A zebrafish model to study and therapeutically manipulate hypoxia signalling in tumorigenesis

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Running Title

Tumors in vhl zebrafish and a novel reporter for HIF signaling

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VHL disease, tumor hypoxia, HIF, prolyl hydroxylase, zebrafish, tumor suppressor gene

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Abstract

Hypoxic signaling is a central modulator of cellular physiology in cancer. Core members of oxygen sensing pathway including the von Hippel-Lindau tumor suppressor protein (pVHL) and the HIF transcription factors have been intensively studied, but improved organismal models might speed advances for both pathobiological understanding and therapeutic modulation. To study HIF signaling during tumorigenesis and development in zebrafish, we developed a unique in vivo reporter for hypoxia, expressing EGFP driven by prolyl hydroxylase 3 (phd3) promoter/regulatory elements. Modulation of HIF pathway in Tg(phd3::EGFP) embryos showed a specific role for hypoxic signaling in the transgene activation. Zebrafish vhl mutants display a systemic hypoxia response, reflected by strong and ubiquitous transgene expression. In contrast to human VHL patients, heterozygous Vhl mice and vhl zebrafish are not predisposed to cancer. However, upon exposure to dimethylbenzanthracene (DMBA), the vhl heterozygous fish showed an increase in the occurrence of hepatic and intestinal tumors, a subset of which exhibited strong transgene expression, suggesting loss of Vhl function in these tumor cells. Compared to control fish, DMBA-treated vhl heterozygous fish also showed an increase in proliferating cell nuclear antigen positive renal tubules. Taken together, our findings establish Vhl as a genuine tumor suppressor in zebrafish and offer this model as a tool to non-invasively study VHL and HIF signaling during tumorigenesis and development.
Introduction

Oxygen homeostasis, a physiological process essential and critical for the normal development and functioning of an organism, requires coordinate regulation of multiple pathways. Reduction in the normal level of oxygen tension in tissues (hypoxia) is observed in a range of disease conditions, including locally in peripheral vascular disease, myocardial infarction and stroke and systemically in pulmonary disease. In addition, hypoxia is a well-described feature of tumor microenvironment. Intratumoral hypoxia is elicited by uncontrolled proliferation of tumor cells coupled with aberrant growth of tumor vasculature. Under hypoxic conditions two distinct mechanisms are activated, that promote cell/tissue survival in low oxygen environments and enhance oxygen delivery to the hypoxic sites. These adaptive responses to hypoxia are induced by a key transcription factor, hypoxia inducible factor (HIF), whose activity is regulated by the availability of oxygen (1). HIF is a heterodimeric protein complex consisting of basic helix-loop-helix Per/Arnt/Sim (PAS) domain containing α and β subunits (HIFα and HIFβ). HIF plays a crucial role in altering the cellular metabolism of tumors to thrive under hypoxic conditions and in facilitating malignant transformation of tumors (2). Several HIF target genes like VEGF, PDGF, TGF, CXCR4, TGF-α, MMP-1 play important roles in the development of cancer (3-5) and significant correlations have been observed between increased HIF-1α levels and patient mortality in many types of cancer (6).

The stability of HIF is regulated in an oxygen-dependent mechanism by the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is encoded by VHL whose biallelic loss lead to the development of a hereditary cancer syndrome involving multiple hemangioblastomas of CNS, clear cell renal carcinoma and pheochromocytoma (7), which occur when heterozygosity
is lost either by mutation on the second allele of *VHL* or via epigenetic silencing (8). pVHL is an essential component of the E3 ubiquitin ligase complex and interacts with the HIFα subunit under normoxic conditions, targeting them for proteasomal degradation. The pVHL-HIFα interaction requires oxygen-dependent hydroxylation of either of two conserved prolyl residues (Pro402 and Pro564) within the oxygen dependent degradation (ODD) domain of HIFα by prolyl hydroxylase domain (PHD) enzymes (9-12).

HIF prolyl hydroxylases belong to the 2OG-dependent dioxygenase superfamily (13), which also require molecular oxygen as a co-substrate for their catalysis resulting in the contribution of one oxygen atom for the hydroxylation reaction and the second oxygen atom towards the oxidative decarboxylation of 2-oxoglutarate yielding succinate and CO2. Prolyl hydroxylation of HIFα generates a binding site for pVHL leading to polyubiquitination of HIFα and subsequent hydrolysis. As oxygen tension decreases below the critical threshold, prolyl hydroxylases become quiescent, allowing HIF-α to stabilize and migrate into the nucleus to form a functional transcription complex by binding with HIF-β subunit. Thus PHD enzymes play a critical role in transducing the physiological signal of hypoxia to the nucleus (14-17). In vertebrates, there are three prolyl hydroxylase (PHD1, PHD2 and PHD3) isoforms capable of hydroxylating HIFα. Interestingly, the *PHD2* and *PHD3* genes are also HIF target genes, providing negative feedback and thus a mechanism to create a flexible threshold for activation of the HIF signaling pathway (17,18).

By negatively regulating HIFs, pVHL plays an important role in the adaptive cellular response to hypoxia. Constitutive stabilization of HIFα subunits and enrichment of *PHD* transcripts have been observed in the *VHL*-deficient renal carcinoma cells under normoxic conditions.
conditions (19,20). We reported earlier the identification of 2 mutant lines carrying null mutations in the zebrafish vhl ortholog. The mutant embryos exhibit a general systemic hypoxic response with up-regulation of genes involved in oxygen sensing and transport, angiogenesis, anaerobic energy metabolism, and display key features of the human VHL-associated disorder “Chuvash polycythemia” (21,22). The vhl mutants also displayed a very strong upregulation of prolyl hydroxylase 3 (phd3) gene expression at various stages (21) suggesting phd3 as an ideal candidate for the readout of Hif activity. We characterized phd3 as a suitable response gene and created a Tg(phd3::EGFP) hypoxia reporter line and describe the utility of this transgene as an in vivo marker for intratumoral hypoxic regions in a zebrafish melanoma model system.

In zebrafish, the effects of vhl heterozygosity on tumor formation have not been analysed in detail, but such fish are generally healthy and fail to show indications of increased susceptibility to tumor formation. Using the Tg(phd3::EGFP) reporter line as a tool we assayed the frequency of LOH in the vhl fish. However, the late average age of diagnosis of ccRCC in human VHL patients suggests that several additional changes are required to turn a VHL LOH cell into a cancer cell, thus the short lifespan of the fish may prevent development of renal cancer. We therefore sought to test their susceptibility when challenged with a wide-spectrum zebrafish carcinogen; 7,12-Dimethylbenz(a)anthracene (DMBA) (23). We report here strong susceptibility of the DMBA-treated vhl+/− fish to hepatic and intestinal tumorigenesis, showing for the first time that Vhl functions as a tumor suppressor in zebrafish. The treatment also led to an increased occurrence of proliferating renal tubules compared to appropriate control fish. Our observations establish that the Tg(phd3::EGFP) line provide a novel in vivo tool to monitor Vhl/Hif/hypoxic signaling under tumor pathological conditions.
Materials and Methods

Zebrafish

Zebrafish (Danio rerio) strains were maintained on 14/10 hour light/dark cycle at 28°C in a UK Home Office approved aquarium facility at the University of Sheffield. The fish embryos were staged according to hours or days post-fertilization (hpf or dpf) (24).

We have utilized two different vhl mutant lines namely, $vhl^{hu2117/+}$ and $vhl^{hu2081/+}$, previously identified and described (21).

phd3::EGFP transgenesis

A EGFP-SV40pA-FRT-Kn-FRT recombineering cassette and red recombineering system in EL250 cells were used to insert EGFP with an SV40 polyadenylation site at the phd3 ATG start site in BAC clone CHORI73-277E22. This modified BAC was used to generate stable transgenic lines according to published protocols (25). We selected two different lines namely, Tg(phd3::EGFP)$^{i144/+}$ and Tg(phd3::EGFP)$^{i146/+}$ with the i144 exhibiting a stronger expression. All the experiments in this study were performed on the i144 line.

We have observed phd3 transgene activation phenotype in the two different vhl mutant allele backgrounds viz. $vhl^{hu2117/hu2117}$ and $vhl^{hu2081/hu2081}$ as well as in the $vhl^{hu2081/hu2117}$ transheterozygous embryos. For this study we have used the $vhl^{hu2117}$ mutant line.

Hypoxia chamber assay
The embryos were raised in E3 medium in a 5% oxygen supplied sealed chamber (INVIVO2 200, Ruskinn Technology Limited, Bridgend, UK) for stipulated periods of time. The chamber was deoxygenized by positive infusion of 5% CO₂ / 95% N₂ gas mixture with >90% relative humidity at 28°C. The continuous O₂ saturation and the pressure inside hypoxia chamber were maintained during the course of the experiment. The transgenic embryos raised under similar conditions in a well-aerated bag inside the chamber served as controls.

**vhl morpholino and mRNA injections**

A splice blocking antisense morpholino (MO) (Genetools, USA) sequence, namely 5’-GCATAATTTCACGAACCCACAAAAG-3’, designed to the exon1 splice-donor site of the vhl gene was utilized to perturb its function. The MO stock solution (10 mg/ml) was diluted appropriately in distilled H₂O and the 1-cell stage transgenic embryos were injected with ~3 ng per embryo. Full length zebrafish vhl gene was subcloned into the pCS2+ expression vector. mRNA from the sequence verified constructs was transcribed *in vitro* after NotI linearization, using SP6 mMESSAGE kit (Applied Biosystems, USA). Dominant active and dominant negative forms of hif-1αα and hif-1αβ were synthesized and injected as previously described (26). For the rescue experiments, 6.5 pg vhl mRNA was injected.

**Dimethyloxaloylglycine treatment**

The 3-dpf wild-type transgenic embryos were treated with 100-μM dimethyloxaloylglycine (DMOG; Frontier Scientific, Logan, UT) or 0.1% dimethylsulfoxide (DMSO; Sigma-Aldrich) for 1-2 days as previously described (21).

**RNA in situ hybridization**
Whole-mount in situ hybridization was performed as per standard protocols (27). The \textit{phd3} (BC066699) antisense digoxigenin-UTP labelled mRNA probe was synthesized from an expressed sequence tag (EST) clone (RZPD/Imagens, Berlin, Germany) (21) and the \textit{in situ} data were collected on a Zeiss Axioplan with a 5x or 20x objective using a Spot4 digital camera.

\textbf{7,12-Dimethylbenz[a]anthracene treatment of vhl$^{+/-}$ fish}

Wildtype and vhl$^{flu/2117/+}$ fry at 3 weeks of age were immersed overnight in aquarium water containing 5 ppm of DMBA (Sigma-Aldrich-Chemie BV, The Netherlands) dissolved in DMSO (23). The next day, all fry were rinsed several times in regular aquarium water and subsequently were returned to the aquarium. Fish were carefully grown and regularly monitored for signs of sickness or evidence of tumor formation. Eventually all fish were culled for analysis at 14 months after the DMBA treatment.

\textbf{Histology and immunostaining}

Adult fish euthanized, culled and fixed in 4\% paraformaldehyde for 3-4 days at 4$^\circ$C. The samples were decalcified in 0.25 M EDTA pH 8 for 2-4 days at RT, embedded in paraffin and 5-\mu m sections were made. For immunostaining the primary antibodies mouse monoclonal anti-PCNA (1:1000, Abcam, UK), rabbit polyclonal anti-HIF1$\alpha$ (1:100) (28) and rabbit polyclonal anti-GFP (1:250, Torrey Pines Biolabs, USA) were used. The stained sections were viewed in a Leica DM 2500 microscope and photographed using Leica DFC 420C digital camera. All the tumor sections were observed and analyzed by a clinical pathologist.

\textbf{Western Analysis}
After the hypoxia exposure treatment, the embryos were harvested in the hypoxic E3 medium itself and protease inhibitor MG132 was added (25 μM final concentration) to prevent proteasomal degradation of Hif-1α protein. The samples were processed as per standard protocols and the antibodies used were anti-Hif-1α (1:500)(28) and anti-acetylated tubulin (1:1000; Sigma-Aldrich, UK).

qRT-PCR

Real time RT-PCR was performed on a BioRad MyiQ machine along with BioRad iQ SYBER Green Supermix according to the manufacturer’s protocol. The sequences of the primers used in the study are as follows: vegf A: F – CAGTGTGAGCCTTGCTGTTC, R – CCATAGGCTCCTGTTCATT; phd 3: F – CGCTGCACCTGTCATT, R – TAGCATACGACGGCTGAAT; vhl: F – ATGTCGCGTCTGGAGATT, R – GATGCACAGGTGTGCTCAGT; βactin: F – GCAGAAGGAGATCACATCCCTGGC, R – CATTGCCGTACCTTCACCGTTC. The primers were optimized using a standard curve of wild type cDNA. The cycling conditions of the PCR program were as follows: step 1: 95°C x 1 min., step2: 40 cycles of the following 95°C x 30sec., 60°C x 30sec., 72°C x 30sec., step3: 95°C x 30sec., step4: 60°C x 30sec., step5: 40 cycles of 55°C x 10sec. The data were analyzed using the ΔΔCt method (29).

Statistical analysis

Statistical analysis and the p-value calculations were done using Chi-square test (30).
Results

phd3 as a potential hypoxia/Hif target gene

In microarray expression analyses of 7 dpf *vhl*<sup>hu2117/hu2081</sup> transheterozygous and 3.5 dpf *vhl*<sup>hu2117/hu2117</sup> homozygous mutant and *vhl*<sup>+/+</sup> sibling larvae, we observed upregulation of several Hif target genes, including *epo*, *vegf*, *phd1-3* in the mutants, with *phd3* consistently exhibiting the strongest increase overall (eg. 23 fold at 7 dpf) (21, FvE unpubl.). When we did western blot analysis of *vhl*<sup>−/−</sup> embryos using Hif-1α antibody, we observed strong deposition of Hif-1α in the mutants (Fig. 1A). Subsequently, when we performed qPCR on 3.5 dpf *vhl*<sup>−/−</sup> mutant embryos and observed 216-fold increase in *phd3* expression in mutant embryos compared to wildtypes (Fig. 1B). In comparison, the classical HIF target *vegf-a* was increased 7-fold (Fig. 1B) in the mutants. To identify the earliest developmental time point at which *phd3* expression is stimulated in the *vhl*<sup>−/−</sup> mutant embryos, we performed whole mount *in situ* hybridization on selected stages of mutant and sibling embryos. In wildtype embryos, *phd3* expression could only be weakly detected in the brain around 30 hpf (data not shown). By 48 hpf, the expression became restricted to a population of cells in the tectum and midbrain-hindbrain boundary (MHB) region and the wildtype fish continued to preserve this confined *phd3* expression pattern during the subsequent embryonic and larval development (Fig. 1C,E,S1A). In contrast, *vhl* mutant embryos exhibited strong *phd3* expression as early as 21.5 hpf (25-somite stage) in the brain and pronephros (Fig.1D) suggesting an induction in Hif activity. By 36 hpf, the mutant embryos showed strong *phd3* expression in the heart and other
visceral organs indicating a systemic response. These embryos continued to exhibit robust expression of the \textit{phd3} gene all through their embryonic (Fig. 1F, S1B) and larval development, until their death at 10-12dpf. Presence of wildtype maternal \textit{vhl} mRNA and Vhl protein during the early embryonic stages in mutant embryos (21), might explain the delay in the activation of \textit{phd3} expression until \textasciitilde21-22 hpf. These observations suggested that, although the physiological and behavioral responses of \textit{vhl} mutants only become evident by 3 dpf, the molecular onset of the hypoxic response can be detected from \textasciitilde22 hpf.

\textbf{Strong induction of a \textit{phd3::EGFP} transgene in \textit{vhl}^- mutants}

In the human \textit{PHD3} gene a hypoxia response element (HRE) was identified in the first intron, but its conservation in fish is unclear and further HREs could be present (31). Therefore, we utilized a recombineering strategy to insert an \textit{EGFP} with an SV40 3’ UTR at the \textit{phd3} ATG start site in the BAC clone CHORI73-277E22. This BAC contains the complete \textit{phd3} gene in a large genomic context (>25 Kb 5’ and >90 Kb 3’). The \textit{phd3} start codon was destroyed by the recombination thus preventing the transgene construct from interfering itself with the hypoxic signaling pathway. This modified BAC was injected to generate two stable transgenic lines and, the stronger line was used in all subsequent experiments.

In wild-type embryos, similar to \textit{phd3} \textit{in situ} stainings, \textit{phd3::EGFP} transgene-mediated \textit{EGFP} fluorescence commenced weakly in the brain and spinal cord from 30 hpf (data not shown). The transgene expression became restricted to MHB region by around 60 hpf and the \textit{EGFP} fluorescence was found to adhere to this region during subsequent stages of development (Fig. 1G). In contrast, the \textit{vhl} mutant embryos commenced transgene expression from 25-somite stage onwards in the brain, spinal cord and pronephros (data not shown). Subsequently,
the mutants began exhibiting strong induction of the transgene in several other organs like the heart, pectoral fins, pancreas and the hatching gland (Fig. 1H). In situ hybridizations for gfp mRNA on phd3::EGFP transgenic embryos correlated well with the endogenous phd3 expression at various stages of development (Fig. S2). We also quantified and compared the level of EGFP fluorescence between the vhl mutants and wildtype siblings at 4 dpf, and found a threefold increase in the amount of fluorescence in the mutants (Table S1).

vhl mRNA rescues phd3::EGFP activation in vhl knockout and knockdown embryos

To prove that phd3::EGFP activation observed in the vhl mutant embryos is specifically affected by the loss of Vhl function, we tried to rescue this phenotype by injecting 6 pg of capped vhl mRNA into single cell stage embryos. By around 28 hpf, both the uninjected and injected wildtype embryos showed no activation of the phd3::EGFP transgene. But while the uninjected vhl mutants exhibited strong expression of the transgene at 28 hpf, the mRNA injected mutant embryos exhibited no EGFP fluorescence at the identical time point (Fig. 2A).

We also stimulated phd3::EGFP expression in the wildtype embryos by knocking down vhl function using antisense morpholino oligonucleotides. Injection of vhl splice morpholino (MO) into 1-cell stage Tg(phd3::EGFP)i144/i144 embryos led to strong induction of the transgene from 60 hpf onwards and morphant embryos continued to display strong EGFP fluorescence until 120 hpf. But when we injected vhl morpholino and vhl mRNA together, persistence of wildtype expression pattern was observed during embryonic and larval development (Fig. 2B), suggesting a complete rescue of the morphant phenotype.

phd3::EGFP activation is Hif dependent
Our observations suggest that loss of function mutations in \textit{vhl} can activate \textit{phd3::GFP} reporter gene expression possibly through the stabilization of Hif\(\alpha\) subunit but Vhl protein is reported to have numerous non-canonical functions as well (32). In order to verify the role of Hif transcription factor in eliciting transgene expression in \textit{vhl}\(^{-/-}\) embryos, we generated dominant active (DA) forms of Hif-1\(\alpha\alpha\) and Hif-1\(\alpha\beta\) by mutating the conserved proline and asparagine hydroxylation sites of Phd and Fih hydroxylases respectively (26). When the DA \textit{hif-1\(\alpha\alpha\)} and \textit{hif-1\(\alpha\beta\)} mRNAs were coinjected into one cell stage \textit{Tg(phd3::EGFP)i144/+} embryos, we observed activation of the transgene in the injected embryos from 24 hpf, as evidenced by strong EGFP fluorescence, mainly in the brain and CNS. The enhanced \textit{phd3::EGFP} expression persisted in the larvae expressing DA Hif-1\(\alpha\) isoforms until at least 48 hpf (Fig. 2C). When the same amount of wild type \textit{hif-1\(\alpha\alpha\)} and \textit{hif-1\(\alpha\beta\)} mRNAs were injected this level of \textit{phd3::EGFP} expression was not observed, confirming that the DA versions of \textit{hif-1\(\alpha\)} have successfully stabilised the protein. These observations clearly show the direct role of Hif-1\(\alpha\) transcription factor in mediating the \textit{phd3::EGFP} transgene activation (Fig. S3A).

**Chemical activation of Hif signaling in wildtype embryos induces \textit{phd3::EGFP} expression**

To further extend this observation, we activated Hif in the wildtype transgenic embryos by inhibiting Phd/Factor inhibiting Hif (Fih) functions using the prolyl hydroxylase inhibitor dimethylfumarate (DMO), and thus preventing the degradation of the Hif\(\alpha\) subunit even in the presence of functional Vhl. Transgenic wildtype embryos at 2.5 dpf were treated with 100\(\mu\)M DMO for 2 days displayed an increase in \textit{phd3::EGFP} expression in the pronephros, gall bladder, liver and brain (Fig. S4A) compared to very mild expression of transgene observed in the 0.1% DMSO treated control embryos. Hif activity and hence the transgene
induction were observed to be restricted to selected tissues/cells of DMOG treated embryos, due to limitations in the mode and timing of the treatment (21). When mRNAs encoding DN forms of hif-1αa and hif-1αb (26) were injected into Tg(phd3::EGFP)i144/+ embryos and subsequently treated with DMOG at 24 hpf, we could block the DMOG mediated induction in EGFP fluorescence at 48 hpf (Fig. S3B, S4B).

Tg(phd3::EGFP) zebrafish as a live reporter of hypoxia

Our results predict that the Tg(phd3::EGFP)i144/i144 embryos should act as a live reporter of hypoxia, and in order to validate this we exposed the transgenic embryos to reduced oxygen environment in a hypoxic chamber for stipulated periods of time. When we subjected 12 hpf transgenic embryos to 5% O2 (hypoxia) for 12 hours, phd3::EGFP expression was triggered indicating the activation of Hif in these embryos (Fig. 3A). In contrast, the normoxia-raised control embryos showed no expression of the transgene at the corresponding stages. High levels of fluorescence observed in the CNS and pronephros of the hypoxia-raised embryos implies vigorous Hif activity in these organ systems in eliciting critical adaptive mechanisms.

To understand whether Hif stabilization could be induced during early embryonic development itself, we subjected 128-cell stage transgenic embryos to hypoxia for 4 hours. We observed strong induction of the transgene in the hypoxia-raised embryos by 70% epiboly stage (Fig. 3B) compared to the normoxia-raised embryos, which showed no induction. When we assayed the hypoxia-raised embryos for Hif-1α levels using western blot analysis, strong presence of Hif-1α was detected in them compared to normoxia-raised embryos, which showed only negligible levels of Hif-1α (Fig. 3C). These observations indicate the usefulness of Tg(phd3::EGFP)i144/i144 embryos as a robust sensor to detect hypoxia.
*phd3::EGFP* transgene functions as an *in vivo* fluorescent marker for intratumoral hypoxia

Since the presence of hypoxic regions are hallmarks of locally advanced solid tumors, we asked whether the *phd3::EGFP* transgene could be used as a novel reporter for detecting intratumoral hypoxic regions in fish tumor models. To test this hypothesis, we induced melanoma in the Tg(*phd3::EGFP*)<sup>i144/i144</sup> fish by injecting a human oncogenic *HRAS<sup>G12V</sup>* (*V12RAS::mCherry*) construct into single cell embryos and misexpressing the oncogene specifically in cells of the melanocyte lineage (33). Few days after injections, the V12RAS injected fish developed several clones of ectopic melanocytes and after few weeks they progressed into melanocytic naevi. Subsequently, after 3 months we observed few of these lesions to form small tumor nodules. As these melanoma nodules became expanded into melanomas we periodically monitored them for *phd3::EGFP* expression. After one year, few fish with large tumors (Fig. 4A) exhibited distinct regions of *phd3::EGFP* mediated fluorescence (Fig. 4C). These EGFP<sup>+</sup> regions are likely to correspond to intra-tumoral hypoxic areas in these melanomas, essentially revealing a subpopulation of tumor cells that possess activated hypoxia-mediated Hif signaling. Histological analysis of these samples using proliferating cell nuclear antigen (PCNA), Hif-1α and GFP antibody staining revealed clear demarcation of the hypoxic regions within these tumors (Fig. 4D,E,F,G). When taking into account the differences in expected half lives of EGFP (Fig S5) and HIF-1α (34) proteins, we observed good correlation between GFP and Hif-1α stainings within the melanoma (Fig. 4F,G), thus validating that the EGFP<sup>+</sup> regions correspond to intratumoral hypoxic regions with activated Hif signaling. These experiments demonstrate the potential of the *phd3::EGFP* transgene in tracking hypoxia under *in vivo* tumor pathologic conditions in zebrafish.
**vhl+/− zebrafish are highly susceptible to DMBA tumorigenesis**

The vhl+/− fish are not obviously predisposed to neoplasia formation. One reason for the lack of tumors might be that the lifespan of the fish is too short to accumulate the necessary changes. Hence we asked whether DMBA treatment of Tg(phd3::EGFP)i144/+;vhl+/− fish would make them susceptible to tumorigenesis. We treated four batches of 21 dpf fish fry with DMBA(23), with each batch consisting of an equal mix of Tg(phd3::EGFP)i144/+;vhl+/+ and Tg(phd3::EGFP)i144/+;vhl+/− genotypes. Two months after the treatment, we observed that a subset of fish in every batch possessed EGFP+ cell clones, primarily in retina (Fig. S6A), skin (Fig. S6B), gills and fins because of easy identification of fluorescent cells in these tissues. When we segregated such fish having EGFP+ cells and performed genotyping analysis: 89% (102/114) was found to be vhl+/− carriers (Table S2). The activation of phd3::EGFP mediated GFP fluorescence in random cell populations in the treated fish suggested DMBA-induced loss of the wildtype vhl allele in these cells. Expectedly, the frequency of occurrence of EGFP+ cells is significantly higher in the vhl+/− heterozygotes where only a single wild type copy needs to be lost.

All the DMBA challenged fish were grown to adulthood and were constantly monitored for tumor formation. Seven to eight months after the DMBA treatment, we observed a small group of fish in all four treated batches to exhibit strong EGFP fluorescence mainly in the trunk region as well as in the region adjacent to cloaca (Fig. 4H,I). At the end of nine months, genotyping and statistical analysis of fish exhibiting strong EGFP fluorescence in all the four batches revealed a significantly (p < 0.002, Chi-square test) greater number (23/27) (Table S3) of them to be vhl+/− heterozygotes.
After growing all the experimental fish for 14 months, they were culled and processed for histological analysis. Serial sagittal sections were made for every sample and subsequently immunostained for the proliferation marker, PCNA. Screening of the PCNA-stained slide preparations showed a significant increase (p < 0.02, Chi-square test) in the occurrence of neoplastic growth in the DMBA treated vhl+/− samples (59%) than the similarly treated wild type samples (23%) (Table 1). In many cases the tumor formation was found in the liver, bile duct and gut tissues. Importantly, the analysis also showed that the fluorescing tissues in all strongly EGFP+ fish to correlate with neoplasias.

The wildtype adult zebrafish intestine showed a regular arrangement of villi and the proliferating epithelial cells were observed to be strictly restricted to intervillus pockets (Fig. 4J) (35,36). However, in the DMBA treated vhl+/− fish we observed a disorganized pattern of intestinal villi architecture (n=8) (Fig. 4K) and these villi were completely encompassed by PCNA+ proliferating epithelial cells (Fig. 4K) analogous to adenoma formation in the human gut. A few of the gut tumors showed abnormally proliferating intestinal epithelial cells in one half of the gut while the other half showed restricted presence of PCNA+ cells (Fig. 4L,M). A few samples exhibited lesions wherein PCNA+ epithelial cells were scattered all through the intestinal villi (Fig. 4M).

The liver tissue in zebrafish consists of hepatocytes, bile ducts and portal vessels (35). In the wildtype liver tissue usually very few proliferating cells are present (Fig. 4N), but in the DMBA treated vhl+/− fish we observed the presence of highly proliferating hepatocytes (n=18) in the liver tissue (Fig. 4O). These tumors were identified histologically as primary hepatocellular carcinoma, wherein either the whole liver or selected regions of liver tissue showed strong staining for PCNA (Fig. 4P,Q). These liver neoplasias showed altered hepatic
architecture as well as abnormal cell morphology along with apoptotic characteristics like presence of fragmented nuclei (Fig. 4R). Similarly, compared to the wild type bile duct tissue, which were not actively proliferating, the DMBA treated fish possessed enlarged, abnormally shaped bile ductules as well as cysts. These bile ducts exhibited the presence of PCNA+ epithelial cells as well as showing abnormal growth of these ducts leading to the development of cholangiocarcinoma (Fig. 4S).

In addition, we also observed occurrence of testicular (Fig. S7A) and ovarian (Fig. S7B) tumors in the DMBA treated wildtype and vhl+/− batches at equal frequencies.

**DMBA induces enhanced proliferation of epithelial cells in vhl+/− renal tubules**

Since ccRCC originates from renal epithelial cells and the human VHL gene is observed to exhibit strong tumor suppressor activity in renal tubules, we asked whether the DMBA treatment of vhl+/− fish could trigger proliferation of renal tubular epithelial cells. Hence, we screened the kidney regions in the PCNA-stained slide preparations of DMBA treated vhl+/− and wildtype groups. In the untreated samples (both wildtype and vhl+/−), the haematopoietic cells in the kidney strongly expressed the proliferation marker, PCNA, and the epithelial cells of the tubules are found to be mostly quiescent. However, the DMBA treatment is observed to induce the transformation of the epithelial cells of renal tubules to a proliferative state as evidenced by robust PCNA+ staining (Fig. 5A-F). A normal renal tubule typically consists of a single organized layer of epithelial cells but, in contrast, most of the proliferating renal tubules exhibited abnormal organization of epithelial cells (Fig. 5A-F) as well as altered cellular morphology (Fig. 5C,E). Statistical analysis showed a highly significant increase in the number of proliferative renal tubules in DMBA treated vhl+/− fish compared with the controls.
These observations suggest that DMBA can induce strong proliferation of renal epithelial cells in the \textit{vhl}^{+/-} fish.

**Vhl exhibits tumor suppressor function in zebrafish**

The observation that after DMBA treatment, \~85\% of the fish with EGFP$^+$ tumors belonged to the \textit{vhl}^{+/-} group suggested that such tumors might have originated from \textit{vhl} null cells and hence most of the tumor cells might be possessing \textit{vhl}^{-/-} genotype. To address this question, we isolated tumor cells, by laser capture microdissection (LCM), from paraffin tissue sections of 5 \textit{vhl}^{+/-} EGFP$^+$ tumor samples, extracted genomic DNA and performed standard sequencing analysis. But, we were unable to identify unambiguously the loss of wildtype \textit{vhl} allele in these samples, either at the \textit{vhl}^{hu2117} locus or elsewhere in the coding region (Fig. S8).

To extend this analysis further, we wanted to quantify the levels of \textit{vhl} mRNA in the \textit{vhl}^{+/-} EGFP$^+$ tumor cells. We dissected tumor tissue from 5 different strongly fluorescing EGFP$^+$ fish (Fig. S9A,B) selected from a DMBA treated \textit{vhl}^{+/-} group, isolated total RNA and performed qRT-PCR analysis. In 4 out of 5 tumor samples we observed a medium to strong decrease in the \textit{vhl} mRNA levels compared to control (Fig. S9C), suggesting that in these samples the wild type Vhl function could be lost/reduced either by mutation(s) or by epigenetic inactivation. Due to technical limitations, we could not assay potential molecular lesion(s) on the wild type \textit{vhl} allele in these neoplastic cells with decreased \textit{vhl} mRNA levels. The fact that DMBA treatment led to tumorigenesis in 55 out of 94 \textit{vhl}^{+/-} fish compared to neoplasias in only 18 out of 77 experimental wild type fish strongly indicate that the Vhl possesses a conserved tumor suppressor function in zebrafish.
Discussion

In this study, we have generated and validated a novel reporter line, Tg(phd3::EGFP), for tracking VHL/HIF signaling in zebrafish. When we subjected the transgenic embryos to hypoxia induced by either genetic, physical or chemical conditions, we could observe strong activation of the transgene. We have also demonstrated the potential of the transgenic line as an in vivo reporter for intratumoral hypoxia in a melanoma model system. These phd3::EGFP transgenic embryos would be ideally suited for small molecule screens to identify novel chemicals that activate or suppress Hif activity in the wild-type or vhl^-/- background, respectively.

Several approaches are being utilized to monitor intratumoral hypoxia like direct measurements of tumor oxygenation, assaying the levels of hypoxia marker proteins like carbonic anhydrase IX, lysyl oxidase etc. and by using small molecule markers like like EF5, pimonidazole, which are derivatives of 2-nitroimidazole, but many of these procedures are invasive and time consuming (37,38). Moreover, these procedures and/or molecular markers have not been successfully employed for measuring hypoxia in zebrafish (39), indicating the need for novel markers for hypoxic signaling. The low background expression and powerful induction by HIF could make the Tg(phd3::EGFP)i144/i144 fish a novel fluorescent marker for in vivo monitoring of hypoxic signaling in many zebrafish tumor model systems.

von Hippel Lindau disease is characterised by formation of a variety of tumor types, most importantly clear cell renal cell carcinoma and retinal and central nervous system haemangioblastomas. Unfortunately, although a variety of mouse models have been made for
this disease, a comparable tumor suppressor activity of VHL homologs has been difficult to show in rodent models. Therefore we tested for such a function of vhl in the zebrafish. Indeed, we report increased susceptibility of the vhl+/− zebrafish to hepatic, bileduct and intestinal tumorigenesis upon exposure to dimethylbenzanthracene (DMBA) compared to the control fish. We also report significantly increased occurrence of proliferating renal tubules in the DMBA treated vhl+/− batch compared to appropriate controls.

We tracked for random occurrence of vhl LOH in live tg(phd3::EGFP)+/−; vhl+/− fish by scoring for cells and/or cell clones emitting strong EGFP fluorescence. While the 6 month old untreated vhl+/− fish had ~1-2 detectable EGFP+ cells in the skin and retinal epithelia, as they become older (~1.5-2 yr old) they accumulated few more LOH events in the skin epithelium (~8-10 GFP+ cells/fish). Upon treatment with DMBA, the vhl+/− fish showed a significant increase in the occurrence of vhl−/− EGFP+ cells in the skin epithelium.

Probably owing to the low rate of spontaneous vhl LOH, vhl+/− heterozygous fish exhibited normal development and showed no detectable signs of tumor or cyst formation. In the case of Vhlh+/− heterozygous mice, depending on the type of genetic background, variable susceptibility to spontaneous tumorigenesis were observed (refs. 40,41,42). Since many of the zebrafish laboratory strains are not highly inbred (43), the strain differences might be less pronounced in fish. However, the lifespan of the zebrafish is only a few years, so even after the spontaneous vhl LOH at few cells, they may not be undergoing enough subsequent mutations in other genes to predispose such fish with a disease phenotype. But, when we challenged the vhl+/− fish with DMBA, we observed a high incidence of tumorigenesis arising from liver, bile duct and gut.
Interestingly, we observed decreased vhl mRNA levels in a set of vhl\(^{+/−}\) EGFP\(^{+}\) tumors, suggesting that in these samples the wildtype vhl allele might have undergone either loss-of-function mutation(s) or epigenetic silencing. Interestingly in human VHL patients, an epigenetic mechanism for inactivation of the wild-type VHL allele, mediated through the transcriptional silencing of the gene via hypermethylation of a 5′-CpG island, has been frequently observed (8,44). DMBA in addition to inducing mutations is also known to cause aberrant methylation of 5′-CpG islands leading to epigenetic silencing of gene(s) (45). Our study exhibit the potential of the vhl\(^{+/−}\) zebrafish as a model to characterize molecular mechanisms involved in VHL induced tumorigenesis.

Though occurrence of ccRCC is the classical VHL disease phenotype, we didn’t observe development of renal cysts or ccRCC in the DMBA-treated vhl\(^{+/−}\) fish within the 14-month experimental period of our analysis. However the presence of significantly increased number of PCNA\(^{+ve}\) renal tubules suggest these proliferating tubules might be at an early stage in the process of renal cyst formation. Some of these proliferating tubules were dysplastic and might be comparable to the VHL mutant foci that are observed in VHL patients (46). Interestingly, presence of duplicated copy of the vhl gene, a vhl-like (vll) gene in zebrafish indicates a possible segregation of VHL functions between these two genes and it would be interesting to assess the combined role of these genes in renal tumor suppression.

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References


Figure Legends

Figure 1

Robust expression of phd3::EGFP transgene in vhl mutants.

(A) Western blot analysis of vhl⁻/⁻ embryos and siblings using Hif-1α antibody. (B) Histogram showing comparison of phd3 and vegfa expression levels between vhl⁻/⁻ and wild type larvae at 3.5 dpf. Each value is an average of three separate biological replicates. (C-D) Whole mount in situ hybridization showing strong onset of phd3 expression in a vhl⁻/⁻ embryo (D) at 21 hpf, while in an identical stage wild type embryo (C) no expression is observed. Arrowheads in the mutant embryo indicate expression in the pronephros. (E-F) Lateral views of 96 hpf wild type (E) and vhl (F) mutant larvae showing persistence of their respective expression pattern. In the mutant larva, we can observe expression in the heart (black arrowhead) and the pectoral fins (white arrowhead). Black arrowhead in the wild-type embryo indicates the staining in the midbrain-hindbrain boundary (MHB) region. (G) A 72 hpf Tg(phd3::EGFP) wild type larva showing transgene mediated EGFP fluorescence only in a population of cells in the MHB region recapitulating the phd3 in situ staining pattern. White arrowhead shows mild fluorescence in the MHB region. (H) In contrast, transgenic larvae in the vhl⁻/⁻ background, showed copious amounts of EGFP fluorescence in the brain, heart, pectoral fins, pronephros, liver etc.

Figure 2

vhl mRNA rescues vhl mutant and morphant phenotypes and Hif mediates phd3::EGFP transgene activation.

(A) Embryos obtained from Tg(phd3::EGFP)¹⁴⁴/¹⁴⁴; vhl⁻/⁻ parents were injected with full-length vhl mRNA at the 1-cell stage and the injected embryos were raised individually in 24-well plates. At 28 hpf, while the uninjected vhl⁻/⁻ mutant embryos showed strong phd3::EGFP mediated EGFP fluorescence, none of the vhl mRNA injected embryos showed activation of transgene expression. Genotypes of the experimental embryos were known by culling the embryos at 96 hpf and performing molecular genotyping assay. (B) Tg
(phd3::EGFP)$^{1144/+}$ embryos were injected with vhl splice morpholino (MO) at the 1-cell stage. At 60 hpf, while the uninjected embryos showed EGFP fluorescence only in the MHB region, the MO injected embryos showed strong induction of transgene mediated EGFP fluorescence. When the vhl MO and vhl mRNA were coinjected into 1-cell stage Tg (phd3::EGFP)$^{1144/+}$ embryos, the injected embryos exhibited no transgene activation, showing rescue of the MO induced phenotype. (C) Tg(phd3::EGFP)$^{1144/1144}$ embryos were coinjected with DA isoforms of hif-1aa and hif-1ab mRNAs at 1-cell stage led to the enhancement of phd3::EGFP mediated EGFP fluorescence in the transgenic embryos by around 48 hpf. The injection of wild type isoforms of hif-1aa and hif-1ab mRNAs into Tg(phd3::EGFP)$^{1144/1144}$ embryos however did not induce transgene expression.

Figure 3

Hypoxia assay.

(A) Tg(phd3::EGFP)$^{1144/1144}$ embryos exposed to hypoxia (5% O2) for 12 hours from 12 hpf showed strong elicitation of transgene expression while the normoxia raised embryos showed hardly any expression at 24 hpf. (B) When the 128-cell stage tg(phd3::EGFP)$^{1144/1144}$ embryos were exposed to hypoxia for 4 hours, the treated embryos similarly showed robust EGFP fluorescence compared to the embryos maintained under normoxic conditions. (C) Western blot analysis of the normoxia-raised and 6 hours hypoxia-raised embryos for the Hif-1α protein.

Figure 4

Immunohistochemical analysis of neoplasias.

(A) A tg(phd3::EGFP)$^{1144/1144}$ fish with V12RAS induced melanoma in the head region showing expression of mCherry by the melanoma cells that facilitates the tracking of tumors. (B) Immunostaining of a melanoma section with PCNA ab showing PCNA+ proliferating tumor cells. (C) Live melanoma fish with intratumoral hypoxic areas or pockets exhibiting phd3::EGFP mediated EGFP fluorescence. (D) Immunostaining of a melanoma section with GFP ab staining displaying distinct hypoxic regions. (E,F,G) Immunostainings on sections of a melanoma tumor with PCNA, GFP and Hif-1α abs reveal
the correlation between the EGFP+ and Hif-1α+ areas. (H-I) Tg(phd3::EGFP)144/144; vhl−/− fish, few months after DMBA treatment, showing development of EGFP+ve neoplasias mainly in the trunk (H-I) region adjacent to anal fin. (J) Section of a wild type adult zebrafish intestine showing organized arrangement of villi and the restricted presence of PCNA+ proliferative cells in the intervillus region. (K) Section of a DMBA treated vhl−/− fish showing abnormal intestinal villi organization. Almost all the cells in these villi are strongly proliferating. (L) Another DMBA treated vhl−/− sample exhibiting neoplasm development in one half of the gut. (M) An example of DMBA treated vhl−/− sample showing abnormal distribution of proliferating cells in the intestinal villi. (N) Section of a wild type adult zebrafish liver showing the presence of bile ducts and portal vessels in addition to hepatocytes. PCNA staining indicates very few proliferating cells in the wild type liver. (O-Q) Sections of DMBA treated vhl−/− samples showing hepatocellular carcinoma, as evidenced by strong PCNA staining, either encompassing the complete organ (P) or few selected regions (Q). (R) An example of a DMBA treated vhl−/− liver sample showing a highly proliferative region possessing cells with apoptotic characteristics like fragmented nuclei and prominent nucleoli. (S) Section of a cholangiocarcinoma arising from bile duct epithelia in a DMBA treated vhl−/− fish, showing abnormal distribution of PCNA+ cells.

Figure 5

Proliferating renal tubules in the DMBA treated vhl−/− fish.

(A-F) Sections of kidney regions in the DMBA treated vhl−/− fish, showing the presence of PCNA+ renal tubules (indicated by white arrowheads). Normally the renal tubules are composed of a single layer of quiescent epithelial cells (indicated by a white box in panel D). The DMBA treatment of the vhl−/− fish transformed many renal epithelial cells to become proliferative. In addition, epithelial cells in the proliferative renal tubules showed aberrant organization (A, C) and altered morphology (D, F).
Table 1. Number of 14 month old DMBA treated wt and vhl<sup>+/−</sup> fish with different types of neoplasias. All the fish were culled, processed for histological analysis and subsequently the sections were screened for tumor formation. (* -The numbers within the brackets indicate GFP<sup>+</sup> neoplasias)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total Fish (n)</th>
<th>Number of fish with neoplasia (n)</th>
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Fig. 5
A zebrafish model to study and therapeutically manipulate hypoxia signaling in tumorigenesis

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