Inhibition of HIF Prolyl Hydroxylase-2 Blocks Tumor Growth in Mice through the Antiproliferative Activity of TGFβ

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Abstract

Virtually all solid tumors are dependent on a vascular network to provide them with the right amount of nutrients and oxygen. In that sense, low oxygen tension or hypoxia leads to an adaptive response that is transcriptionally regulated by the hypoxia-inducible factors (HIF), which are tightly controlled by the HIF prolyl hydroxylases (PHD). In this study, we show that inhibition of the oxygen sensor PHD2 in tumor cells stimulates vessel formation but paradoxically results in a profound reduction of tumor growth. This effect relies on the antiproliferative nature of the TGFβ signaling pathway, in a largely HIF-independent manner. Moreover, our findings reveal that PHD2 has an essential function in controlling the dual nature of TGFβ during tumorigenesis and may offer an alternative opportunity for anticancer therapy. Cancer Res; 71(9); 3306–16. ©2011 AACR.

Introduction

Tumors can grow only when they have sufficient nutrients to sustain their metabolic needs for which they require a functional vascular system. This blood supply is the rate-limiting factor for tumor growth beyond a certain size. Moreover, many tumors proliferate so quickly that they exceed their own vascular supply, resulting in micro-environments characterized by low oxygen tension (hypoxia), a phenomenon that consequently leads to the expression of genes that promote survival of the cells, new vessel formation, and anaerobic metabolism (1). This transcriptional response is, to a large extent, regulated by the hypoxia-inducible factor (HIF) family, which comprise complexes composed of a constitutive HIFα subunit and an oxygen-sensitive HIFα subunit that is degraded under normoxic conditions (2). The overexpression of HIF has been detected in a wide variety of tumor types and directly correlates with tumor invasiveness and metastasis, as well as overall survival of patients (3). In addition, HIFα ablation in various tumor models often resulted in slower tumor growth accompanied by reduced vascular density (4, 5). In this context, it is not surprising that this system is tightly regulated by a family of enzymes known as prolyl hydroxylase domain proteins (PHD; refs. 6–8). Of the 3 major PHDs, PHD2 has been proposed to be the key oxygen sensor during normoxia and mild hypoxia (9). The importance of this enzyme is underscored by the fact that inactivation of PHD2 severely deregulates normal embryonic development resulting in embryonic lethality by E14.5, whereas PHD1−/− or PHD3−/− mice develop normally (10). Moreover, conditional somatic inactivation of PHD2 in mice causes an increase in erythropoietin production in the kidney, consequently leading to polycythemia, as has been observed in hypomorphic mutations in humans (10–13). Furthermore, heterozygous deficiency of PHD2 in mice has been shown to promote normalization of the endothelial lining and vessel maturation—a feature that redirected the specification of the endothelial tip cell to a more quiescent cell type during tumor development (14). In addition to the role of PHD2 as an inhibitor of HIFα, it has been suggested that PHD2 can regulate the NFκB pathway via hydroxylation of the inhibitor of κB kinase (IKKκb; ref. 15). Overall, these data highlight the importance of PHD2 and its various functions. In the present study, we describe a number of mouse tumor cell lines that show a significant reduction in growth when PHD2 is silenced, regardless of the fact that the enzyme still plays a critical role in regulating angiogenesis. Not only do we provide evidence for a link between PHD2 and the TGFβ signaling pathway but also our data suggest that changing PHD2 levels in tumor cells results in the transformation of TGFβ from a tumor promoter to a tumor suppressor, also known as the "TGFβ paradox" (16, 17).

Materials and Methods

Cells

The murine osteosarcoma cell line LM8 (a gift from Dr. C. Beltinger, University Children’s Hospital, Ulm, Germany; received in 2005) was grown in MEM-α medium. Lewis lung carcinoma (LLC) 3LL-D122 cells and a B16BL6 melanoma...
subline (a gift from Dr. C. Libert, DMBR-UGent, Belgium; received in 2005) were grown in Dulbecco’s modified Eagle’s medium (DMEM). After arrival, cells were cultivated and stored in numerous batches in liquid nitrogen. Stable cell lines (see further) were made from wild-type (WT) cells not passaged more than 5 times. After selection and cultivation, cells were stored in the same manner as WT cell lines. All cells were mycoplasma negative and tested with regard to morphology and growth rate in vitro and in vivo. No other authentication has been done. Cells were typically used before the fifth passage. All media were supplemented with 10% heat-inactivated FBS, NEAA, and l-glutamine.

**Mice and tumor models**

Animal experiments were approved by the state of Saxony. All experiments were conducted with female C57BL/6 and C3H mice between 8 and 12 weeks of age (Taconic or the Dresden University of Technology). All mice received between $1 \times 10^6$ (B16BL6) and $2 \times 10^6$ (LLC and LMS) tumor cells injected into both flanks. All tumors were measured with calipers every 2 to 3 days, and the volume of each measurement was calculated as follows: $(\text{width}^2 \times \text{length})/2$. Tumors were isolated unless explicitly mentioned otherwise (anti-TGFβ treatment) and processed immediately or stored at $-80^\circ$C. Lung tissues for the detection of metastasis were prepared as formalin-fixed paraffin-embedded (FFPE) material. Circulating EGFP+ tumor cells were isolated at day 14 and counted from 1 mL of peripheral blood after the removal of red blood cells and adherence to a culture dish (ACK lysis; Sigma). Specific antibodies used included PHD2 (Novus Biologicals), HIF1α (Cayman chemical), β-actin (Sigma), c-Myc (Santa Cruz), phosphorylated Smad2/3 ([pSmad2/3]; Chemicon Int.), and IKKα and β (Cell Signaling). Specific bands on radiographic films were quantified using a BioRad densitometer and analyzed via the Quantity One analysis software.

**In vitro doubling time**

Cells were plated in triplicate in 6-well dishes and, every 24 hours, were trypsinized and counted using a CASY cell counter. On the basis of the total cell numbers per time point, the doubling time for each cell line was calculated.

**Immunohistochemistry and histology**

To visualize the functional vasculature, mice were intravenously injected with Hoechst33342 prior to tumor isolation. Frozen sections measuring 8 μm were stained with platelet endothelial cell adhesion marker (PECAM/CD31) to visualize vessels. Nonfunctional vessels were identified on displaying PECAM+/Hoechst− staining. Vessel density was determined by manually counting PECAM+ staining in viable tumor areas. Detection of apoptotic cells in tumor sections was done using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) detection system (Roche). Proliferating tumor cells were detected via Ki67 (DAKO) staining, paraffin-embedded lung sections (3 μm) were stained with hematoxylin and eosin, and metastatic nodules were counted in 4 different sections throughout the organ. Fluorescent and light microscopy was done with an Axioplan-2 imaging microscope and plan Apochromat lenses (Carl Zeiss). The cameras and acquisition software were either Q Imaging Retiga 2000R and Image pro MC6.0 (fluor) or AxioCam MRc5 and Axiovision (light). Representative images are presented from experiments with similar results. All quantifications were done using the NIH ImageJ software.

**Short hairpin RNA constructs**

Oligos were cloned into lentivector pLVTHM and packed with psPAX2 and envelope vector pMD2.G (vectors kindly provided by Dr. D. Trono, Lausanne, Switzerland). All experiments refer to the use of individual constructs and not mixtures. To avoid clonal preferences, all in vivo experiments were repeated with different individual clones from the same short hairpin RNA (shRNA; shPHD2 or shScr) or with a pool of EGFP+ sorted cells (shHIF or shHIF/shPHD2#2; for details, see Supplementary Table S1).

**Quantitative RT-PCR**

Total RNA was extracted from individual tumors using the RNeasy Mini Kit (Qiagen). cDNAs were synthesized using Superscript First-Strand synthesis (Invitrogen). Relative gene expression was determined using Maxima SYBR Green QPCR Master Mix (Fermentas) on an iCycler iQ (BIO-RAD). The ΔΔCT method was used with normalization to both mTBR and EF2 as reference genes (see Supplementary Table S2).

**NFκB reporter assays**

The NFκB Ready-To-Glow Luciferase Reporter Assay Kit (Clonetech) was used to monitor NFκB signal transduction. All LMS cell lines were grown in culture medium and transfected with a pNFκB–MetLuc2–reporter vector. Metridia luciferase was measured 24 hours after transfection.

**Statistics**

Data and graphs represent mean ± SEM of representative experiments. Statistical significance was calculated by the Student’s $t$ test (Prism v4.02), with $P < 0.05$ considered statistically significant.

**Results**

**PHD2 inhibition results in reduced tumor growth**

The essential physiologic role of PHD2 in regulating HIF in vivo inspired us to examine its function in depth during tumor development. However, recent evidence showed that its tasks are not restricted to the inhibition of HIF alone (reviewed in ref. 18). Furthermore, using a complete collection of human cancer microarray data (Oncomine Database), we found that significantly more tumor types (12.2%) overexpress PHD2
mRNA versus nontumoral tissue, than tumor types that underexpress the gene (2.2%; Supplementary Fig. S1A; ref. 19). Therefore, we conducted PHD2 knockdown studies using different mouse tumor cell lines in immunocompetent mice. Initially, we selected 2 independent shRNA sequences against PHD2. Both constructs stably overexpress shPHD2 (#1 or #2) and significantly knockdown PHD2 in a mouse osteosarcoma cell line (LM8). Of these, shPHD2#2 shows the most profound inhibition compared with shPHD2#1 (Fig. 1A and Supplementary Fig. S1B). In addition, stabilization of HIF1α in the PHD2 knockdown cells is already apparent under normoxic conditions and much more profound at 5% O2. On the other hand, sustained incubation at very low oxygen levels (1%) shows no significant difference in HIF1α levels compared with control LM8 cells, which confirms the limited activity of the PHD2 protein under permanent low O2 conditions (ref. 9; Fig. 1A). HIF1α could barely be detected under harsh conditions and was, therefore, not considered in the subsequent studies (data not shown).

To assess the development of these cells in vivo, we injected them in C3H mice and found that both independent shPHD2 clones grew significantly slower than the control cell lines [scramble (shScr) or nontransfected WT; Fig. 1B]. In addition, at later stages, we detected a significant difference in growth pace between both shPHD2 clones that reflects their differential knockdown efficiency (Fig. 1C). Moreover, virtually all shPHD2#2 LM8 tumors stopped growing and regressed over time. This resulted in complete loss of 75% of the tumors (9 of 12) and several tumor-free mice (4 of 6 animals were followed up until 3 months after the experiment). The differences observed in the mice, however, were not related to any fundamental defect of the cells themselves, because they all displayed similar growth rates in vitro (Supplementary Fig. S1C). Western blot analysis of representative tumor sample lysates confirmed a clear reduction of PHD2 in LM8shPHD2 tumors 3 weeks after inoculation (Supplementary Fig. S1D). Although our findings are in agreement with information obtained via the Oncomine Database, a recent publication showed inhibition of PHD2 in several human tumor samples, indicating that HIF1α acts as a tumor suppressor. In addition, simultaneous knockdown of HIF1α and PHD2 drastically reduced tumor growth, again to a level not significantly different from shPHD2 tumors, showing that tumor growth retardation by silencing PHD2 is mainly HIF1α independent (Fig. 2A and Supplementary Fig. S2B). Consequently, we decided to investigate the role of PHD2 in the NFκB pathway. Not only does this transcription factor play a pivotal role in many different types of cancers (21) but also it was shown that PHD1 and PHD2 can hydroxylate/inactivate IKKβ and, therefore, reduce NFκB activity (15). However, using an in vitro reporter assay, we found that PHD2 silencing in LM8 cells significantly blocks the NFκB pathway (Fig. 2B). This result is surprising but may be directly caused by reduced amounts of IKKα and IKKβ proteins in the LM8shPHD2 cells, suggesting an alternative and probably indirect PHD2 regulation of these kinases (Fig. 2C).

**Inhibition of PHD2 induces vessel density**

To investigate how the reduction of PHD2 in tumor cells blocks tumor growth, we defined the vessel density on tumor sections via immunohistochemistry. PECAM (CD31) staining revealed that LM8shPHD2 tumors display significantly higher vessel density (>35%) than scrambled tumors—a difference that we could also confirm in LLC tumors (Fig. 3A and Supplementary Fig. S3A). The finding that a reduction in tumor growth is related to an increase in blood vessels is contrary to the classical idea that angiogenesis promotes tumor growth. One plausible explanation is that the vessels in the shPHD2 tumors are not functional due to nonproductive angiogenesis, as described previously (22). We, therefore, injected Hoechst33342 prior to isolation but found no difference in perfused (CD31+/Hoechst+) versus nonperfused (CD31+/Hoechst−) vessels between shPHD2 and control tumors (Fig. 3B). In addition, we checked for extravasation of tumor cells. Compared with controls, we found significantly less number of circulating EGFP+ tumor cells in the peripheral blood of shPHD2 tumor–bearing mice, and this is in agreement with the difference in volume of the primary tumors (Fig. 3C). Moreover, metastatic nodules in the lungs of the latter mice were hardly detectable (Supplementary Fig. S3B). Furthermore, knockdown of PHD2 in tumor cells did not induce more programmed cell death in vivo (apoptosis), as shown by TUNEL staining, nor did it lead to more necrosis (Fig. 3D and E).

**PHD2 silencing reduces cell proliferation in a TGFβ-dependent manner**

Because of the discrepancy between the elevated vessel density and the reduced growth of the tumor itself, we examined the proliferation rate of the tumor cells by means of Ki67 staining on tumor sections. These results clearly show that the proliferation rate of the tumor cells themselves, in both PHD2-inhibited tumor clones, is significantly reduced compared with control tumors (Fig. 4A). In an attempt to identify regulators involved in this decreased proliferation, we examined the role of TGFβ in our model. This family of cytokines exerts pro- as well as antiproliferative effects on
PHD2 Silencing Inhibits Tumor Development

Figure 1. PHD2 inhibition leads to stabilization of HIF1α and diminishes tumor growth. A, LM8
shRNA constructs (#1 or #2) were grown in 21% O2 (normoxia), 5% O2, or 1% O2 for 24 hours and examined for PHD2, HIF1α, and β-actin via Western blot assay. B, shPHD2#1 and #2 or control LM8 (shScr or WT) were inoculated and measured every 2 to 3 days (n = 12). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 represent the difference for both shPHD2#1 and #2 versus shScr and WT samples. C, differential effect of both shPHD2 clones over a longer time period (n = 12). ***, P < 0.01; and ***, P < 0.001 represent the difference for both shPHD2#1 and #2 versus shScr and from day 26 on for shPHD2#2 versus shPHD2#1. shScr tumor-bearing mice were sacrificed at day 21 and shPHD2#1 at day 33. LLC (D) and B16BL6 (E) tumor cells knocked down for PHD2 (shPHD2#2) and control cells (shScr) were inoculated in immunocompetent C57BL/6 mice. Tumors were measured every 2 to 3 days (n = 12). *, P < 0.05; **, P < 0.01.

different cell types and is an important regulator of the transcriptional repression of c-Myc through the canonical Smad-signaling pathway (23, 24). Expression analysis of TGFβ isoforms and their main receptors in LM8 tumor lysates revealed a significant induction for most of them in all shPHD2 tumors compared with controls (Fig. 4B–F). Furthermore, we were able to show induced activation of the TGFβ pathway by means of pSmad2/3 (Fig. 4G) as well as a significant repression of c-Myc in all LM8shPHD2 tumor lysates (Fig. 4H and I). Downstream, at the level of cell-cycle regulation, we found a clear up-regulation of cyclin-dependent kinase (CDK) inhibitors p15INK4B and p27KIP1 (in particular, for shPHD2#2), but not p21Waf1/Cip1 (Fig. 4J and L). In LLC tumors, comparable differences were detected for c-Myc and the CDK inhibitors (Supplementary Fig. S4A–C) but not for the members of the TGFβ family or pSmad2/3 (data not shown).

Anti-TGFβ treatment induces growth in PHD2-inhibited tumors

To determine the impact of TGFβ on growth inhibition of these tumors, we developed an in vivo treatment method by which mice were injected daily (at different sites but near the tumor) with a pan-specific TGFβ antibody that neutralizes all 3 TGFβ isoforms (Supplementary Fig. S5A). In all mice containing one of the shPHD2 tumor cell lines, the anti-TGFβ treatment led to a significant induction of tumor growth compared with the isotype controls, showing the antiproliferative nature of TGFβ in PHD2 silenced LM8 tumors (Fig. 5A and B). Congruent with these enlarged tumors, we observed a clear upregulation of proliferating tumor cells (Ki67 staining; Fig. 5C). In addition, we evaluated the effect of the treatment on tumors of the best shRNA sequence (shPHD2#2) and found a significant drop of pSmad2 (no pSmad3 was detected on day 16; Fig. 5D), upregulation of c-Myc (Fig. 5E), and a partial reduction of p15INK4B (Fig. 5F) but not of p27KIP1. Furthermore, inhibition of TGFβ led to a moderate induction of vessel density, suggesting that the activity of the endogenous TGFβ does not promote the induced angiogenesis seen in shPHD2 tumors (Supplementary Fig. S5B). On the other hand, an anti-TGFβ treatment of shScr tumor–bearing mice resulted in a significant reduction of growth compared with the
isotype controls, suggesting a pro-oncogenic effect of endogenous TGF\(\beta\) in control LM8 tumors (Fig. 5G). Furthermore, we carried out the same treatment in C57BL/6 mice bearing different LLC tumors. Similarly, the 10-day treatment resulted in a significant induction of tumor growth in shPHD2#2 tumors as well as significant growth retardation in the control tumors (Fig. 5H and I). Moreover, if we combine the growth curves of LM8shPHD2#1, #2, and the LM8shScr tumors or those from LLCshPHD2#2 and LLCshScr, all treated with the TGF\(\beta\) antibody, it emerges that all the groups grow evenly fast and show no significant difference throughout the treatment (see Supplementary Fig. S5C and D), suggesting that the TGF\(\beta\) pathway covers the entire growth difference induced by PHD2 silencing. In conclusion, these results not only show that the anti-TGF\(\beta\) treatment reaches exactly as far as the PHD2 silencing effect, in LM8 as well as in LLC, but also TGF\(\beta\) is an antiproliferative factor in tumors with reduced levels of PHD2 and is pro-oncogenic in the WT tumors.

Discussion

Recent evidence has shown that inhibition of PHD2 in human tumor cell lines leads to the induction of angiogenesis and thus enhanced blood supply and faster tumor growth (20). In addition, in our study, using several mouse tumor cell lines, we found that the ablation of PHD2 resulted in an increase in vessel density, but that this was accompanied by an impressive decrease in tumor growth. Moreover, we found no evidence that this was due to nonproductive angiogenesis, as we have shown in the context of PHD4 overexpression (Klotzsche-von Ameln and colleagues, unpublished data) or in relation to the inhibition of delta-like ligand 4 (Dll4), as shown by others (22). In contrast, the augmented vascularization was completely overruled by the antiproliferative effect of TGF\(\beta\) on the tumor cells themselves and not by the induction of apoptosis or necrosis, as has been shown for this growth factor in other settings (16, 25). Moreover, this is the first time a direct relation between PHD2 silencing and regulation of the TGF\(\beta\) pathway has been shown in vivo. The impact of these findings was further supported by the repression of the proto-oncogene c-Myc in shPHD2 tumors. TGF\(\beta\) itself can only induce cell-cycle arrest at G1 (26) by activating various antiproliferative responses, such as the transcriptional upregulation of CDK inhibitors, including p15\(^{INK4b}\), p21\(^{CIP1/WAF1}\), and p27\(^{KIP1}\), which specifically requires the downregulation of c-Myc (16, 27–29). In addition, our results provide evidence that p15\(^{INK4b}\) and p27\(^{KIP1}\) were induced in shPHD2 tumors.

The importance of TGF\(\beta\) as an antiproliferative marker in different shPHD2 tumors was shown by the significant induction of tumor growth during a 10-day anti-TGF\(\beta\) treatment.
Figure 3. Loss of PHD2 leads to induction of vessel density. A, PECAM staining of tumor sections expressing shPHD2 (#1 and #2) compared with control tumors (shScr). Vessels were counted in different areas of individual tumor (n = 5). *, P < 0.05; ***, P < 0.001. Error bars represent mean ± SEM. Scale bar represents 50 μm. B, PECAM staining (red; vessels) and Hoechst33342 (blue; in vivo perfusion). PECAM+/Hoechst+ staining, perfused vessels; PECAM+/Hoechst− staining, nonperfused vessels (n = 4). Scale bar represents 50 μm. C, 1 mL of peripheral blood was isolated from tumor-bearing mice (n = 5) and plated out in cell-culture dishes after RBC lysis. EGFP+ cells were counted for the different groups. *, P < 0.05; **, P < 0.01. D, TUNEL staining on tumor sections. Photographs represent typical views. Scale bar represents 100 μm. E, necrotic cells per viable area was calculated using ImageJ software.
Figure 4. PHD2 silencing blocks tumor growth via the antiproliferative TGFβ pathway. A, Ki67 staining on tumor sections expressing shPHD2 (#1 and #2) compared with control tumors (shScr) representing cell proliferation (n ≥ 6). Bars represent means ± SEM. **, P < 0.01. Scale bar represents 50 μm. B to F, shPHD2 (#1 and #2) and control tumors were isolated 21 days after inoculation and quantitative PCR (qPCR) was carried out (n = 4). Average of all shScr samples is set as 1 ± SEM. *, P < 0.05; **, P < 0.01; and ***, P < 0.001. G, immunoblot on tumor lysates. All samples represent randomly chosen samples. Ratios are calculated as the band intensity of pSmad2/3 versus β-actin (n = 4). *, P < 0.05; **, P < 0.01. Average of shPHD2 samples is set as 1 ± SEM. H, qPCR on shPHD2 and shScr samples (n = 4). *, P < 0.05. I, a representative immunoblot on shPHD2#2 and control tumors for c-Myc and β-actin (n = 4). Graph represents quantification of relative amount of c-Myc in the different groups. *, P < 0.05; **, P < 0.01; n = 3, for shPHD2#1 and n = 4 for others. Average of shPHD2 samples is set as 1 ± SEM. J to L, qPCR on shPHD2 and control tumors samples (n ≥ 4). Average of all shScr samples is set as 1 ± SEM. **, P < 0.01; N.S., not significant.
Figure 5. In vivo anti-TGFβ treatment induces growth in shPHD2 tumors. A and B, LM8 tumors (shPHD2#1 or #2) treated with a pan-TGFβ antibody for 10 consecutive days. Controls received an equal amount of isotype IgG antibody. n = 10; *, P < 0.05; **, P < 0.01. C, Ki67 staining on LM8 osteosarcoma sections from shPHD2 tumors (n ≥ 6) treated with or without the pan-TGFβ antibody. Mean of individual tumors ± SEM. *, P < 0.05. Scale bar represents 50 μm. D, pSmad2 immunostaining on tumor lysates of individual shPHD2#2 tumors with or without anti-TGFβ antibody treatment. *, P < 0.05. Ratios for pSmad2 versus β-actin. E and F, qPCR analysis on shPHD2#2 tumor samples treated with or without anti-TGFβ for c-Myc and p15LNK4B. *, P < 0.05. Average of all untreated shPHD2#2 samples is set as 1 ± SEM. G, shScr control cells treated either with a pan-TGFβ antibody or an IgG isotype control as described (n = 10; *, P < 0.05). H and I, LLC cells (shPHD2#2 or shScr) were treated as in A, B, and G. n = 10; *, P < 0.05.
This was accompanied by an upregulation of c-Myc and reduction of pSmad2 and p15\(^{ink4b}\). Conversely, the antibody treatment of control tumors resulted in significantly smaller tumors, suggesting that the knockdown of PHD2 switched the nature of TGF\(\beta\) from a pro-oncogenic to an antiproliferative factor. This dual role of TGF\(\beta\) has been the subject of several previous studies. Indeed, in normal cells and even during the early stages of tumor development, TGF\(\beta\) has been shown to act as a potent inhibitor of proliferation. However, during tumor progression, and for rather unclear reasons, TGF\(\beta\) can turn into an oncogenic factor, inducing cell proliferation and invasion (24, 30–32). Nevertheless, our results imply that it is feasible to switch the nature of TGF\(\beta\) in aggressive and highly metastasizing cancer cells by knocking down the expression of PHD2, leading to slower growing as well as regressing tumors (Fig. 6). The importance of this finding is underscored by the fact that the anti-TGF\(\beta\) treatment has a similar effect in the more aggressive LLC tumors. However, the LLChshPHD2 tumors do not express more TGF\(\beta\) ligands/receptors or pSmad2/3 than their controls, although they display TGF\(\beta\)-related reduction of c-Myc and higher CDK inhibitor levels (p15\(^{ink4b}\) and p27\(^{kip1}\)). This implies that there are other cofactors, we have not revealed, that directly or indirectly regulate the antiproliferative TGF\(\beta\) pathway after inhibition of PHD2. Indeed, it is known that transcription factors E2F/4/5 and DP1, together with corepressor p107, act as Smad2/3/4 cofactors, and thus link the TGF\(\beta\) pathway to c-Myc repression (33). On the other hand, p15 repression can be directly regulated by c-Myc, similar to other factors such as Miz1 and KLF5. Induction of its expression can then, again, be supported by p300, acetylated KLF5, and FOX-O in combination with the R-Smads (34). The difference between both cell lines might, therefore, lie in the expression or activity of one or more of these cofactors or in the difference in one of the TGF\(\beta\) members.

Moreover, our in vivo experiments suggest that the effect of inhibiting PHD2 in LM8 tumor cells is largely HIF-independent, an effect that has been previously described (20, 35). For example, inhibition of PHD2 in human tumor cells was suggested to induce the NFXB pathway and lead to the overexpression of IL-8 (interleukin 8) and angiogenin, resulting in an enhanced influx of bone marrow–derived cells (BMDC) and angiogenesis (20). We did not find any evidence of a differential influx of BMDCs in our tumors (S.M., B.W., unpublished results), nor did PHD2 silencing induce the NFXB pathway. On the contrary, we detected significantly less NFXB activity in LM8shPHD2 cells and reduced amounts of the NFXB activators, IKK\(\alpha\), and/or \(\beta\) (36), suggesting that, in our study, regulation of NFXB via PHD2 modulation does not occur through the classical pathway (15). Whether these findings are directly associated to the PHD2-induced antiproliferative TGF\(\beta\) pathway still needs to be revealed.

Although it has been shown that TGF\(\beta\) can play a critical role in regulating both the activation and resolution phases of angiogenesis (37), we found that endothelial cell proliferation in shPHD2 tumors was still partially inhibited by TGF\(\beta\). Indeed, anti-TGF\(\beta\) treatment in shPHD2 tumors resulted in a significant induction of K\(i\)67 in tumor cells and an upregulation of vessel density. The factors responsible for the overall induced vascularization in our PHD2 knockdown tumors are unknown but not detrimental for the growth retardation in our study.

Silencing of PHD2 may induce tumor-suppressive effects as was implied by the analysis obtained via the Oncomine
Database and our own tumor studies (20, 38, 39). Indeed, although the discrepancy between our work and that of Chan and colleagues may be the difference in experimental setup (mouse tumor cell lines in immunocompetent mice vs. human xenografts in immunocompromised mice), it is more plausible to assume that the individuality of each tumor cell line accounts for the differences observed. Moreover, the role of PHD2 in supporting stromal cells must be considered, as it has been shown that PHD2 downregulation in endothelial cells normalizes the tumor vasculature, resulting in reduced malignancy and metastasis (14).

Finally, our findings may have interesting medical implications because the TGFβ pathway is currently being evaluated as a therapeutic target in an attempt to restore its normal function (40–42). By using specific PHD2 inhibitors, certain tumors may transform to a less malignant and metastasizing phenotype by the re-induction of the antiproliferative TGFβ pathway. However, more research is necessary in spontaneous developing tumors before any conclusions can be drawn regarding a useful therapy. At this stage, our results provide evidence for an HIF-independent role of PHD2 in the malignancy transformation of TGFβ, which will hopefully lead to a better understanding of the dual and complex role of this growth factor in oncogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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