Tumour hypoxia promotes the accumulation of the otherwise oxygen-labile hypoxia-inducible factor (HIF-α) subunit whose expression is associated with cancer progression, poor prognosis and resistance to conventional radiation and chemotherapy. The oxygen-dependent degradation of HIF-α is carried out by the von Hippel–Lindau (VHL) protein-containing E3 that directly binds and ubiquitylates HIF-α for subsequent proteasomal destruction. Thus, the cellular proteins involved in the VHL–HIF pathway have been recognized as attractive molecular targets for cancer therapy. However, the various compounds designed to inhibit HIF-α or HIF-downstream targets, although promising, have shown limited success in the clinic. In the present study, we describe the bioengineering of VHL protein that removes the oxygen constraint in the recognition of HIF-α while preserving its E3 enzymatic activity. Using speckle variance–optical coherence tomography (sv–OCT), we demonstrate the dramatic inhibition of angiogenesis and growth regression of human renal cell carcinoma xenografts upon adenovirus-mediated delivery of the bioengineered VHL protein in a dorsal skin-fold window chamber model. These findings introduce the concept and feasibility of 'bio-tailored' enzymes in the treatment of HIF-overexpressing tumours.

INTRODUCTION

Tumour growth invariably outstrips its blood supply as the diffusional capacity of oxygen from the nearest blood vessels is surpassed, leading to regions of hypoxia within the tumour mass. In addition, tumour cells close to a blood vessel can experience hypoxia due to disruptions in blood flow, a common characteristic of malformed tumour vasculature (Brown & Wilson, 2004). The transcription factor HIF is activated under hypoxia and triggers the transcription of a large number of genes that promote various adaptive cellular responses ranging from anaerobic metabolism, erythropoiesis and angiogenesis to cell survival. HIF-induced genes are known to drive oncogenesis and as a result, HIF overexpression is frequently associated with increased phenotypic aggressiveness and poor prognosis in numerous tumour types including brain, breast, lung, colon, skin, prostate and kidney cancers (Kim & Kaelin, 2004; Semenza, 2003).

HIF is a heterodimeric transcription factor composed of two subunits, HIF-α and aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-α and ARNT are members of the basic-helix–loop–helix (bHLH) Per/ARNT/Sim (PAS) family of
transcription factors. The basic domain is essential for DNA binding, whereas the HLI and PAS domains are necessary for heterodimerization and DNA binding. HIF-α contains two transactivation domains (NAD and CAD, located in the amino (N) and carboxy (C) termini, respectively), whereas ARNT binds one transactivation domain (TAD) in the C-terminus. Under low oxygen tension, HIF-α recruits transcriptional co-activators p300/CBP and binds ARNT. The active HIF complex binds to hypoxia-responsive elements (HREs) in the promoters/enhancers of the numerous HIF-target genes such as vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT1), transforming growth factor-α (TGF-α) and erythropoietin (EPO) to initiate their transcription (Kim & Kaelin, 2004; Semenza, 1999). Under normal oxygen tension, HIF-α is hydroxylated on conserved proline residues in the oxygen-dependent degradation domain (ODD) by prolyl hydroxylase domain-containing family of enzymes (PHD1-3) (Brück & McKnight, 2001; Epstein et al, 2001; Ivan et al, 2001; Jaakkola et al, 2001; Masson et al, 2001). This oxygen-dependent modification of HIF-α permits recognition by the VHL tumour suppressor protein, which functions as the substrate-recognition component of an E3 ubiquitin ligase EC (Elongins BC/Cul2/VHL) that polyubiquitylates HIF-α for subsequent proteasomal degradation (Cockman et al, 2000; Kamura et al, 2000; Maxwell et al, 1999; Ohh et al, 2000; Tanimoto et al, 2000). Unlike HIF-α, ARNT is constitutively expressed and stable, and thus the regulation of HIF is at the level of HIF-α stability (Kim & Kaelin, 2004).

VHL protein has two major functional domains: the α domain is required for the nucleation of the Elongins BC/Cul2 in VHL complex and the β domain is required for prolyl-hydroxylated HIF-α recognition (Stebbins et al, 1999). Furthermore, functional inactivation of the VHL protein is the cause of hereditary VHL cancer syndrome—characterized by the development of hypervascular tumours in multiple organs including the brain, spine, retina and kidney, and is also associated with the development of the vast majority of sporadic clear-cell renal cell carcinoma (CCRCC), which is the most common form of kidney cancer (Kim & Kaelin, 2004). Notably, all CCRCC-causing VHL mutants tested-to-date have shown a failure in either assembling into an ECV complex or binding to HIF-α (Kim & Kaelin, 2004). Concordantly, tumour cells including CCRCC devoid of functional VHL protein have enhanced expression of HIF-target genes irrespective of oxygen tension. In addition, growth factors binding to their cognate receptor tyrosine kinases (RTKs) and ensuing activation of the phosphoinositide 3-kinase and MAPK signalling pathways can regulate HIF-1α protein levels in an oxygen-independent fashion (Maynard & Ohh, 2007; Semenza, 2003). Both pathways can activate mTOR-mediated cap-dependent translation of HIF-1α mRNA, and PI3K can also increase translation of HIF-1α mRNA through an internal ribosomal entry site (IRES)-dependent mechanism (Maynard & Ohh, 2007). Thus, tumour-associated mutations impinging on the PI3K or MAPK pathways including gain-of-function mutations in RTKs and Ras or loss-of-function mutations in phosphatase and tensin homolog and tumour suppressor complex 1/2 tumour suppressor genes, increase HIF-1α synthesis. In addition to mutations in various oncogenes and tumour suppressor genes, the most common mechanism of HIF-1α stabilization in cancer arguably involves the general oxygen-sensing pathway in regions of tumour hypoxia, in which, for example, a functional VHL protein would be rendered ineffectual in negatively regulating HIF-α stability.

Overexpression of HIF-1α or HIF-2α has been strongly associated with tumour progression and resistance to therapy, implicating HIF-1α and HIF-2α as compelling therapeutic targets for anti-cancer therapy (Kondo et al, 2002; Semenza, 2003). Currently, there are a number of compounds either in clinical development or approved by the Food and Drug Administration that have been shown to block HIF-1α activity. For example, gefitinib and erlotinib, trastuzumab, and everolimus and temsirolimus have been shown to inhibit HIF-1α synthesis by blocking upstream oncogenic epidermal growth factor receptor, human epidermal growth factor receptor 2/Neu and mammalian target of rapamycin signalling pathways, respectively (Melillo, 2007). A topoisomerase I inhibitor topotecan and a microtubule polymerization inhibitor 2ME2 have also been found to interfere with HIF-1α mRNA translation. DNA-binding molecule echinomycin interrupts the DNA binding of HIF-1α, whereas HIF-1α-mediated transcription is reduced by the proteasome inhibitor velcade and the antifungal agent amphoterocin B. In addition, inhibition of the chaperone Hsp90 by 17-AAG and 17-DMAG, as well as inhibition of HDAC by LAQ824, has been found to induce HIF-1α protein degradation (Melillo, 2007; Semenza, 2007). However, none of the above agents directly targets HIF-1α and each drug has multiple functions other than blocking HIF-1α. Moreover, the inhibitory effect of these agents on HIF-2α is largely unknown, despite an increasing evidence supporting an important role of HIF-2α in tumourigenesis. For example, HIF2 has recently been shown to transactivate Oct-4, a transcription factor essential for maintaining stem cell pluripotency, and angiopoietin-1 receptor, Tie-2 and VEGFR2 have been established as HIF2-target gene products (Covello et al, 2006; Duan et al, 2005; Elvert et al, 2003; Tian et al, 1997). HIF2 also has a higher transactivation activity than HIF1 on the promoters of VEGF, TGF-α and EPO (Gunaratnam et al, 2003; Warnecke et al, 2004; Wiesener et al, 1998). In addition, several lines of evidence have shown the stabilization of HIF-2α, but not HIF-1α, to be the critical oncogenic event upon the loss of VHL protein in CCRCC (Kondo et al, 2002, 2003).

In the present study, we demonstrate that a bioengineered VHL protein can engage and degrade HIF-1α and HIF-2α irrespective of oxygen tension, eliminating the necessity for prolyl-hydroxylation of HIF-α for degradation. We further show that adenovirus-mediated delivery of a bioengineered VHL protein dramatically inhibits angiogenesis and regresses CCRCC xenografts in vivo. This is the first report illustrating the feasibility of an E3 ubiquitin ligase designed to remove the oxygen constraint as an alternative mode to directly and constitutively target and destroy HIF-α for rational anti-cancer therapy.
RESULTS

Unlike binding to VHL protein, prolyl-hydroxylation of HIF-α is not required for binding ARNT since heterodimerization occurs under hypoxic conditions. Thus, we sought to generate a VHL–ARNT chimaera containing the minimal region of ARNT required for binding HIF-α fused to the α domain of VHL known to bind elongin C, which bridges the VHL protein to the rest of the ECV complex (Fig 1A). A prediction is that the VHL–ARNT chimaera would bind HIF-α irrespective of oxygen to promote its degradation.
VHL–ARNT fusion proteins bind HIF-α and form an ECV complex

The bHLH, PAS A and PAS B domains of ARNT are required for dimerization with HIF-1α and HIF-2α. C-terminal to the PAS B domain is the PAS C-terminal domain (PAC) that is less well-described, but has been proposed to play a similar role in heterodimerization (Maynard & Ohh, 2004). To define the minimal region of ARNT required for strong dimerization with HIF-1α, we generated T7-tagged truncation plasmids encoding the following domains: HLH, PAS A, PAS B (T7-HPAS, residues 103–419); bHLH, PAS A, PAS B (T7-bHPAS, residues 90–419); HLH, PAS A, PAS B, PAC (T7-HPAC, residues 103–467); and bHLH, PAS A, PAS B, PAC (T7-bHPAC, residues 90–467) (Fig 1B). An in vitro binding assay was performed with 35S-labelled in vitro translated HA-HIF-1α mixed with 35S-labelled in vitro translated T7-tagged ARNT truncation mutants. The reaction mixtures were immunoprecipitated with an anti-T7 antibody, resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography (Fig 1C). The ARNT truncation mutants containing the PAC domain showed increased binding to HIF-1α (Fig 1C, compare lanes 5 and 8 with lanes 11 and 14). Based on these findings, T7-HPAC and T7-bHPAC were used to generate the VHL–ARNT chimaera.

VHL–ARNT chimaeras were generated by fusing the VHL α domain (residues 151–194) C-terminal to T7-HPAC and T7-bHPAC with or without a 6-Glycine flexible linker between the two heterologous protein fragments, giving rise to the following constructs: T7-HPACV, T7-HPACGV, T7-bHPACV and T7-bHPACGV (Fig 1D). We next tested the ability of the fusion proteins to bind HIF-1α by performing an analogous in vitro binding assay (Fig 1E). The addition of VHL α domain did not diminish the ability of ARNT truncation mutants to bind HIF-1α, and T7-HPACV and T7-HPACGV chimaeras displayed stronger interaction with HIF-1α than T7-bHPACV or T7-bHPACGV containing the basic DNA binding sequences (Fig 1E, compare lanes 9 and 12 with lanes 18 and 21).

We explored whether the VHL–ARNT fusion proteins bound HIF-1α in vivo. HEK293A cells were transiently co-transfected with the mammalian expression plasmids encoding HA-HIF-1α and empty plasmid (MOCK) or T7-HPACV, T7-HPACGV, T7-bHPACV or T7-bHPACGV. Cells were treated with the proteasome inhibitor MG132 to stabilize the oxygen-labile HIF-1α. Cells were then lysed, immunoprecipitated with an anti-HA antibody and visualized by immunoblotting (Fig 2A). T7-VHL served as a positive control showing co-precipitation of the scaffold component Cul2 (Fig 2B, lane 3), while the ARNT truncation mutant T7-bHPAC lacking the VHL α domain served as a negative control showing a failure in co-precipitating Cul2 (Fig 2B, lane 4). VHL–ARNT chimaeras, when normalized for expression, exhibited Cul2 binding with efficiency comparable to that of wild-type VHL protein, indicating their ability to form an ECV complex.

VHL–ARNT chimaeras promote HIF-α degradation and inhibit HRE-mediated transcription under hypoxia

We asked whether T7-HPACV or T7-HPACGV could degrade HIF-1α under hypoxia. HEK293A cells were transiently co-transfected with plasmids encoding HA-HIF-1α and empty plasmid or T7-VHL, T7-HPACV, T7-HPACGV, T7-bHPACV or T7-bHPACGV. Prior to lysis,
cells were treated with or without proteasome inhibitor MG132 and maintained under hypoxia (1% oxygen). Cells were lysed and equal amounts of the whole cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-HIF-1α antibody (Fig 3A). As expected, co-transfection of T7-VHL had negligible effect on HIF-1α protein levels, evidenced by the similar amounts of HIF-1α detected with or without MG132 (Fig 3A, compare lanes 3 and 4). Notably, the endogenous VHL protein in HEK293A cells had likewise no discernable effect on HA-HIF-1α expression under hypoxia (Fig 3A, lane 2). In contrast, the expression of either T7-HPACV or T7-HPACGV caused dramatic attenuation of HIF-1α levels in the absence of MG132 (Fig 3A, lanes 6 and 8). These results indicate that both T7-HPACV and T7-HPACGV effectively promote HIF-1α for proteasome-dependent degradation under hypoxia. Consistent with this notion, non-hydroxylated HIF-1α(Pro564Ala) (Ivan et al, 2001; Jaakkola et al, 2001), which has been shown to be stable in the presence of wild-type VHL protein under normoxia, was effectively degraded by T7-HPACGV in a proteasome-dependent manner (Fig S1 of Supporting Information).

To assess the binding of VHL–ARNT chimaeras to HIF-1α under hypoxia, an analogous experiment in HEK293A cells was performed with anti-HA and anti-T7 immunoprecipitations of the whole cell lysates. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-HIF-1α and anti-T7 antibodies (Fig 3B). As expected, T7-VHL failed to co-precipitate HA-HIF-1α under hypoxia even in the presence of MG132 (Fig 3B, lane 3). In contrast, both T7-HPACV and T7-HPACGV co-precipitated HA-HIF-1α in the presence of MG132 (Fig 3B, lanes 5 and 7). These results strongly suggest that VHL–ARNT chimaeras bind HIF-1α under hypoxia to promote its proteasome-dependent degradation. AhRryl Hydrocarbon Receptor (AhR) is another well-characterized ARNT binding partner ubiquitously expressed and involved in endo- and xenobiotic metabolism (Kewley et al, 2004). AhR as expected bound ARNT, but failed to bind HPACGV in an in vitro binding assay (Fig S2A and S2B of Supporting Information). Accordingly, HPACGV failed to downregulate the expression of AhR in vitro or in vivo (Fig S2C and D of Supporting Information). Thus HPACGV, which was generated and optimized for binding and degrading HIF-α, does not interact with arguably the next best-characterized ANRT-binding partner AhR.

Under hypoxia, HIF-α dimerizes with ARNT to form an active transcription factor HIF, which engages HREs in the promoters of a myriad of hypoxia-inducible genes to initiate their transcription. We sought to determine the effect of T7-HPACV and T7-HPACGV on HRE-driven transcription by performing a dual-luciferase assay using a firefly luciferase reporter driven by five contiguous HRE elements from the phosphoglycerate kinase-1 promoter (Fig 3C). HEK293A cells were transiently co-

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transfected with plasmids encoding (HRE)$_5$-Luc and T7-VHL, T7-HPACV or T7-HPACGV. Cells were maintained at either normoxia (21% oxygen) or hypoxia (1% oxygen) for 16 h prior to lysis. The transactivation activity from the HRE promoter was markedly higher under hypoxia than normoxia, as expected. Also, the ectopic expression of T7-VHL did not influence HRE-driven transactivation under hypoxia, since a VHL protein is ineffective in targeting HIF-α for destruction under hypoxia. In contrast, T7-HPACV and T7-HPACGV significantly reduced the transactivation from the HRE promoter under hypoxia (Fig 3C), indicating a marked loss of endogenous HIF function. Notably, T7-HPACGV was reproducibly more potent in attenuating HIF-mediated transcription than T7-HPACV (Fig 3C), and thus the T7-HPACGV chimaera was selected for subsequent experimentation. Furthermore, HPAC in the absence of a VHL protein diminished HIF-driven transcription under hypoxia by forming an inactive transcripational complex, whereas HPACGV in comparison, dramatically attenuated HIF-driven transcription (Fig S3 of Supporting Information), suggesting that the potency of HPACGV is due to the rapid E3 enzymatic activity causing the degradation of HIF-α upon binding.

Adenoviral delivery of T7-HPACGV attenuates HIF-α and HIF-target gene expression independent of oxygen tension

A cardinal feature of CCRCC is the overexpression of hypoxia-inducible genes. This is principally due to the inactivation of VHL protein that is observed in the vast majority of CCRCC. Interestingly, CCRCC harbouring wild-type VHL protein also displays strong hypoxia signatures and several lines of evidence suggest the stabilization of HIF-2α to be a critical oncogenic event in the pathogenesis of CCRCC (Kondo et al., 2002). We generated recombinant adenoviruses expressing enhanced green fluorescence protein (EGFP) alone or in combination with T7-VHL (Ad-EGFP-T7-VHL) or T7-HPACGV (Ad-EGFP-T7-HPACGV) and tested their ability to form an ECV complex in the CCRCC cell line 786-O (VHL−/−; HIF-1α−/−), a widely used cell system for CCRCC with constitutive activation of HIF-2α. Upon high-efficiency infection of 786-O cells with the indicated adenoviruses (>95%, as determined by EGFP fluorescence; data not shown), cells were lysed and immunoprecipitated with anti-T7 antibody. Bound proteins were resolved by SDS–PAGE and immunoblotted with anti-T7, anti-Cul2 and anti-Elongin B antibodies (Fig 4A). Both Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, co-precipitated Cul2 and Elongin B to comparable levels, suggesting equal ability of ECV complex formation (Fig 4A).

We next determined the effect of Ad-EGFP-T7-HPACGV on HIF-2α protein levels under normoxia and hypoxia. 786-O cells were uninfected (MOCK) or infected with Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV and maintained at 21 or 1% oxygen for 48 h. Cells were then lysed, resolved by SDS–PAGE and immunoblotted with anti-HIF-2α and anti-T7 antibodies (Fig 4B). Cells infected with Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, showed near-complete loss of HIF-2α expression under normoxia (Fig 4B, left panel). Importantly, while HIF-2α protein level was unaffected by Ad-EGFP-T7-VHL or Ad-EGFP infection, Ad-EGFP-T7-HPACGV dramatically attenuated the expression of HIF-2α under hypoxia (Fig 4B, right panel). These results demonstrate that, unlike Ad-EGFP-T7-VHL, which is only capable of degrading HIF-2α under normoxia, Ad-EGFP-T7-HPACGV degrades HIF-2α irrespective of oxygen tension.

We next generated 786-O cells stably expressing a firefly luciferase reporter gene driven by five HRE elements from the VEGF promoter (786-HRE-Luc). Infection of 786-HRE-Luc cells with Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, significantly decreased HIF-dependent HRE-driven luciferase activity under normoxia (Fig 4C). Under hypoxia, only Ad-EGFP-T7-HPACGV infected cells showed marked loss of luciferase activity (Fig 4C). Intriguingly, Ad-EGFP-T7-HPACGV had noticeably greater effect on the attenuation of HIF transactivation activity than Ad-EGFP-T7-VHL even under normoxia. This is likely a reflection of the restricted binding of VHL protein to prolyl-hydroxylated HIF-α, which is critically dependent on the Fe$^{2+}$ ion, 2-oxoglutarate and oxygen-dependent activity of prolyl hydroxylase domain (PHD) enzymes compared to the unrestricted capacity of VHL–ARNT fusion protein to bind any and all unmodified or modified HIF-α.

We next assessed the endogenous HIF transcriptional activity in 786-O cells infected with Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV. Consistent with the aforementioned biochemical analyses, Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV, but not Ad-EGFP, significantly decreased the protein level of GLUT1 under normoxia (Fig 4D, left panel). Under hypoxia, only Ad-EGFP-T7-HPACGV resulted in the marked reduction in GLUT1 (Fig 4D, right panel). Moreover, HIF-target gene transcripts such as GLUT1, VEGF and BNIP3L were reduced upon Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV infection under normoxia (Fig 4E). In contrast, only Ad-EGFP-T7-HPACGV was effective at decreasing GLUT1, VEGF and BNIP3L mRNA levels under hypoxia (Fig 4E).

Treatment of CCRCC xenografts with Adeno-EGFP-T7-HPACGV results in decreased angiogenesis and significant tumour necrosis

Optical coherence tomography (OCT) is a non-invasive near-infrared imaging technique that provides depth-resolved microstructural information in biological tissue at near-histology resolution (1–10 μm) (Huang et al., 1991). Depending on tissue optical properties, optical coherence tomography imaging depth ranges from 2 mm in highly scattering samples such as solid tumors to >20 mm in the eye. Recent advances in spectral domain OCT made three-dimensional in vivo imaging feasible. We developed a method of mapping normal microvascularity with high sensitivity and tracking photodynamic therapy-induced microvascular changes within the window chamber using an interframe speckle variance (SV) technique (Mariampillai et al., 2008). We apply, for the first time, the speckle variance technique to monitor tumour angiogenesis in response to the various recombinant adenoviral treatments within the skin-fold window chamber.

On day 2, 3 and 8, post-implantation of 786-O cells stably expressing red fluorescent protein DsRed2 (786-dsRed) in the dorsal skin-fold window chambers on severe combined
immunodeficiency mice (see Fig S4 of Supporting Information), intratumoural injections of Ad-EGFP, Ad-EGFP-T7-VHL or Ad-EGFP-T7-HPACGV were administered at $2 \times 10^8$ ifu. The adenovirus infections were monitored by the area and intensity of EGFP expression as measured by green fluorescence imaging, and tumour xenografts were visualized using red fluorescence microscopy, while angiogenesis was assessed using white-light microscopy and sv-OCT (Fig. 5). Adenoviruses infected the tumours with high efficiency and importantly, there was a complete lack of EGFP signal in the normal mouse tissues surrounding the xenografts. This allowed the observed effects to be directly attributed to specific adenoviral infection of the tumour (Fig. 5, second rows). Ad-EGFP treatment had negligible effect on the growth of tumours, which showed extensive angiogenesis within the tumour mass (Fig 5A). Ad-EGFP-T7-VHL treatment had a modest negative effect on tumour growth with some angiogenesis occurring within the tumour as compared to the Ad-EGFP treated group (Fig 5B). In contrast, Ad-EGFP-T7-HPACGV treatment dramatically inhibited tumour angiogenesis and showed significant tumour regression, especially in the central core region by day 8 post-implantation and most noticeably by day 10, the final day of the assay (Fig 5C).

To further examine the regression in Ad-EGFP-T7-HPACGV-infected tumours, we performed analogous experiments in which the tumours were resected 7 days post-implantation, corresponding to 4 days post-first adenoviral treatment, for immunohistochemical analysis (Fig 6). Immediately prior to sacrificing the mice, fluorescent, white-light and svOCT images were collected for analysis. Consistently, Ad-EGFP-infected tumours were highly angiogenic, while Ad-EGFP-T7-HPACGV-infected tumours exhibited markedly lower levels of neovascularization in the tumour core (Fig 6A). Green fluorescence
Figure 5. Ad-EGFP-T7-HPACGV treatment inhibits human CCRCC tumour xenograft angiogenesis in a dorsal skin-fold window chamber model. 786-dsRed cells were implanted into dorsal skin-fold window chambers in SCID mice. Tumours were intratumourally injected with
A. Ad-EGFP on day 2 post-implantation;
B. Ad-EGFP-T7-VHL on day 3 post-implantation;
C. Ad-EGFP-T7-HPACGV on day 8 post-implantation. Tumours were visualized by red fluorescence microscopy and positivity of adenoviral infection was monitored by green fluorescence microscopy. Tumour angiogenesis was visualized by white-light microscopy and sv-OCT. Four mice received treatments per recombinant adenovirus. Representative images are shown from each treatment group.
microscopy and anti-GFP immunohistochemical analysis of the resected tumours revealed positive GFP expression throughout Ad-EGFP-infected specimens (Fig 6A). However, while green fluorescence microscopy showed similar GFP expression in the Ad-EGFP-T7-HPACGV-infected tumours, anti-GFP immunohistochemical staining from numerous Z-stacked sections of the tumour revealed striking absence of GFP staining in the tumour core (Fig 6A). Consistent with this observation, hematoxylin and eosin (H&E) staining showed viable tumour cells throughout the Ad-EGFP-treated tumour mass, while Ad-EGFP-T7-HPACGV-treated tumours displayed an interface of viable to necrotic tumour cells in which the tumour periphery contained mostly viable cells with admixed early necrotic changes at the viable–necrotic interface to a largely necrotic tumour core with infiltrating inflammatory cells (Fig 6A and B). These results show that adenovirus-mediated expression of the VHL–ARNT fusion protein dramatically attenuates tumour angiogenesis and growth in a mouse xenograft model.

DISCUSSION

Accumulation of HIF-1α due to tumour hypoxia promotes progression and aggressiveness of cancer, and is associated with resistance to conventional cancer therapies. In addition, cancer-causing mutations on a growing list of oncogenes and tumour suppressor genes have been identified to enhance the expression of HIF-α, underscoring the significance of HIF in oncogenesis (Roberts & Ohh, 2008). Perhaps, the most direct and convincing association between tumour-causing mutations and HIF activation is the tumour suppressor VHL protein, which functions as a substrate-binding component of an E3 that polyubiquitylates prolyl-hydroxylated HIF-α for immediate destruction via the 26S proteasome (Kim & Kaelin, 2004). Moreover, CCRCC that frequently harbours mutations that inactivate the VHL protein or cause its loss, overexpresses HIF-2α and is one of the most resistant tumours to radiation or chemotherapies (Cohen & McGovern, 2005). Thus, the molecular understanding of the VHL–HIF oxygen-sensing...
pathway has been invaluable for conceptualizing new and targeted anti-cancer therapeutics. Surgery by radical or partial nephrectomy is the most effective treatment option for localized renal cancer. However, in one-third of patients, tumours recur post-operatively as distant metastases, and only 4–6% of these tumours respond to chemotherapy. The standard non-surgical treatment for advanced CCRCC has been the administration of interleukin-2 (IL-2) or IFN-α. However, the response rates were generally low with considerable toxicity. For example, high-dose IL-2 regimen has a response rate of only 21% and causes significant toxicities (Cohen & McGovern, 2005). Recently, clinical trials of RTK inhibitors, such as VEGFR2 and PDGFR-β inhibitors sorafenib and sunitinib have yielded promising results by prolonging progression-free survival in approximately 70% of patients with metastatic CCRCC. However, neither drug has had a significant effect on overall patient survival (Brugarolas, 2007; Oudard et al, 2007). Sorafenib and sunitinib as well as bevacizumab (a humanized monoclonal anti-VEGF antibody) are anti-angiogenic agents that curtail tumour neovascularization (Brugarolas, 2007; Oudard et al, 2007). However, while angiogenesis is one, albeit a major cellular response initiated by HIF, there are other important HIF-regulated functions, including anaerobic metabolism and cell survival, unlikely to be affected by these drugs. Thus, some of the strategies are aimed at targeting HIF-α itself rather than the downstream targets of HIF. For example, the most recent FDA approval was granted to temsirolimus, an injectable mTOR inhibitor as a first-line therapy for advanced CCRCC (Costa & Drabkin, 2007). The antitumour effects of temsirolimus are presumed to be through the inhibition of cap-dependent translation of HIF-1α. However, mTOR-mediated translation decreases under hypoxia as an energy-conserving measure. Under these circumstances, HIF-1α mRNA translation is ensured through IRES-dependent mechanisms (van den Beucken et al, 2006). This alternative method for HIF-1α synthesis under hypoxia questions the activity of mTOR inhibitors in hypoxic tumours. Moreover, numerous other small molecule inhibitors such as gefitinib, trastuzumab or topotecan in comparison to HDAC inhibitor LAQ824 have been shown to reduce the expression of HIF-1α (Melillo, 2007; Semenza, 2007). However, none of these agents directly targets HIF-1α with each drug having additional functions other than blocking HIF-1α. Thus, new strategies to inactivate HIF-1α, as well as HIF-2α, exclusively and directly would constitute a major conceptual advancement in anti-cancer therapeutics.

In the present study, we describe the re-engineering of an E3 ligase ECV containing the VHL–ARNT chimaeric F-box component to remove the oxygen constraint in the recruitment of HIF-α to promote its rapid degradation under any oxygen tension. The expression of VHL–ARNT fusion protein in hypoxic tumour cells with constitutive stabilization of HIF-1α and/or HIF-2α leads to a dramatic and highly efficient downregulation of HIF activity, ultimately suppressing the growth of CCRCC xenografts. Notably, the strategy of chimaeric F-box proteins in the context of SCF (Skp1/Cul1/F-box protein) E3 ligase has been described for targeted proteolysis of cellular proteins including Rb (Zhou et al, 2000), β-catenin (Cong et al, 2003; Liu et al, 2004; Su et al, 2003), c-myc (Cohen et al, 2004) and cyclin A–Cdk2 complex (Chen et al, 2004). For example, Cong et al showed that an SCF complex containing a chimaeric protein with the β-catenin binding domain of E-cadherin fused to the F-box protein βTrCP targets the stable β-catenin mutant for ubiquitin-mediated destruction (Cong et al, 2003). Hence, the replacement of the substrate-binding interface of any F-box protein with the HIF-α-binding region of ARNT (i.e., HPAC) may have been sufficient to promote oxygen-independent degradation of HIF-α. However, emerging evidence suggests that HIF-α triggers its own degradation by initiating the conjugation of ubiquitin-like NEDD8 onto Cul2, which enhances the E3 activity of ECV (Sufan & Ohh, 2006). SCF was also enriched in neddylated Cul1 upon SCF-specific substrate recruitment (Read et al, 2000), suggesting coordination between substrate-SCF binding and neddylation. The precise mechanism(s) governing substrate-dependent triggering of E3 function remains incompletely understood, but suggests molecular interplay between substrates and substrate-specific E3s. While we have presented evidence of feasibility in promoting human CCRCC xenograft regression and cell death using the bioengineered VHL–ARNT chimaera in an animal model system, it remains an outstanding question whether such a treatment would lead to the growth suppression of other tumour types exhibiting strong hypoxic signatures with overexpression of HIF-α.

MATERIALS AND METHODS

Cells

786-O (VHL−/−; HIF-1α−/−) CCRCC cells and human embryonic kidney cells (HEK293A) were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA) at 37°C in a humidified 5% CO2 atmosphere. 786-O cells stably expressing DsRed2 (786-dsRed) were generated by nucleofecting (Amaxa, Gaithersburg, MD, USA) the mammalian expression plasmid encoding DsRed2 (Clontech, Palo Alto, CA, USA). Clones were selected with 500 μg/ml G418 (Sigma–Aldrich, Oakville, ON, Canada) and red fluorescence was confirmed using a fluorescence microscope (Nikon Eclipse TE200). Similarly, 786-HRE-Luc cells were generated by nucleofecting 5xHRE-Luciferase mammalian expression plasmid (kindly provided by Dr R. P. Hill) in combination with an empty mammalian expression plasmid pCDNA3.1 encoding the neomycin resistance gene. Positive clones were selected with 500 μg/ml G418 and luciferase expression was confirmed by luciferase assay.

Antibodies

Monoclonal anti-T7, anti-HA (12CA5), anti-α-tubulin, anti-vinculin, anti-GFP and anti-AhR antibodies were obtained from Novagen (Madison, WI, USA), Boehringer Ingelheim (Laval, QC, Canada), Sigma–Aldrich (Oakville, ON, Canada), Millipore (Billerica, MA, USA), Covalence (Berkeley, CA, USA) and Abcam (Cambridge, MA, USA), respectively. Monoclonal anti-HIF-1α and polyclonal anti-HIF-2α were obtained...
**PROBLEM:**
Under normal oxygen tension, von Hippel–Lindau (VHL) tumour suppressor protein promotes the degradation of a key transcription factor called the hypoxia-inducible factor (HIF), which governs cellular adaptation to hypoxia or low oxygen tension. Tumour hypoxia, a common feature of solid tumours, or mutations in the VHL gene cause inappropriate accumulation of HIF, the extent of expression of which correlates with disease progression, poor prognosis and resistance to radiation and chemotherapy. Thus, various drugs designed to block the activity of HIF have been recognized as attractive strategies for cancer therapy, but have shown limited success in the clinic.

**RESULTS:**
In the present study, the authors describe the bioengineering of a VHL protein that can recognize HIF and promote its degradation in the absence of oxygen. The study goes further and demonstrates the potency of this engineered VHL on the growth of arguably one of the most resistant tumours to conventional cancer therapy, kidney cancer, in a mouse model system.

**IMPACT:**
This report introduces the concept and feasibility of ‘bio-tailored’ proteins in the treatment of tumours that overexpress HIF and highlights the potential of this novel anti-cancer strategy.

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**Plasmids**
The ARNT truncation plasmids T7-bHPAS (residues 90-419), T7-HPAS (103-419), T7-bHPAC (90-467) and T7-HPAC (103-467) were generated using the following primers introducing 5' BamHI and 3' XhoI sites. ARNT basic region forward primer: 5'-GCGCGGATCCATGAAGATTGATTGCCAGGGAAAATCAC-3', ARNT HLH forward primer: 5'-GGCGCGGATCCATGAACAGATGAAGATTGATTGCCAGGGAAAATTACAC-3', ARNT PAS B reverse primer: 5'-GGCGAGGATCCATGAACAGATGAAGATTGATTGCCAGGGAAAATTACAC-3', ARNT PAC reverse primer: 5'-CCGCGCTAGATCAAAGTTCTTACACTAC-3'. The polymerase chain reaction (PCR) products were digested with BamHI and XhoI and ligated into pcDNA3-T7 cut with these restriction enzymes. T7-HPAS, T7-bHPAC and T7-HPAC were generated using the above-mentioned forward primers, a reverse PAC primer XbaI site without a Stop codon: 5'-GCGCGGATCCATGAACAGATGAAGATTGATTGCCAGGGAAAATTACAC-3', and the appropriate combination of the following VHL primers, introducing a 5' XhoI site and a 3' Apol site VHL forward primer: 5'-GGCGAGGATCCATGAACAGATGAAGATTGATTGCCAGGGAAAATTACAC-3', VHL forward primer including the Gly(y) linker: 5'-GGCGAGGATCCATGAACAGATGAAGATTGATTGCCAGGGAAAATTACAC-3' and VHL reverse primer: 5'-CCGCGCTAGATCAAAGTTCTTACACTAC-3'. The corresponding PCR products were ligated into pcDNA3-T7 cut with BamHI and Apol by a three-way ligation. All plasmids were confirmed by DNA sequencing. pcDNA3-HA-HIF-1a, pcDNA3-HA-HIF-1a (P564A) pcDNA3-T7-VHL, pcDNA3.1-

**Plasmid encoding DsRed2** was obtained from Clontech.

**Immunoprecipitation and immunoblotting**
Immunoprecipitation and immunoblotting were performed as described previously (Evans et al, 2007).

**In vitro binding assays**
In vitro binding assays were performed as described previously (Ivan et al, 2001; Maynard et al, 2007; Ohh et al, 2000).

**Hypoxia treatment of cells**
Cells were maintained at 1% O2 in a ThermoForma (Marietta, OH, USA) hypoxia chamber. Cell lysates were prepared in the chamber in a hypoxic environment before further experimentation.

**Luciferase assays**
The dual-luciferase assay was performed as described previously (Evans et al, 2007).

**Infection of 786-O or 786-HRE-Luc cells with recombinant adenovirus**
Ad-EGFP (where EGFP stands for enhanced green fluorescence protein), Ad-EGFP-T7-VHL and Ad-EGFP-T7-HPACGV were generated by the Viral Vector Core Facility (Neuroscience Research Institute, University of Ottawa, Ottawa, ON, Canada). 786-O or 786-HRE-Luc cells were infected at 70% confluency with a final multiplicity of infection (MOI) of 300. Cells were lysed 48 h after infection for further experimentation.

**Quantitative real-time PCR**
Quantitative real-time PCR was performed and results were interpreted as described previously (Evans et al, 2007). The following primer sets were used: GLUT 1 5'-CACCACCTCAGGTGTTACTT-3' and...
CAAAATGTT-3

diameters as small as 25

This technique allows for blood vessels with

motion artefact removal and all images were normalized to the

previously (Mariampillai et al, 2008). Low pass Gaussian filter was used

to produce two-dimensional projection maps of microvasculature

acquired with 1600

stacks over a 5

m m3 volume of the window chamber were

covered 1.0), and represented as the mean value ± standard deviation of

three independent experiments performed in triplicate.

Mouse dorsal skin-fold window chamber assay

All animal experiments were performed in accordance with the

institutional animal care guidelines (University Health Network, ON,

Canada). SCID mice were anaesthetized by intraperitoneal injection of a

mixture of ketamine and xylazine (80 and 5 mg/kg, respectively). Dorsal

window chambers were installed as described previously (Algire &

Legallais, 1949). Briefly, a circular incision of 1 cm diameter was made in

the dorsal skin and the titanium chamber was surgically implanted.

7 × 10^5 786-dsRed tumour cells were implanted in the dermis on the

right dorsal side of the mouse using an 18G syringe. A circular glass

coverslip was positioned over the incision allowing visualization of the

tumour and longitudinal monitoring of the associated vasculature.

Adenoviral intratumoral injections with Ad-EGFP, Ad-EGFP-T7-VHL or

Ad-EGFP-T7-HPACGV were performed using 2 × 10^8 infectious units (ifu).

In vivo imaging

Fluorescence and white-light imaging were performed using an MZ

FLIII, Leica stereomicroscope. Tumours, as identified by red fluorescence

and white-light stereomicroscope images, were also imaged non-

invasively using OCT (Huang et al, 1991). A 36 kHz 1.3

m swept source

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invasively using OCT (Huang et al, 1991). A 36 kHz 1.3 μm sweep source

OCT system was used to probe tissue microstructure and microvascu-

lature with approximately 10 μm resolution. Three-dimensional image

stacks over a 5 × 5 × 2 mm^3 volume of the window chamber were

acquired with 1600 × 2000 × 512 pixels. Image stacks were processed to

produce two-dimensional projection maps of microvasculature

within the volume using the interframe sv algorithm as described

previously (Mariampillai et al, 2008). Low pass Gaussian filter was used

for motion artefact removal and all images were normalized to the

same false colour map. This technique allows for blood vessels with

diameters as small as 25 μm to be visualized without the use of any

exogenous contrast agents during longitudinal studies.

Immunohistochemical staining

Sample preparation and immunohistochemical staining were per-

formed as described previously (Evans et al, 2007).

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The authors declare that they have no conflict of interest.

For more information

VHL Family Alliance:

http://www.vhl.org

References

Algire GH, Legallais FY (1949) Recent developments in the transparent-

chamber technique as adapted to the mouse. J Natl Cancer Inst 10;

225-253 incl 228 pl.


Nat Rev Cancer 4: 437-447


Brunick RK, McNight SL (2001) A conserved family of prolyl-4-hydroxylases

that modify HIF. Science 294: 1337-1340


cyclin a/cyclin-dependent kinase 2 complex suppresses tumor cell growth in

vitro and in vivo. Cancer Res 64: 3949-3957

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER,

Pugh CW, Ratcliffe Pj, Maxwell PH (2000) Hypoxia inducible factor-alpha

binding and ubiquitylation by the von Hippel-Lindau tumor suppressor


2477-2490


knockout (Tiuko) of C-Myc affects late lung and intestinal development in the

mouse. BMC Dev Biol 4: 4


to study the function of beta-catenin in tumorigenesis. BMC Mol

Biol 4: 10


molecular biology and potential for targeted therapies. Oncologist 12:

1404-1415

Covello KL, Kehler J, Yu H, Gordon JD, Arsham AM, Hu CJ, Labosky PA, Simon MC,

Keith B (2006) HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell

function, embryonic development, and tumor growth. Genes Dev 20:

557-570


for HIF-2alpha during vascular development. Circulation 111:

2227-2232


inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional

activation of vascular endothelial growth factor receptor-2 (Fik-1). J Biol

Chem 278: 7520-7530

Epstein AC, Gleadle JM, McNeill LA, Hewston KS, O’Rourke J, Mole DR, Mukherji


mammalian homologs define a family of dioxygenases that regulate HIF by

prolyl hydroxylation. Cell 107: 43-54

Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW, Kim WY, Saravanan A,


egl-9 and mammalian homologs define a family of dioxygenases that regulate HIF by

prolyl hydroxylation. Cell 107: 43-54

Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW, Kim WY, Saravanan A,


cadherin transcription by HIF-mediated regulation of SIP1 and snail. Mol Cell

Biol 27: 157-169

Gunaratnam N, Marley M, Franovic A, de Paulsen N, Mekhail K, Parolin DA,

Nakamura E, Lormier IA, Lee S (2003) Hypoxia inducible factor activates the

transforming growth factor-alpha/epidermal growth factor receptor growth

stimulatory pathway in VHL(−/−) renal cell carcinoma cells. J Biol Chem 278:

24496-44974
Oxygen-independent degradation of HIF-α


Sufian RI, Ohh M (2006) Role of the NEDD8 modification of Cul2 in the sequential activation of ECV complex. Neoplasia 8: 956-963


