Factor inhibiting HIF (FIH-1) promotes renal cancer cell survival by protecting cells from HIF-1α-mediated apoptosis

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BACKGROUND: Clear cell renal cell carcinoma (CCRCC) is the commonest form of kidney cancer. Up to 91% have biallelic inactivation of VHL, resulting in stabilisation of HIF-α subunits. Factor inhibiting HIF-1 (FIH-1) is an enzyme that hydroxylates HIF-α subunits and prevents recruitment of the co-activator CBP/P300. An important question is whether FIH-1 controls HIF activity in CCRCC.

METHODS: Human VHL defective CCRCC lines RCC10, RCC4 and 786-O were used to determine the role of FIH-1 in modulating HIF activity, using small interfering RNA knockdown, retroviral gene expression, quantitative RT–PCR, western blot analysis, Annexin V and propidium iodide labelling.

RESULTS: Although it was previously suggested that FIH-1 is suppressed in CCRCC, we found that FIH-1 mRNA and protein are actually present at similar levels in CCRCC and normal kidney. The FIH-1 inhibition or knockdown in the VHL defective CCRCC lines RCC10 and RCC4 (which express both HIF-1α and HIF-2α) resulted in increased expression of HIF target genes. In the 786-O CCRCC cell line, which expresses only HIF-2α, FIH-1 attenuation showed no significant effect on expression of these genes; introduction of HIF-1α resulted in sensitivity of HIF targets to FIH-1 knockdown. In RCC4 and RCC10, knockdown of FIH-1 increased apoptosis. Suppressing HIF-1α expression in RCC10 prevented FIH-1 knockdown from increasing apoptosis.

CONCLUSION: Our results support a unifying model in which HIF-1α has a tumour suppressor action in CCRCC, held in check by FIH-1. Inhibiting FIH-1 in CCRCC could be used to bias the HIF response towards HIF-1α and decrease tumour cell viability.

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Clear cell renal cell carcinoma (CCRCC) often presents late and remains a significant cause of morbidity and mortality. Recently there have been advances in identifying signalling pathways that are activated in CCRCC, and the introduction of treatments targeting these pathways has shown useful activity in patients with the disease (Linehan et al., 2009). At a genetic level, biallelic inactivation of VHL occurs in the majority of CCRCC and normal kidney. The FIH-1 inhibition or knockdown in the VHL defective CCRCC lines RCC10 and RCC4 (which express both HIF-1α and HIF-2α) resulted in increased expression of HIF target genes. In the 786-O CCRCC cell line, which expresses only HIF-2α, FIH-1 attenuation showed no significant effect on expression of these genes; introduction of HIF-1α resulted in sensitivity of HIF targets to FIH-1 knockdown. In RCC4 and RCC10, knockdown of FIH-1 increased apoptosis. Suppressing HIF-1α expression in RCC10 prevented FIH-1 knockdown from increasing apoptosis.

which there are two isoforms HIF-1α and HIF-2α (Maxwell et al., 1999). Although it is likely that other actions of VHL contribute to its tumour suppressor action in the kidney, activation of HIF (and more specifically HIF-2α) has been shown to be necessary and sufficient for growth of VHL defective CCRCC cells in xenograft assays (Kondo et al., 2002, 2003). The HIF activation has a range of effects, which could contribute to tumour progression, including enhancing glucose uptake, and increasing expression of glycolytic enzymes and angiogenic mediators (Semenza, 2007). When VHL is present, HIF activation is dramatically downregulated in the presence of oxygen through oxygen-dependent enzymatic hydroxylation of specific prolyl residues by the prolyl hydroxylase domain (PHD) enzymes in the central part of HIF-α subunits, which leads to capture by VHL and ubiquitylation (Epstein et al., 2001). In VHL defective cells, HIF-α subunits are stable in the presence of oxygen (Maxwell et al., 1999). Studies in mice and humans have established that VHL loss-of-function alone is not sufficient for tumourigenesis (Mandriota et al., 2002; Rankin et al., 2006; Frew et al., 2008). The additional events that are required for tumour development are incompletely understood, but there is evolution from exclusive HIF-1α expression in normal renal epithelium and very early lesions to a predominant or exclusive HIF-2α response in tumours, which is likely to be important (Raval et al., 2005).
Co-activator recruitment by HIF-2α subunits is regulated by oxygen via FIH-1. This hydroxylates a conserved asparagine residue (Asn 803 in human HIF-1α) within the C-terminal transactivation domain (CTAD) of HIF-α, thereby preventing binding of the co-factor p300 and inhibiting HIF transcriptional activation (Freedman et al., 2002; Hewitson et al., 2002; Lando et al., 2002a,b; Elkins et al., 2003). The role of FIH-1 in regulating the HIF response has been less extensively investigated than that of the PHD enzymes and VHL, but it is established that attenuating FIH-1 increases expression of HIF target genes across a wide range of oxygen tensions (Stolze et al., 2004). Importantly, it has recently been established that FIH-1 hydroxylates ankyrin repeats in other proteins besides HIF-2 subunits (Cockman et al., 2006; Linke et al., 2007).

Here we investigate the role of FIH-1 in modulating HIF activity in VHL defective CCRCC. Previous studies of two renal cancer cell lines, A498 and 786-O, suggested that FIH-1 expression was specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically repressed by a mechanism involving phosphatidylinositol 3-kinase (PI3K) and the atypical protein kinase C, PKCζ, specifically 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with 7.5 μg ml⁻¹ polybren (Sigma-Aldrich, Poole, UK), and added to cells that had been plated the day before on p100 dishes at 30–40% confluence. After 20 h, cells were washed, and fresh media were added for 20 h before performing a second round of infection.

An active form of HIF-1α carrying the substitutions P402A and P564A, which is resistant to hydroxylation by PHD enzymes, was cloned into pBMNz-HIF1α-neo (Raval et al., 2005). Following infection as described, 786-0 cells were selected with G418.

Retroviral vectors of pFIH-1

The coding sequence for human FIH-1 with and without a C terminal Pκ tag (V5 epitope from paramyxovirus) was inserted into pCMVR-Neo using standard manoeuvres. Following infection with retroviruses, cells were selected with G418.

Cell proliferation and apoptosis assays

Cell culture expansion was over a period of 3 days by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich). Cells were transfected as described above and after 15–20 h, were plated at a density of 3 × 10⁵ cells per well into a 24-well tissue culture plate. After 24, 48 and 72 h, 50 μl of 5 mg ml⁻¹ MTT solution were added to the cell cultures in 0.5 ml of medium. After 4 h, media were removed and precipitated formazan crystals formed in viable cells were solubilised with 200 μl of isopropanol-triton (0.1%). Product formation was quantified by absorbance at 550 nm.

Cell culture expansion was also assessed by manual counting. Transfected cells were plated at a density of 2 × 10⁴ cells per well into a six-well tissue culture plate, or 1 × 10⁵ cells per well into a 24-well tissue culture plate and viable cells were counted using a hemocytometer after trypan blue staining.

Apoptosis of siRNA-transfected cells was measured by the Annexin-V-FITC Detection Kit I (BD Biosciences, Oxford, UK) according to the manufacturer’s instructions. After staining, cells were analysed on a Becton Dickinson FACS Caliber flow cytometer with CellQuest software (BD Biosciences).

Apoptosis was also measured using the Cell Death Detection ELISA Plus kit (Roche, Burgess Hill, UK). Cell pellets of transfected cells were placed into 200 μl of lysis buffer provided by the manufacturer for 30 min and centrifuged. Aliquots of the supernatant (20 μl) were used in an ELISA with anti-DNA and anti-histone antibodies to detect the presence of cytoplasmic nucleosomes.

Statistical analysis

Data are presented as the mean (± s.e.m.) of three independent experiments. ANOVA or Student’s t-test were used to evaluate differences and the level of statistical significance is indicated by the use of asterisks in the figures: *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Factor inhibiting HIF-1 is expressed in renal cancer

The expression of FIH-1 in CCRCC has not been directly examined to our knowledge. We therefore analysed expression of FIH-1 in CCRCC samples and adjacent uninvolved kidney samples from the same patient. Specimens were obtained at the time of nephrectomy for CCRCC. Although the levels detected were variable, Figure 1 shows that there was no significant difference in HIF-1α mRNA or protein levels between tumour specimens and adjacent kidney (Figures 1A and B). As expected for cancers where the HIF pathway is constitutively activated, GLUT1 mRNA levels were increased in the tumour samples (data not shown).

Factor inhibiting HIF-1 functions in VHL defective CCRCC cell lines

The presence of equivalent amounts of FIH-1 in tumour and adjacent kidney suggested that FIH-1 may actually be reducing HIF activity in this setting: HIF-α subunits would be abnormally stabilised (because VHL is absent), but HIF transcriptional activity would be limited by the action of FIH-1 in the presence of oxygen, which would hydroxylate the CTAD and reduce co-activator recruitment. To examine this we first exposed VHL defective RCC10 cells to reduced oxygen, as a means to reduce FIH-1 enzymatic activity. As Figure 2A shows, we found increased mRNA levels of four well-characterised HIF-α target genes GLUT1, PHD3, VEGF and BNIP3 in RCC10 and RCC4 at 0.1 and 1% oxygen (Figure 2A). Expression of each of these genes was further increased in hypoxia from the high levels observed in the absence of VHL. Importantly, similar results were obtained in a second VHL defective cell line, RCC4 (Figure 2A). As reducing oxygenation could have other effects besides attenuating FIH-1 activity, we used DMOG, a small molecule inhibitor that is an analogue of the co-substrate 2-oxoglutarate as an alternative method of inhibiting FIH-1. Figure 2B shows that treatment of CCRCC cell lines with DMOG increases expression of HIF target genes.

These results would be consistent with FIH-1 exerting a negative effect on HIF activation in these VHL defective cell lines. However hypoxia and DMOG are not specific inhibitors of FIH-1; they will inhibit other 2-oxoglutarate-dependent oxygenases including the PHD enzymes (Epstein et al., 2001). In the absence of VHL, prolyl hydroxylation of HIF-1α and HIF-2α by the PHD enzymes has been reported to decrease transactivation by the N-terminal transactivation domain (NTAD) (To and Huang, 2005), providing a potential mechanism by which hypoxia and DMOG would increase expression of HIF targets in a manner independent of FIH-1 and

Figure 1 The FIH-1 is expressed at a similar level in renal cancer and uninvolved kidney. (A) The FIH-1 mRNA levels in renal cancer samples and uninvolved kidney from the same patients. n = 10. (B) Representative immunoblots showing FIH-1 protein in renal cancer samples and uninvolved kidney from the same patients.
CTAD activity. To directly examine whether FIH-1 inhibits HIF activity we used RNA interference (RNAi). By using either of two different non-overlapping siRNA sequences independently, we achieved a significant reduction of FIH-1 at the mRNA (~70% attenuation) and protein level. The FIH-1 knockdown resulted in a significant increase in HIF target gene mRNA levels as well as GLUT1 protein levels (Figures 3A and B). Taken together, these results provide clear evidence that FIH-1 is acting to reduce expression of HIF target genes in RCC10 and RCC4 cells under normoxic conditions.

Increasing FIH-1 expression has little effect on the expression of HIF target genes

The inhibition of HIF transcriptional activity by FIH-1 in RCC10 and RCC4 cells is clearly incomplete as HIF exerts potent effects on gene expression in these cells, as demonstrated by the effects of siRNA for HIF-α subunits (Raval et al., 2005). One explanation for this incomplete inhibition would be that the amount of FIH-1 enzyme in these cells is insufficient to achieve maximal down-regulation of HIF. We therefore examined the effect of increasing
expression of FIH-1. A retroviral vector containing cDNA encoding for FIH-1 was prepared and used to infect RCC10 cells. The FIH-1 sequence was tagged with the Pk epitope to allow detection of the exogenous FIH-1 (Figure 4A). Analysis of mRNA levels showed a six-fold increase in FIH-1 mRNA when compared with levels in RCC10 cells infected with an empty vector (Figure 4B). In case the Pk tag might reduce enzymatic activity, we also performed this experiment with untagged FIH-1, with similar results (data not shown). This lack of effect of augmenting FIH-1 contrasts with the effect of introduction of VHL, which leads to marked suppression of HIF target genes (Maxwell et al., 1999). This implies that a substantial proportion of HIF activity is resistant to the action of FIH-1; probably this involves transactivation mediated by the NTAD of HIF-2α subunits, which is not regulated by FIH-1.

**Attenuating FIH-1 does not reduce HIF target gene expression in 786-O cells, which only express HIF-2α**

To investigate this further we examined the effect of FIH-1 knockdown in another well-characterised VHL defective cell line, 786-O, which expresses HIF-2α, but not HIF-1α (Maxwell et al., 1999). We found that FIH-1 siRNA has no effect on HIF target gene expression in 786-O cells (Figure 5A). This contrasts with our observations in RCC10 and RCC4 cells, but is in line with a previous study (Datta et al., 2004). Possible explanations for this would be either that FIH-1 was inactive in these cells (as was suggested in the previous study), or that the HIF-2α they contain is not susceptible to inactivation by FIH-1. To distinguish these possibilities, we expressed HIF-1α in 786-O cells, and then performed RNAi against FIH-1. Western blot analysis confirmed exclusive expression of the HIF-2α isoform in a pool of parental 786-O cells infected with empty vector (pBMNz) and expression of HIF-1α in cells infected with pBMNz-HIF-1α (Figure 5B). Using siRNA, we attenuated FIH-1 in both pools of 786-O cells (Figure 5B). In the pBMNz transfected pool, in which there is exclusive expression of HIF-2α, attenuation of FIH-1 did not affect HIF target gene expression. In contrast, in pBMNz-HIF-1α, FIH-1 attenuation augmented HIF target gene levels of PHD3, VEGF and the pro-apoptotic gene BNIP3 (Figure 5C). These results show that active FIH-1 is present in 786-O cells, and introducing HIF-1α can reveal this activity. Furthermore, HIF-2α (at least in 786-O cells) is insensitive to inactivation by FIH-1.

**Attenuating FIH-1 reduces growth of renal cancer cells expressing HIF-1α and induces apoptosis**

Previously it has been shown that HIF-1α has anti-proliferative effects in VHL defective renal cancer cells (Raval et al., 2005). This raises the interesting possibility that in cells that lack VHL and express HIF-1α, FIH-1 may favour tumour growth by decreasing the anti-proliferative consequences of HIF-1α activation and shifting the balance of HIF activation towards HIF-2α. To test this, we examined the effect of FIH-1 siRNA on cell population expansion and apoptosis of RCC10 and RCC4 cell cultures. Knockdown of FIH-1 significantly reduced expansion of RCC10 cell cultures using either of the two siRNAs (Figure 6A). The RCC4 and RCC10 cells showed reduced population expansion as assessed by counting the number of viable cells or by MTT assays (Figure 6B). In contrast, population expansion of 786-O cells that do not express HIF-1α was not reduced. This suggested that the effect of FIH-1 knockdown on population expansion might be mediated via increasing the activity of HIF-1α. To test this possibility, RCC10 were transfected with shRNA targeting HIF-1α before FIH-1 knockdown. This prevented the effect of FIH-1 knockdown on population expansion (Figure 6C). Interestingly, suppression of HIF-1α expression resulted in modest, but statistically significant, increase in cell numbers in comparison with control, consistent with HIF-1α suppressing proliferation and/or enhancing cell death.

To investigate the mechanism(s) by which cell numbers were decreased, we assayed cytological histone-associated DNA fragments to assess apoptotic cell death (Figure 6D). This was increased by attenuating FIH-1 in VHL defective RCC10 and RCC4 cells. However, no significant increase was observed in 786-O cells (which express only HIF-2α) or in RCC10 cells in which VHL was stably expressed, resulting in suppression of HIF-1α and HIF-2α. Independent evidence for increased apoptosis was provided by flow cytometry analysis (Figure 6E). The FIH-1 knockdown in RCC10 cells resulted in an increase in early apoptotic (Annexin-V positive) cells, compared with the control shLuc transfection (46.0% vs 9.6%). Taken together, the results are consistent with FIH-1 decreasing apoptosis through a decrease in the activity of HIF-1α. The 786-O cells showed a much less marked effect, but interestingly there was some increase in early apoptotic cells, on FIH-1 knockdown (16.59% with siRNA for FIH-1 vs 5.7% in controls) (Data not shown). This may reflect a HIF-α, independent action of FIH-1 mediated by one of a number of other identified substrates, which includes components of the Notch or NFkB pathways (Cockman et al., 2006; Coleman et al., 2007; Zheng et al., 2008).

**DISCUSSION**

The major findings of this study are that FIH-1 is present at similar levels in normal kidney and CCRCC, that FIH-1 inhibition in CCRCC cells can increase expression of HIF targets, and that...
inhibiting FIH-1 can increase apoptosis in these cells. It is noteworthy that FIH-1 expression is maintained in CCRCC compared with normal kidney, as it was previously suggested that an important step in evolution to CCRCC following the loss of VHL function was suppression of FIH-1. In that model, suppression of FIH-1 was considered necessary to achieve HIF activation. Our study shows that FIH-1 is present and active in CCRCC, but it only partially inactivates HIF. This is consistent with the fact that biallelic inactivation of vhl in mouse and human renal epithelium (and other cell types) is associated with marked activation of HIF target genes, even though FIH-1 has not been inactivated (Mandriota et al., 2002; Rankin et al., 2006).

Evidence for the model in which suppression of FIH-1 was a pivotal aspect of CCRCC included the fact that decreasing FIH-1 did not influence HIF activity in 786-O cells. Our study confirms this observation, but implies a different mechanism in which FIH-1 is present but the HIF-2α in these cells is resistant to its action. In the previous studies it was suggested that FIH-1 suppression was achieved in CCRCC via PKCζ suppressing FIH-1 at the level of transcription. Our finding that FIH-1 expression is similar in normal kidney and CCRCC implies that any negative effect of PKCζ is likely to be present in normal renal epithelium as well as CCRCC. This is supported by the fact that similar suppression of FIH-1 via PKCζ was also observed in HEK 293 cells, a non-malignant renal cell line (Li et al., 2007).

Regardless of the mechanism(s) by which a substantial proportion of HIF activity in CCRCC is resistant to FIH-1, we show that FIH-1 is protecting RCC10 cells from apoptosis and this is mediated by its effect on HIF-1α. That increased activity of HIF-1α subunits may be resistant to the action of FIH-1. As HIF-1α is known to be a substantially better substrate for FIH-1 than HIF-2α (Bracken et al., 2006) it is likely that this resistant fraction is predominantly HIF-2α. This is further supported by data that mutating the FIH-1 target residue in HIF-2α does not increase HIF target gene expression. An increase in expression of HIF target genes following FIH-1 inhibition is seen following introduction of HIF-1α. Data are presented as the mean of three independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 5 Introducing HIF-1α in 786-O cells reveals the ability of FIH-1 to regulate HIF activity. (A) The 786-O cells were treated with siRNA for FIH-1 or control. No increases in HIF target gene mRNA were observed, contrasting with results from RCC10 and RCC4 (Figure 2). Data are presented as the mean of three independent experiments. (B) The 786-O cells were infected with retrovirus-encoding HIF-1α in which the two prolyl residues that are targets for hydroxylation were mutated (left panels), or empty vector (right panels). Following selection with G418, cells were treated with luciferase control siRNA or FIH-1 siRNA. Immunoblots show expression of FIH-1, HIF-1α and HIF-2α. (C) Analysis of expression of the indicated HIF target genes. Inhibition of FIH-1 in cells infected with pBMNz empty vector (EV) does not increase HIF target gene expression. An increase in expression of HIF target genes following FIH-1 inhibition is seen following introduction of HIF-1α. Data are presented as the mean of three independent experiments. (*p < 0.05, **p < 0.01, ***p < 0.001).
biallelic inactivation of VHL in human kidney, which show HIF-1α activation, are not associated with a net increase in proliferation (Mandriota et al., 2002). This suggests that evolution to CCRCC involves a number of additional steps, and there is evidence that a progressive increase in HIF-2α relative to HIF-1α is important. Consistent with this, in xenograft assays of CCRCC cells, active HIF-2α is both necessary and sufficient for tumour growth (Kondo et al., 2002, 2003), whereas active HIF-1α is insufficient (Maraniche et al., 2002). An attractive possibility is that by exerting more effect on HIF-1α than HIF-2α, FIH-1 contributes to VHL defective cells evading apoptosis. This is also consistent with the fact that mutations in FIH-1 have not been reported in CCRCC (Morris et al., 2004).

The intersection of the effects of VHL, HIF and FIH-1 on patterns of gene expression and on cell proliferation have recently been examined in murine embryonic fibroblasts with genetic deletions of each gene, and multiple combinations thereof (Zhang et al., 2010). In murine embryonic fibroblasts (MEFs) loss of FIH-1 was shown to have significant and complex effects on expression of HIF target genes in the absence of VHL under normoxic conditions. Loss of VHL in MEFs was associated with reduced plating efficiency and there was an additive negative effect of loss of FIH-1, consistent with the effects that we observe of FIH-1 knockdown on population expansion in CCRCC cells. Strikingly, removal of HIF-1α restored plating efficiency to that of controls.

The crystal structure of FIH-1 has been solved and there are consider that other FIH-1 substrates have been identified besides HIF-1α. In particular, it has been shown that asparagine residues in ankyrin repeat domains are hydroxylated by FIH-1 including the intracellular domain (ICD) of the Notch receptor, the
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1αB family of inhibitory proteins and tankyrase (Cockman et al., 2006, 2009; Coleman et al., 2007; Ferguson et al., 2007; Zheng et al., 2008). Therefore FIH-1 inhibition is likely to have wide-ranging effects that would have to be investigated before FIH-1 can be considered a suitable target for inhibition.

Useful insight into this is provided by the recently reported knockout mouse, which is viable but exhibits hypermetabolism (Zhang et al., 2010). Our study raises the possibility that FIH-1 would be a useful therapeutic target in clear cell renal carcinoma.
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