S1). Increased levels of both fumarate and argininosuccinate were observed in the tumors (Figures 3F and 3G).

Argininosuccinate Is Generated in FH-Deficient Cells via Reversal of Argininosuccinate Lyase

Argininosuccinate can be generated either from citrulline and aspartate (through argininosuccinate synthetase [ASS1]) or from fumarate and arginine (through reversal of argininosuccinate lyase [ASL]) (Figure 1E; Wheatley, 2005). To confirm independently the route by which argininosuccinate accumulates in FH1 deficiency, we cultured WT and FH1KO MEFs in medium containing uniformly labeled [U-15N4,U-13C6] arginine and analyzed the cells using heteronuclear single quantum correlation nuclear magnetic resonance (HSQC-NMR) (Ludwig and Günter, 2011). We observed labeled argininosuccinate in FH1KO cells but not in WT cells (Figure 4A), consistent with a reversed ASL reaction in these cells. We then analyzed two panels of isogenic FH-deficient lines; FH1KOMEFs reconstituted with either FH or FHcyt (O’Flaherty et al., 2010) and UOK262 (derived from a metastasis from an FH mutant renal cancer (Yang et al., 2010b), which we have genetically complemented with the same FH or FHcyt constructs (Figure S2). CE-TOFMS analysis demonstrated that re-expression of either FH- or FHcyt-suppressed fumarate (Figure 4B) and argininosuccinate (Figure 4C) accumulation in both FH-deficient cell lines. We observed argininosuccinate m+10 as the predominant isotopomer (Figure 4C), indicating the direct formation of argininosuccinate from arginine and fumarate, thus corroborating the data from the [D5]-glutamine labeling (Figure 1E).

FH-Deficient Cells Exhibit Increased Sensitivity to Arginine Deprivation

Recent studies have demonstrated that depletion of arginine in ASS1-negative tumors inhibits their growth and predicts clinical benefit (Feun et al., 2012; Kelly et al., 2012; Yang et al., 2010a).

Figure 3. Metabolite Analysis of Kidneys from FH1KO- and FH-Reconstituted Mice

(A) Heatmap showing relative levels of specific metabolites in kidneys from control, FH1 KO, and reconstituted (FH1 KO+FH and FH1 KO+FHcyt) mice. For each group, kidneys from six mice (aged 15 weeks) were analyzed using CE-TOFMS (Soga et al., 2009), and the data were filtered to select only metabolites where there was a 2-fold difference between any group and a p value <0.05 (one-way ANOVA). Relative metabolite levels are indicated by red (high) and green (low). (B–E) Concentrations (µM) of the specific urea cycle metabolites fumarate (B), argininosuccinate (C), aspartate (D), and citrulline (E) measured in kidneys from mice of each of the above four genotypes. (F and G) Analysis of FH mutant renal cancers (n = 3) and adjacent normal tissue (n = 2) indicates accumulation of fumarate (F) and aspartate (G). Error bars indicate SEM. See also Table S1.
Figure 4. Argininosuccinate Is Produced from Arginine in FH-Deficient Cells

(A) Sections of HSQC spectra for the C4 (top panel) and C5 (bottom panel) resonances of arginine and argininosuccinate detected in FH1WT (red) and FH1KO (blue) MEFs. Argininosuccinate was detected in FH1KO MEFs only, which also correlates with the appearance of the fumarate resonance at 6.5/138 ppm.

(B and C) Cells of the indicated genotype were cultured in media containing 15N4,13C6-arginine for 12 hr prior to metabolite extraction and analyses of fumarate (B) and argininosuccinate (C) levels by CE-TOFMS (Soga et al., 2009). The m+10 isotopomer comprises >90% of total argininosuccinate detected in FH1KO MEFs and UOK262 cells, suggesting direct conversion of argininosuccinate from arginine and fumarate. Three independent cultures were used for each condition, and data shown here are representative of two independent experiments.

(D–G) Concentrations (fmol/cell) of fumarate (B), argininosuccinate (C), citrulline (D), and aspartate (E) were determined in FH1WT and KO MEFs by CE-TOFMS, confirming the metabolic profile observed in equivalent mouse kidneys of significantly elevated fumarate and argininosuccinate and significantly depleted aspartate.

(H) The manganese-containing enzyme arginase catalyzes the conversion of arginine and water to ornithine and urea. Levels of arginine and ornithine (μM) measured in medium by HPLC-MSMS following treatment with bovine arginase show that arginine levels are significantly reduced with time with a concomitant increase in ornithine.

(I–L) WT and KO FH1 MEFs were cultured in medium treated with arginase for 1, 2, 4, and 6 hr compared to no treatment (0 hr). Cells were then returned to standard DMEM (containing arginine) and assayed separately for proliferative capacity (I and J) and colony-forming capacity (K and L).

(I and J) Reduced proliferative capacity of FH1KO MEFs compared to WT controls is observed following culture in arginine-depleted medium for as little as 2 hr. 1.5 × 10^4 cells/well of 96-well plate were plated for each experimental group and measurements made using CyQuant. Fluorescence units were measured and normalized to those of cells cultured in standard untreated medium.

(K) Representative photographs of colony assays for WT and KO FH1 MEFs 10^5 cells/10 cm plate were cultured for 10 days for each experimental group. Three separate dilutions were plated for each experimental group, and the experiments were repeated in triplicate.

(L) The number of colonies formed by FH1WT and KO MEFs was counted and expressed relative to the colony count of cells cultured in standard untreated medium (0 hr). The graph of plating efficiency versus time cultured in arginine-depleted medium shows that FH1KO MEFs are acutely sensitive to reduced arginine even for a very short time period (2 hr).

See also Figure S2.
Given that FH-deficient cells exhibit a reversal of ASL activity in the urea cycle, we hypothesized that transient arginine depletion might selectively inhibit the growth of FH-deficient cells. Comparison of metabolite concentrations in FH1WT and KO MEFs confirmed the profile observed in equivalent mouse kidneys, of significantly elevated fumarate and argininosuccinate and significantly depleted aspartate (Figures 4D-4G; Table S1). Hence, we cultured FH1KO and WT MEFs for 1, 2, 4, and 6 hr in standard Dulbecco’s modified Eagle’s medium (DMEM) depleted of arginine by addition of bovine arginase and then returned them to standard DMEM. Since L-arginine is metabolized to L-ornithine and urea by arginase (Shambaugh, 1977), the effectiveness and time course of arginine depletion and parallel increase of ornithine in the medium was confirmed by HPLC-MSMS (Figure 4H). The consequences of arginine depletion for cell proliferation and survival in colony assays were determined and show clearly that culture of FH1KO MEFs for as little as 2 hr in medium depleted of arginine results in loss of proliferative capacity, which increases significantly with time (Figures 4I and 4J). Similarly, there is a significant reduction in the number of FH1KO cells surviving depletion of arginine from the culture medium to form colonies compared to untreated cells or WT control MEFs (Figures 4K and 4L). This further confirms the dependence of FH1KO MEFs on arginine for survival and growth.

DISCUSSION

Here, through global metabolite analyses, we have shown that the urea cycle and arginine biosynthesis are significantly perturbed in FH-deficient cells, cysts, and tumors. Furthermore, arginine biosynthesis occurs predominantly in the cytosol (Salway, 1999) and therefore questions the relative role(s) of mitochondrial and cytosolic FH in oncogenesis. To expand our findings and to assess directly if cytosolic FH has a functional role in vivo, we generated two FH-expressing transgenic mouse lines (including a cytosolic variant, FHcyt), which we have previously demonstrated to suppress both HIF-1α and NRF2 expression (Adam et al., 2011; O’Flaherty et al., 2010), activation of the NRF2 antioxidant pathway (Shan et al., 2009; Yang et al., 2010b), there are also clear differences in their metabolism; for example, the reductive carboxylation pathway is used by UOK262 cells (Mullen et al., 2009) but is not utilized by either FH1KO MEFs or FH1-deficient murine proximal tubular epithelial cells (Frezza et al., 2011b). It is possible that the mouse and human cell lines are models for different stages in the pathogenesis of HLRCC. We would suggest that the FH1 mouse model (both in vivo and in vitro) is a particularly valid model of the early stages of FH deficiency that lead as far as cyst development in vivo, and that factors yet to be defined may then act to drive cells toward neoplasia and further dysregulated metabolism. However, studies using the model have also highlighted pathways that are clearly evident in FH-deficient human tumors, such as succination (Bardella et al., 2011); activation of the NRF2 antioxidant pathway (Adam et al., 2011; Ooi et al., 2011), and, as indicated here, alteration of urea cycle metabolism. The UOK262 cell line perhaps reflects better the later stages of renal neoplasia and metastasis associated with this. UOK262 are quite abnormal cells, and re-expression of either FH or FHcyt, at least in our hands, exacerbates their abnormal morphology and, unlike reconstituted MEFs, does not affect a full “rescue,” exemplified by incomplete ablation of normoxic HIF-1α (Figure S2). It is also possible that the UOK262 cells have acquired additional mutations subsequent to loss of FH activity.

The relatively low success rate in treating renal cancer has been attributed in part to high levels of heterogeneity within tumors (Fisher et al., 2012; Gerlinger et al., 2012; Yap et al., 2012), and therefore treatment of HLRCC with arginase could be more effective if explored in combination with other therapies. Thus, tumor and/or metastasis-derived cell lines such as UOK262 will clearly reflect only a subset of the pathological features of such heterogeneous renal cancers.

In summary, we have utilized a murine model of early HLRCC to demonstrate that renal cyst development is independent of mitochondrial FH activity. These studies have highlighted the previously unrecognized importance of the urea cycle and arginine metabolism for FH-deficient cells and tumors and offer a potential Achilles’ heel for such cells.
**EXPERIMENTAL PROCEDURES**

See Extended Experimental Procedures for additional information.

**Generation of FH and FH**<sup>trans</sup>** Transgenic Mice**

The transgenic constructs consisted of the ubiquitous CAG promoter (Niwa et al., 1991) driving the expression of either full-length (FH) or mitochondria leader sequence deleted (−MLS) (FH<sup>trans</sup>) human FH cDNA, both of which carried a V5 tag at the C terminus (Figure 3A), together with a rabbit beta-globin polyadenylation sequence. The two transgenic constructs were cloned into a PhiC31 integrase-mediated exchange vector, pCB92, which was assembled by modifying pExchange4-CB9 (derived from pRMCE (Hitz et al., 2007)) with a V5 tag at the C terminus (Figure 3A), together with a rabbit beta-globin polyadenylation sequence. The two transgenic constructs were cloned into a PhiC31 integrase-mediated cassette exchange in murine IDG26.10-3 ES cells as previously described (Chen et al., 2011). Correctly integrated ES cell clones were identified by PCR (Figure S1) and injected into mouse C57BL/6J blastocysts, and the resulting chimeric males were mated to C57BL/6 females. Genotypes of transgenic F1 mice were established by PCR (Figure S1). All procedures were conducted in keeping with AAGR guidelines and under UK Home Office regulations after approval by the Local Ethical Review process in Oxford University.

**Human Tissue Samples**

Anonymized human tumor and normal samples were collected with full ethical approval (MREC 05/Q1605/66) as approved by the Oxford Centre for Histopathology Research (OCHR).

**Statistics**

All statistical analyses are indicated in the text in the relevant sections. One-way ANOVA and t tests were performed using GraphPad Prism version 5.0d for Macintosh (GraphPad Software, La Jolla, CA; http://www.graphpad.com).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.006.

**LICENSING INFORMATION**

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