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Expression of Prolyl-Hydroxylase-1 (PHD1/EGLN2) Suppresses Hypoxia Inducible Factor-1α Activation and Inhibits Tumor Growth

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ABSTRACT

Hypoxic stress is one of the major selective pressures in the microenvironment of solid tumors, and overcoming this restriction is essential for tumor progression. One of the key factors driving the cellular response to lack of oxygen is hypoxia inducible factor (HIF), a key transcriptional factor. The level of the α subunit of HIF-1 is regulated by rapid degradation that is controlled by a family of prolyl hydroxylases (PHDs/EGLNs), the activity of which depends on oxygen availability. Our study shows that ectopic expression of mPHD1 suppressed accumulation of HIF-1α and secretion of Vascular Endothelial Growth Factor after treatment of cells with a hypoxia-mimetic drug. Furthermore, when colon carcinoma cells expressing mPHD1 were injected into nude mice, tumor growth was inhibited, and the inhibition of tumor growth was correlated with increased necrosis and a striking decrease in microvessel density. These data demonstrate that inhibition of hypoxia-induced activation of HIF-1α through activation of HIF-hydroxylase can provide a novel therapeutic strategy for inhibition of tumor growth and neovascularization and support the development of gene transfer approaches based on the activation of HIF-prolyl hydroxylases.

INTRODUCTION

The ability of cells to respond to environmental changes in oxygen levels is an important physiological mechanism that requires tight regulation of sensing and adaptation responses. Hypoxia inducible factor (HIF)-1 is a transcriptional complex that plays a central role in oxygen homeostasis and in coordinating the cellular response to hypoxia. After hypoxic stress, HIF-1 activates the transcription of >40 genes important for survival and adaptation to hypoxia (1). HIF-1 is a heterodimer consisting of two subunits: HIF-1β is expressed constitutively in the nucleus and also participates in other transcriptional pathways, and HIF-1α, which is specific for the hypoxia response. In normoxia, HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway, and its low levels do not allow heterodimer formation and transcriptional activation (2, 3). When hypoxia occurs, this degradation is suppressed and HIF-1α is stabilized rapidly. The degradation of HIF-1α is mediated by the product of the *Von Hippel-Lindau* tumor suppressor gene, which acts as the recognition component of a ubiquitin E3 ligase complex. *Von Hippel-Lindau* interacts specifically with an oxygen-dependent degradation domain within HIF-1α. *Von Hippel-Lindau* recognition requires enzymatic hydroxylation of specific prolyl residues within the HIF-1α oxygen-dependent degradation domain, which depends on the availability of molecular oxygen (reviewed in Ref. 4).

Recently, several groups have shown that this oxygen-dependent hydroxylation of HIF-1α is mediated by a family of three prolyl-hydroxylases belonging to the super-family of 2-oxoglutarate-dependent iron-dependent dioxygenases, designated prolyl-hydroxylase domain-containing proteins (PHDs) or HIF-prolyl-hydroxylases (Refs. 5–7). A bioinformatics study identified the same family of proteins in both the human and mouse genome and named them EGLN1–3, for their homology to the *Caenorhabditis elegans* protein Egl-9 (8). Recently, a fourth member of the group was identified (9). The identification of the HIF prolyl-hydroxylases offers a link between oxygen sensing and the activation of HIF-1α, which, in turn, regulates the cellular response to hypoxia.

After hypoxia, HIF-1 modulates a range of effects that include promoting adaptive changes in cellular metabolism, such as increased glucose uptake and glycolysis, along with the production of several angiogenic factors and their receptors (10). The control of angiogenic processes has great significance in both ischemic and neoplastic diseases. The ability to induce the formation of new blood vessels was shown to be a key factor determining tumor growth (11), and, thus, the regulation of HIF-1α activity is a very attractive target for therapeutic manipulation.

Interestingly, two members of the prolyl-hydroxylase family were identified previously as cell growth regulators. The rat *SM-20* is homologous to *PHD3* and was first identified as a growth-factor responsive gene in smooth muscle cells (12) and, later, as a mitochondrial apoptotic factor in neuronal cells (13). *PHD1* was cloned as an estrogen-induced gene in a breast cancer cell line and was designated *EIT-6* (14). In addition, we recently identified *Falkor*, the mouse homologue of *PHD1*, as a DNA damage related growth regulator in mouse embryo fibroblasts (15).

On the basis of the finding that the human homologue of *Falkor*, *PHD1*, is a regulator of HIF-1α, we set out to study the implications of this regulation on HIF-1α activity and on cell growth both *in vitro* and *in vivo*.

Here we show that expression of *Falkor*, hereafter referred to as *mPHD1* (*mouse PHD1*), results in inhibition of HIF-1α accumulation after hypoxia and in repression of the transcriptional activity of endogenous HIF-1α under hypoxic conditions. Moreover, although p53 was shown in several studies to be an inhibitor of HIF-1α (16, 17), the mPHD1-dependent inhibition of HIF-1α seems to be p53-independent. The repression of HIF-1α activity by expression of *mPHD1* resulted in decreased survival of HCT116 cells after treatment with the hypoxia-mimetic drug desferrioxamine mesylate (DFO). When injected s.c. into nude mice, cells expressing *mPHD1* formed smaller tumors than control cells, which had increased necrosis and decreased vascularization.

MATERIALS AND METHODS

Cell Culture and Infections. HCT116 cells were cultured in McCoy's 5A medium (Biological Industries, Kibutz beit Haemek, Israel) with 8% FCS and 1% penicillin-streptomycin (Biological Industries). Stable cell lines expressing pBabe-mPHD1 or pBabe empty vector were generated by viral infection as described previously (15), except that only Phoenix-Ampho packaging cells were used, and selection was done with 1 μg/ml puromycin for 48 h.

Luciferase Assays. For luciferase assays, 3×10^5 HCT116 or H1299 cells were seeded per well of 24-well plates and transfected with 50 ng of hypoxia-responsive luciferase constructs, HIF-1α expression vector, 100 ng of cyto-

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megalovirus-β-galactosidase expression vector and mPHD1-pCDNA3 expression vector. The total amount of DNA in each transfection was kept constant by complementing with control vector DNA. All transfections were done with Fugene (Roche). Twenty-four hours later, DFO was added and incubated overnight. Forty-eight hours after transfection, cells were rinsed twice with PBS and lysed, and luciferase assays were performed following instructions by the manufacturer (Promega). Transfections were done in triplicate and normalized to β-galactosidase activity as an internal transfection control.

β-Galactosidase Enzyme Assay. Five microliters of cell lysate were mixed with 200 μl of Lac Z buffer (60 mM Na_2CO_3, 40 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4$) and 20 μl of substrate [2 mg/ml 2-Nitrophenyl b-D-galactopyranoside (Sigma, Rehovot, Israel) in Na_2CO_3, 40 mM NaH_2PO_4]. The reaction was incubated at 37°C until yellow color was visible. Results were read at A_{420} using a multiwell ELISA reader.

Protein Assays. The p53 protein assay was performed as described previously (18). For protein assays of HIF-1α, nuclear extracts were prepared as described (19). Extracts were analyzed for protein concentration by a bicinchoninic acid protein assay kit (Pierce). HIF-1α was detected by a monoclonal antibody that was the kind gift of Dr. Eric Huang.

Semi-quantitative and Real-Time Reverse-Transcription PCR. Total RNA was prepared from HCT116 cells using TriReagent (MRC Inc.). The cDNA was reverse-transcribed from 1 μg of RNA using EZ-First Strand cDNA Synthesis Kit (Biological Industries) according to the protocol of the manufacturer. Semi-quantitative PCR for vascular endothelial growth factor (VEGF) was done with the following primers: 5′-GAACTTTCTGCTGTCTTGGG and 3′-TCACCGCCTCGGCTTGTC.

A cDNA loading control was done using glyceraldehyde-3-phosphate dehydrogenase primers. Real-time PCR was performed using TaqMan Universal PCR Master Mix and VEGF Assays-On-Demand Gene Expression Mix in an ABIprism 7000 instrument (Applied Biosystems). Results were normalized to those of glyceraldehyde-3-phosphate dehydrogenase for each reaction.

VEGF ELISA Assay. Cells were plated in a 24-well plate and treated were either treated in duplicates overnight with 200 μM DFO or left untreated. The supernatants of cells were collected and subjected to an ELISA assay with a kit specific for human VEGF according to the manufacturer (Oncogene). The results were normalized to the number of cells evaluated by using the WST-1 reagent (Roche).

Histology and Immunohistochemistry. For tumor necrosis evaluation, tumors were excised and fixed in 4% buffered formaldehyde. Paraffin-embedded 7-μm sections were stained with H&E.

CD-31 staining was done on 14-μm cryostat sections with an antimouse CD-31 monoclonal antibody diluted to 1:100 (PharMingen). Antibody distribution was visualized using the avidin-biotin complex technique (Vectastatin ABC Elite kit; Vector) and 3,3′-Diaminobenzidine tetrahydrochloride substrate (Sigma), followed by counterstaining with hematoxylin.

For quantitative analysis, capillaries, identified by positive staining for CD31, were counted and their density was expressed as the percentage of capillaries of total section area. For necrosis quantitation, necrotic areas of serial paraffin sections stained with H&E were analyzed, and the percentage of necrotic area of total section area was calculated using the Image-Pro plus 4.1 software.

BSA-Fluorescein Labeling. BSA (Sigma) was labeled with 5(6)-carboxyfluorescein succinimidyl ester (Molecular Probes Inc.) and purified using centrifugal filtration (Amicon Centriprep YM30; Millipore Corporation). Fluorescein-labeled BSA was injected into the tail vein at a dose of 3 mg in 100 μl/mouse.

Xenograft Growth Assay. Female CD1-nude mice (7–9 weeks of age) were housed in a barrier facility, and cell injections were done under a laminar flow cabinet. For inoculation, HCT116-pBabe, or HCT116-mPHD1 cell suspensions were prepared in PBS. Cells (5×10^6) in 200 μl of PBS were injected s.c. into the dorsal region. Tumors were measured every 3–4 days with calipers, and tumor volumes were calculated by the formula $4/3 \times \Pi xr^3$ (r = larger diameter + smaller diameter/4). Tumor growth was measured for 30 days in all experiments, unless tumors reached a size of 15 mm in any diameter, in which case the mouse was sacrificed.

Statistical Analysis. Statistical analyses were done by Student's t test. Statistical significance was defined as $P < 0.05$.

RESULTS

mPHD1 Inhibits HIF-1α Accumulation and Transcriptional Activity. It has been shown by several groups that hydroxylation of HIF-1α by either PHD1 or the other two members of the prolyl-hydroxylases family promotes degradation of HIF-1α (5–7). We wanted to test whether mPHD1-mediated hydroxylation would affect the transcriptional activity of HIF-1α. To that end, H1299 lung carcinoma cells were transiently transfected with a luciferase reporter gene driven by three tandem repeats of the hypoxia-response element and with HIF-1α alone or in combination with a plasmid encoding mPHD1 (15). A plasmid encoding for b-galactosidase was cotransfected in each sample, and all luciferase experiments were normalized to b-Galactosidase activity as internal transfection control. As seen in Fig. 1A, mPHD1 inhibits HIF-1α dependent transcription in a dose dependent manner.

To see whether overexpression of mPHD1 will inhibit endogenous HIF-1α activity under hypoxic conditions, we transiently transfected H1299 cells with mPHD1 and a luciferase reporter gene driven by the hypoxia-response element. Twenty-four hours after transfection, cells were treated overnight with 100 μM the hypoxia-mimetic drug DFO, an iron chelator that has been shown to mimic hypoxia in cells (20), and a luciferase assay was performed. Expression of mPHD1 inhibited activation of endogenous HIF-1α in a dose dependent manner (Fig. 1*B*).

Because it has been shown that p53 inhibits the transcriptional activity of HIF-1α (16, 17), we further asked whether the inhibitory effect of mPHD1 on HIF-1α was p53-dependent. To that aim, we performed the same assay described in Fig. 1*B* in wild-type p53

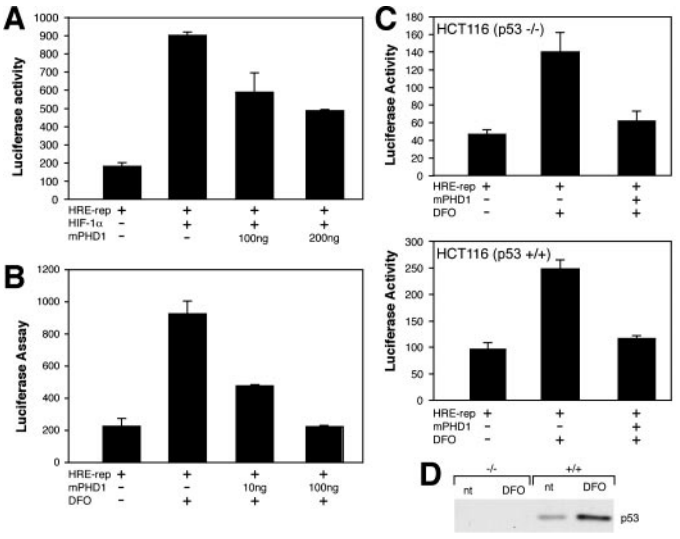

Fig. 1. Inhibition of hypoxia inducible factor 1α (*HIF-1α*) transcriptional activity by mouse PHD1 (*mPHD1*). *A*, H1299 cells were transfected with 50 ng of hypoxia-response element-pGL3 reporter (*HRE-rep*) alone or in combination with 100 ng of HIF-1α, 100 ng, or 200 ng mPHD1-pCDNA3, as indicated. Luciferase assay was performed 48 h after transfection and normalized to β-galactosidase activity as an internal transfection control. The results represent the average of triplicate samples, with *error bars* corresponding to SDs. *B*, H1299 cells were transfected with 50 ng of hypoxia-response element-pGL3 (*HRE-rep*) alone or in combination with increasing amounts of mPHD1-pCDNA3 as indicated. Twenty-four hours after transfection, cells were treated with 100 μM desferrioxamine mesylate (*DFO*) overnight or left untreated, as indicated, and luciferase assays were performed the next day. *C*, HCT116 cells (*p53+/+* or *p53−/−*, as indicated), were plated at 3×10^5 cells/well in a 24-well plate. Cells were transfected in triplicates with 50 ng of hypoxia-response element-reporter (*HRE-rep*) alone or in combination with 50 ng mPHD1-pCDNA3. Twenty-four hours after transfection, cells were treated with 200 μM DFO overnight or left untreated, as indicated, and luciferase assays were performed the next day. *D*, HCT116 cells were untreated or incubated with 200 μM DFO for 6 h. Cells were collected and lysed in TLB. Fifty micrograms of protein lysate from each sample were resolved by 10% SDS-PAGE, followed by immunoblotting with p53-specific antibodies. *nt*, not treated; *TLB*, tris lysis buffer.

(*p53*+/+) or p53 deficient (*p53*−/−) HCT116 colon cancer carcinoma cells (21). As seen in Fig. 1*C*, the inhibition of HIF-1α by mPHD1 in HCT116 cells seems to be p53 independent, and we observed no difference in the mPHD1-mediated transcriptional inhibition between the two cell lines, although p53 was stabilized in $p53^{+/+}$ HCT116 cells after DFO treatment (Fig. 1*D*).

Loss of p53 in tumor cells enhances HIF-1α levels and augments HIF-1-dependent transcriptional activation of the *VEGF* gene in response to hypoxia. This implies that amplification of normal HIF-1α-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis (20). Additionally, it was shown in various human tumors that HIF-1α expression correlates with aberrant p53 accumulation and cell proliferation (22). With these reports in mind, and considering that the effect of mPHD1 on HIF-1α seemed to be p53 independent, the rest of the experiments presented below were performed in p53-null HCT116 cells, which seemed to be a more stringent environment to further study mPHD1-mediated inhibition of HIF-1α.

To further analyze the role of mPHD1 in the cellular response to hypoxia mediated by HIF-1α, we prepared stable cell lines of p53−/− HCT116 cells by retroviral infection with the retroviral vector pBabe or with the same vector encoding mPHD1 (mPHD1-pBabe). Western blot analysis with anti-mPHD1 antibodies in these cells confirmed overexpression of mPHD1 (Fig. 2*A*).

Because it was shown that the PHDs mediate the degradation of HIF-1α, we next asked whether the mPHD1-dependent inhibition of HIF-1α transcriptional activity was a result of decreased levels of HIF-1α protein. As shown in Fig. 2*B*, there was decreased HIF-1α accumulation after 5 h of treatment with DFO in HCT116-mPHD1 stable clones, as compared with HCT116-pBabe control cells. In agreement with the results of the luciferase assays, the inhibition of HIF-1α accumulation in cells overexpressing mPHD1 resulted in decreased induction of the HIF-1α downstream target VEGF. The results of a reverse-transcription PCR assay performed on cells treated with DFO for 5 h are shown in Fig. 2*C*. The level of VEGF in HCT116-mPHD1 cells was lower than that observed in HCT116-pBabe cells after hypoxia. The primers we used detected both $VEGF_{165}$ and $VEGF_{121}$ isoforms. As seen in Fig. 2*C*, there was a reduction in the basal level of VEGF transcription in mPHD1-expressing cells; however, this decrease was not as reproducible as the inhibition of VEGF induction after DFO treatment. To quantitate the inhibition of VEGF transcription by mPHD1 expression, we performed real-time PCR with cDNA prepared from nontreated or DFO-treated cells. Shown in Fig. 2*D* is the mean fold induction of VEGF mRNA after DFO treatment from three separate experiments, normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase transcription as an internal control in each experiment. Expression of mPHD1 resulted in an average inhibition of 28% ($P = 0.025$) on induction of VEGF transcription after treatment with DFO, when compared with the induction in control cells. To verify this inhibition at the protein level, we performed an ELISA assay to measure secretion of VEGF into the culture medium. The results were normalized to cell number. As expected, cells expressing mPHD1 secreted less VEGF after treatment with DFO as compared with control cells. The results of a typical experiment are shown in Fig. 2*E*. The average inhibition of VEGF secretion by mPHD1 expression was 30%, which was calculated for combined data from four separate experiments.

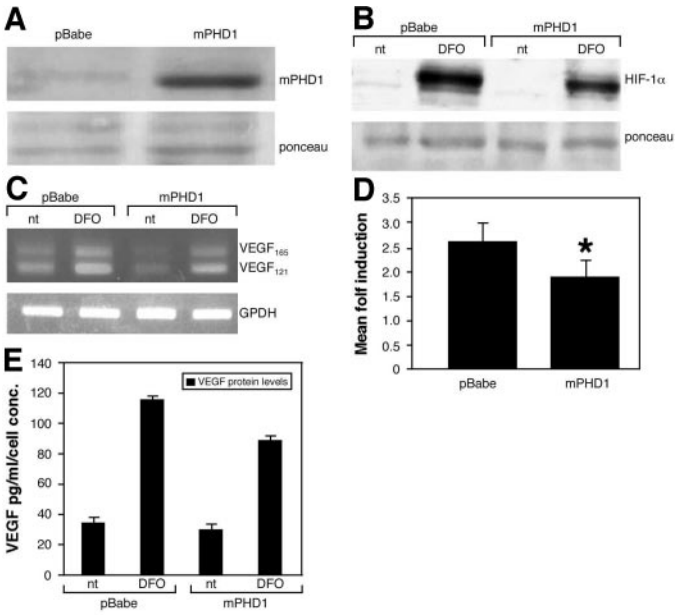

Fig. 2. Overexpression of mouse PHD1 (*mPHD1*) in HCT116 cells inhibits stabilization of HIF-1α and vascular endothelial growth factor (*VEGF*) induction after hypoxia. *A*, p53−/− HCT116 cells were infected with mPHD1-pBabe or pBabe alone. After 48 h of puromycin selection, cells were collected, and 50 μg of nuclear extracts were resolved by 10% SDS-PAGE. mPHD1 was immunoblotted with a rabbit polyclonal antibody. Ponceau staining is shown as a loading control. *B*, HCT116 stable cell lines were treated with 200 μM desferrioxamine mesylate (*DFO*) for 5 h, or were left not treated (nt) collected, and lysed as in *A*. Fifty micrograms of nuclear extracts were separated on a 7.5% gel and immunoblotted with a monoclonal anti-HIF-1α antibody. Ponceau staining is shown as a loading control. *C*, reverse-transcription PCR of cDNA prepared from HCT116 stable clones treated with 200 μM DFO. VEGF165 and 121 variants were amplified. PCR with glyceraldehyde-3-phosphate dehydrogenase primers is shown as a loading control. *D*, cDNA, as in *C*, was used for real-time PCR. Shown is the fold induction VEGF after treatment with DFO relative to nontreated levels. The *graph* shows a mean with SD of three separate experiments. The difference between mPHD1-expressing cells and controls was found to be significant ($P = 0.025$; indicated by *). *E*, VEGF ELISA. Cells were plated in a 24-well plate and treated overnight with 200 μM DFO. Supernatants of cells were collected and subjected to a VEGF ELISA assay. Results were normalized to cell number. The results show a representative experiment of four separate experiments performed. *nt*, not treated.

mPHD1-Mediated Inhibition of Cell and Tumor Growth. To determine whether the inhibition of HIF-1α transcriptional activity had an effect on cellular growth, we examined survival of cells after treatment with DFO in HCT116 cells. Cells were treated overnight with DFO and plated the next day at a limiting dilution. One week later, cells were fixed and stained with crystal violet, and the number of colonies in each plate was counted. Shown in Fig. 3*A* is reduced survival of cells after hypoxia treatment in HCT116-mPHD1 cells, as compared with HCT116-pBabe cells. In the absence of hypoxia-mimetic treatment, no difference in colony formation was observed in mPHD1-expressing *versus* control cells (not shown).

We, therefore, hypothesized that the *in vitro* inhibition of the HIF-1α-mediated response after treatment with DFO might predict an *in vivo* repression of tumor growth by mPHD1 expression. To that end, we injected the HCT116 cell lines into CD1-Nude mice and followed the tumor growth and survival of the mice. HCT116-pBabe or HCT116-mPHD1 cells were injected s.c. into the flanks of nude mice. Tumor diameters were measured twice a week with a caliper. As shown in Fig. 3*B*, the average tumor volume in mice injected with HCT116-pBabe cells was significantly larger than that of tumors in mice injected with HCT116-mPHD1 cells ($n = 9$; $P < 0.05$). When tumors reached a size of 15 mm in any diameter, the mouse was sacrificed. Strikingly, a higher percentage of mice that received HCT116-mPHD1 injections survived until the experiment was terminated, as compared with mice that received injections of control cells: only one mouse died in the HCT116-mPHD1-injected group ($n = 11$), whereas five animals died in the control group ($n = 10$) . A representative survival curve is shown in Fig. 3*C*.

To examine the possible mechanism underlying these differences in tumor growth, tumors of ~6 mm were excised and stained with H&E. Histological analysis indicated that tumors from mice receiving

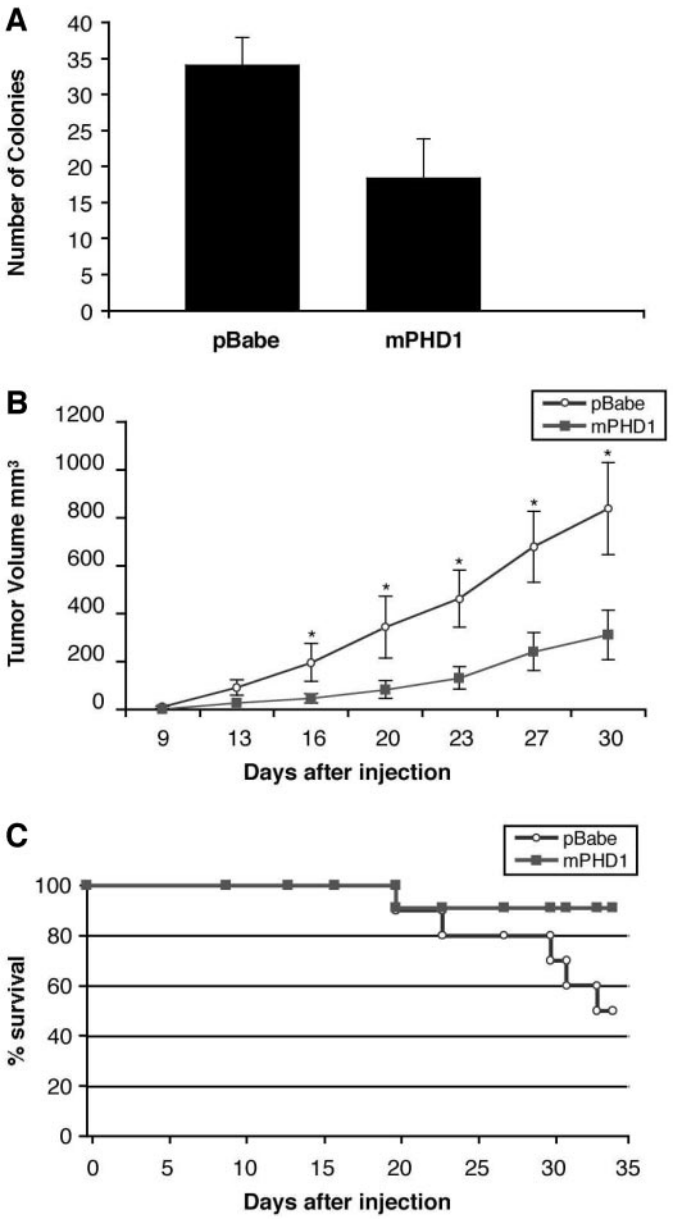

Fig. 3. Expression of mouse PHD1 (*mPHD1*) inhibits colony survival and tumor growth. *A*, colony survival assay. HCT116 stable clones were treated with 200 μM desferrioxamine mesylate (DFO) overnight. The next day, 5×10^3 cells were plated in triplicate in 6-cm plates. Ten days later, cells were fixed and stained with crystal violet, and colonies were counted. Shown is the average of triplicates with *error bars*. *B*, growth of tumors formed from HCT116-pBabe or HCT116-mPHD1 cells injected s.c. into CD1-nude mice as described in "MATERIALS AND METHODS." All values are expressed as mean ± SE. A significant difference in tumor size between the two groups was detected beginning from day 16 ($P < 0.05$, indicated by ∗). *C*, survival of mice receiving injections as in *B*. When tumors reached a diameter of 15 mm at any diameter, the mouse was sacrificed. HCT116-pBabe: $n = 10$; HCT116- mPHD1: $n = 11$.

HCT116-mPHD1 injections exhibited more necrosis than those derived from control cells, as shown in Fig. 4*A*. This difference was even more prominent when larger tumors (>10 mm) were analyzed (Fig. 4*B*). A schematic representation of the necrotic areas in the tumor sections shown in Fig. 4, *A* and *B*, is depicted in Fig. 4*C*. Furthermore, quantitation of necrotic areas in three different sections of each 6-mm tumor, which was calculated for three different tumors, revealed that the necrosis in tumors from HCT116-mPHD1-injected mice was 35–50% higher than the percentage of necrosis found in control tumors of the same size and time after injection.

To grow beyond a critical mass of several millimeters, solid tumors must activate the "angiogenic switch" (11), which allows them to develop autonomous vasculature. We have shown that mPHD1 expression resulted in inhibition of HIF-1α accumulation, transcriptional activity, and a decrease in the expression of its target gene, *VEGF*. Thus, we hypothesized that the inhibition of tumor growth and the increased necrosis caused by expression of mPHD1 is a result of decreased tumor vascularization. To assess the effect of mPHD1 expression on tumor vascularization, mice with tumors at a size of 6 mm received i.v. injections of fluorescently labeled BSA; the animals were sacrificed immediately, and the tumors were excised. As shown in Fig. 5*A*, tumors obtained from mice that received injections of control cells (*left panel*) were more vascularized than tumors from HCT116-mPHD1-injected mice (*right panel*).

Further evaluation of tumor vascularization was achieved by immunostaining with an antibody specific for the endothelial marker PECAM (CD31). A typical staining pattern is shown in Fig. 5*B*. Microvessel density was lower in sections excised from mPHD1-expressing tumors (*right panel*) than that of controls (*left panel*). The *lower panel* depicts an enlargement of the framed area in the *upper panel*.

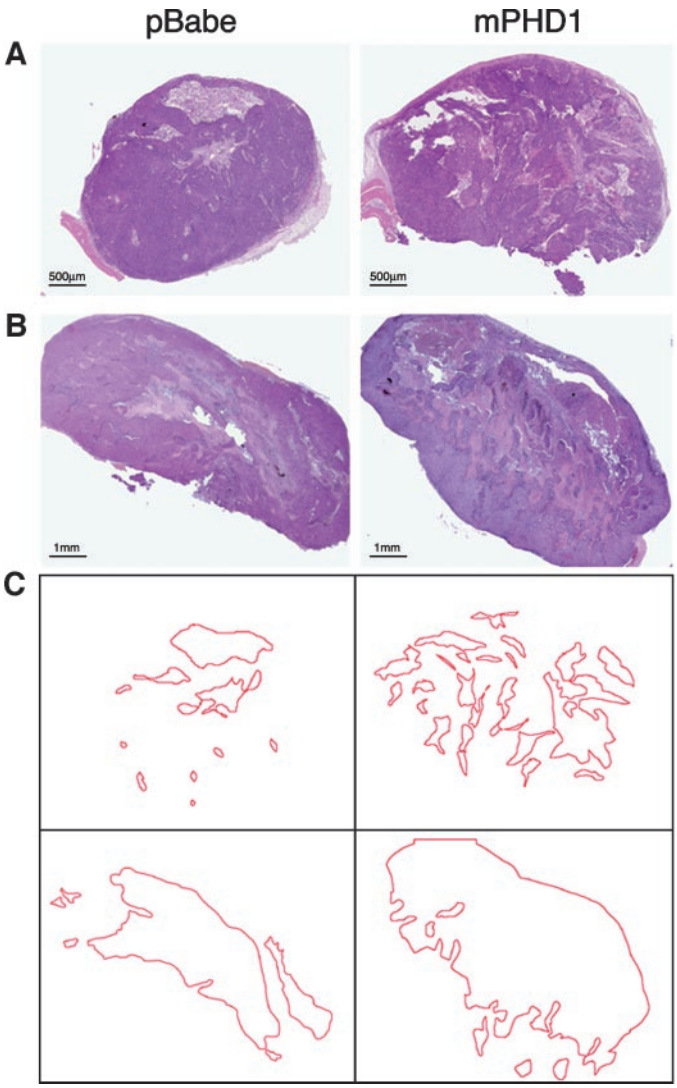

Fig. 4. Necrosis is increased in mouse PHD1 (*mPHD1*)-expressing tumors. H&E staining of tumors reveals necrotic areas in tumors formed from HCT116-mPHD1 cells, as compared with HCT116-pBabe cells in small tumors of ~6 mm (*A*) and in larger tumors of about 12 mm (*B*). *C*, a schematic representation of necrotic areas in the tumors shown in *A* and *B*.

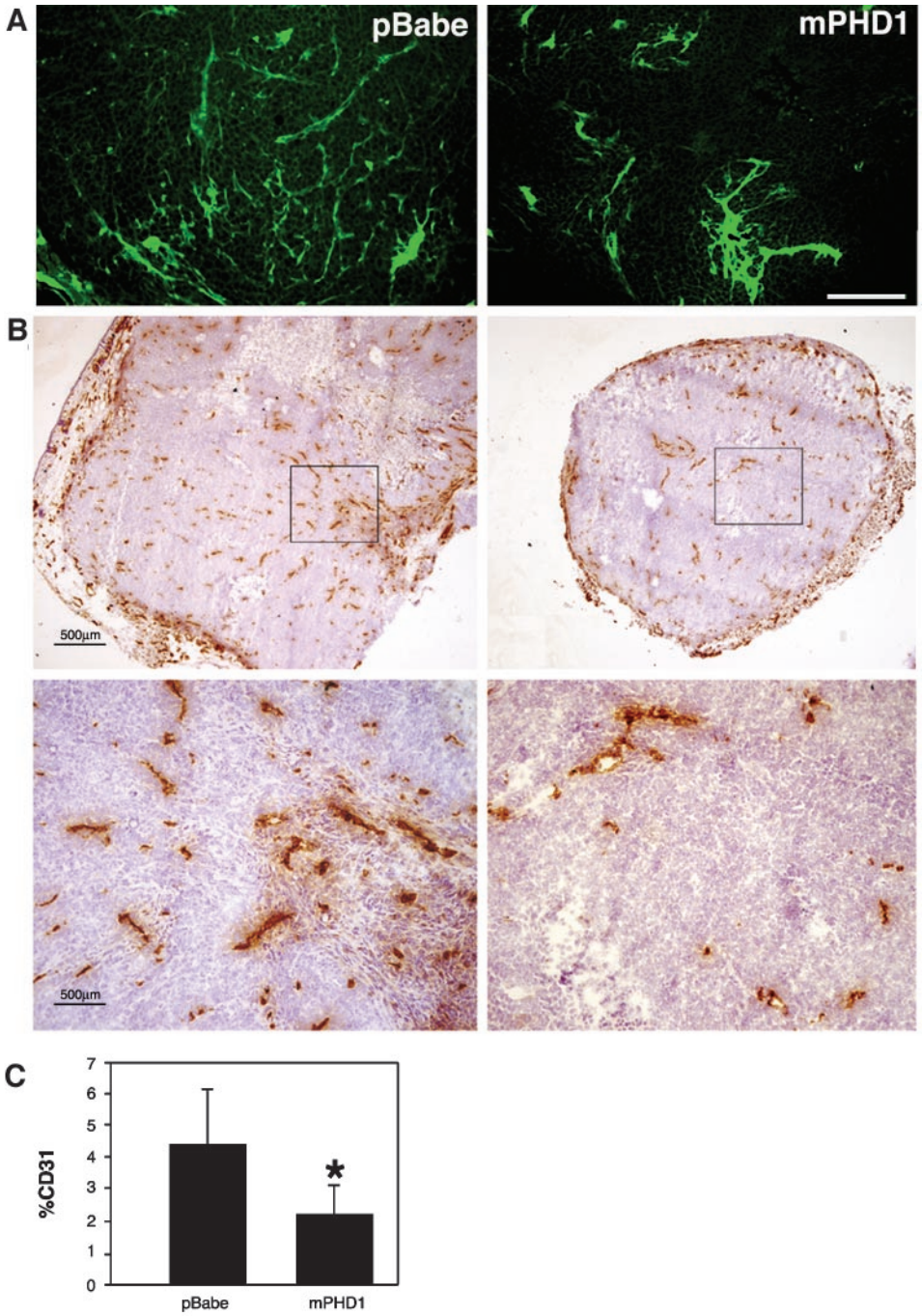

Fig. 5. Decreased vascularization in mouse PHD1 (*mPHD1*)-expressing tumors. *A*, fluorescein-conjugated BSA was injected i.v. into mice to assess tumor vascularization. *Left panel*, HCT116-pBabe; *right panel*, HCT116- mPHD1. *Scale bar* = 100 μM. *B*, CD-31 (PECAM) immunostaining was used to visualize capillary density in tumors. The *lower panels* show ×10 magnifications of the framed area in the respective *upper panels*. *C*, quantitation of capillary density is shown as the percentage of CD-31 positive areas of the total section area. Analysis was done on three tumors and at least five different sections per tumor. The difference was found to be significant ($P = 0.014$; indicated by ∗).

Quantitative analysis of the difference in capillary density was performed on three tumors and at least five different sections per tumor, and the results are shown in Fig. 5*C*. Microvessel density of tumors resulting from mPHD1-expressing cells was remarkably reduced by 50%, as compared with that of tumors resulting from control cells ($P = 0.014$).

DISCUSSION

In previous work, we identified mPHD1 as a novel cell growth regulator. By using a functional genetic screen, we isolated a dominant negative fragment from the COOH terminus of mPHD1 (15). We showed that the dominant negative fragment from the COOH terminus of mPHD1 enhances cell growth and predicted that the full-length protein would have a growth-suppressive effect. The same gene was cloned by others and was shown to be part of a family of prolyl-hydroxylases that regulate the stability of HIF-1α, thus acting as "molecular sensors" for oxygen (5–7). Interestingly, the dominant negative fragment we isolated contained the conserved residues of the jelly roll motif typical of the 2-oxoglutarate-dependent oxygenase family (5). In this study, we set out to study the outcome of mPHD1 expression on cell growth under hypoxia. Our results show that, when overexpressed, mPHD1 can inhibit the stabilization of HIF-1α after hypoxia. Although the PHDs were predicted to be inactive under hypoxia, because their hydroxylation activity is iron and oxygen-dependent (23), expression of mPHD1 in our system was sufficient to inhibit the transcriptional activation of HIF-1α after treatment with DFO, as

measured by the hypoxia-response element-luciferase assay. These results are in agreement with a recent study by Metzen *et al.*, who showed that transient expression of HIF-1α hydroxylases inhibits transcription from the hypoxia-responsive element under hypoxic conditions (24).

When measured at the protein level, the accumulation of HIF-1α after treatment with DFO was decreased in cells expressing mPHD1. Although partial, this inhibition resulted in decreased levels of HIF-1α, which led to reduced transcriptional activation of *VEGF*, a target gene of HIF-1, and one of the most potent angiogenic factors (25). The results of the colony assay showed that inhibition of HIF-1 transcriptional activation led to reduced survival of cells after hypoxic stress.

Hypoxia is the main physiological inducer of angiogenesis and, hence, a major selective force for the genetic changes that result in neovascularization of tumors. A large body of data shows that overexpression of HIF-1α and repression of HIF-1α-negative regulators, such as *p53* and *Von Hippel-Lindau*, are important steps in tumorigenicity and in progression of tumors (20, 26–28). HIF-1α is overexpressed in >70% of human cancers and their metastases, compared with normal adjacent tissues, and the expression of HIF-1α is correlated with poor prognosis (29, 30). We, therefore, hypothesized that the *in vitro* inhibition of the HIF-1α-mediated response that we showed after treatment with DFO might predict an *in vivo* repression of tumor growth by mPHD1 expression. Indeed, our results show that the colorectal carcinoma cell line HCT116 cells stably expressing mPHD1 grew significantly slower and formed smaller tumors in nude mice as compared with control cells.

Solid tumors are less oxygenized than normal tissues, and the prolonged hypoxia of the tumor tissue leads to the formation of necrotic regions (31). Our results show that tumors from mPHD1 overexpressing cells had more necrosis than control tumors, suggesting that they were subjected to more severe hypoxic conditions, most likely attributable to decreased vascularization. Our findings indicate that the inhibitory effect of mPHD1 expression on tumor growth was attributable to inhibition of angiogenesis. Indeed, when we analyzed the tumors for capillary and blood-vessel density, the vascularization of tumors resulting from mPHD1 expressing cells was strikingly reduced by 50%, as compared with that of tumors resulting from control cells.

It is interesting to note that the effects of mPHD1 expression we showed *in vivo* were more dramatic than the ones we observed *in vitro*; the inhibition of VEGF transcription and secretion and the decrease in colony survival were ~30%, whereas in the *in vivo* experiments, there was a dramatic difference in tumor growth and mice survival and a 50% reduction in tumor vascularization. This probably reflects the fact that the *in vitro* results showed cell intrinsic effects of HIF-1α activity, whereas the *in vivo* observations were a consequence of combined cell intrinsic and extrinsic effects resulting from the >40 genes transcriptionally regulated by HIF-1 (1). Several of these genes are associated with the relationship between the tumor and its microenvironment, including *VEGF*, as well as the *met* proto-oncogene, which promotes invasive growth (32).

The fact that all tumors depend on neovascularization to support their growth beyond a critical size has made the angiogenic process an attractive target for therapy in recent years. Mediators of angiogenesis, such as VEGF and the endothelin A receptor, have been the target of several recent antiangiogenic protocols (33, 34). Suppression of tumor growth through inhibition of HIF-1α-induced transcription has been suggested in several studies (35, 36). Our data suggest an approach that targets the stabilization of HIF-1α following hypoxic stress. Through overexpression of a protein that mediates the degradation of HIF-1α, we could abrogate the activation of HIF-1α and take advantage of its selective induction in hypoxic cells. This is supported by our previous observation that overexpression of mPHD1 in primary cells or in tumor cell lines did not change their growth under normal conditions (15).

A similar strategy has been described in a recent report suggesting activation of angiogenesis by pharmacological inhibition of PHDs as a treatment for ischemic diseases (37). The advantage of targeting the activation of HIF-1, rather than specific downstream genes such as *VEGF*, is by inhibiting a whole range of survival and angiogenic pathways activated by HIF-1.

In conclusion, our data show that expression of the HIF-prolyl-hydroxylase-1 can inhibit stabilization of HIF-1α under hypoxia, decrease VEGF secretion, and inhibit tumor growth attributable to the inhibition of vascularization. These findings support the use of gene-transfer approaches to inhibit solid tumor growth through enhancement of the prolyl-hydroxylase-mediated degradation of HIF-1α.

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