

# Tumor Vasculature Is Regulated by PHD2-Mediated Angiogenesis and Bone Marrow-Derived Cell Recruitment

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## SUMMARY

Sustained angiogenesis, through either local sprouting (angiogenesis) or the recruitment of bone marrow-derived cells (BMDCs) (vasculogenesis), is essential to the development of a tumor. How BMDCs are recruited to the tumor and their contribution to the tumor vasculature is poorly understood. Here, we demonstrate that both IL-8 and angiogenin contribute to the complementary pathways of angiogenesis and BMDC mobilization to increase tumor growth. These two factors are regulated by PHD2 in a HIF-independent but NF- $\kappa$ B-dependent manner. PHD2 levels are decreased in human cancers, compared with corresponding normal tissue, and correlate with an increase in mature blood vessels. Thus, PHD2 plays a critical role in regulating tumor angiogenesis.

## INTRODUCTION

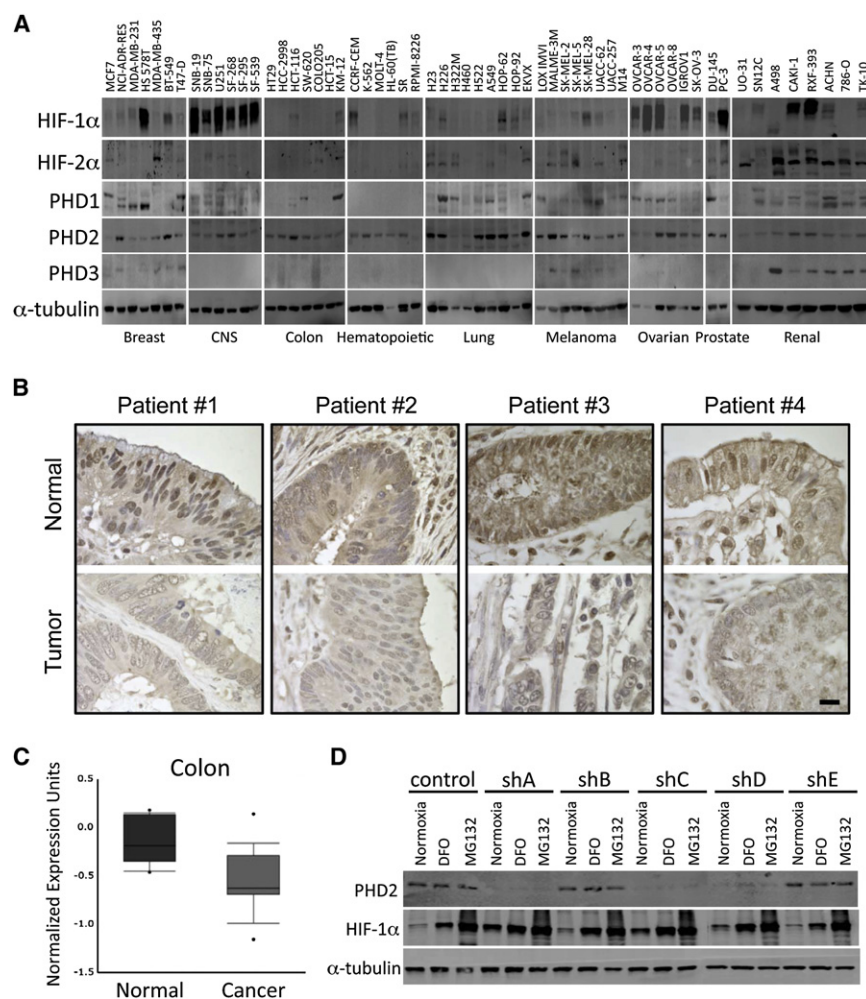
Tumor growth is highly dependent on its vascular supply. However, tumor growth often exceeds the ability of the existing vasculature to supply it with nutrients and oxygen (Hanahan and Weinberg, 2000). Continued tumor growth therefore requires new blood vessels. This induction of angiogenesis by solid tumors encompasses two complementary processes: sprouting angiogenesis and neovascularization (Carmeliet, 2000). Sprouting angiogenesis is a local response, involving existing blood vessels. Neovascularization, or vasculogenesis, is the de novo formation of new blood vessels through the mobilization and recruitment of bone marrow-derived cells (BMDCs).

This angiogenic switch is mediated in part by the hypoxia-inducible factor (HIF) family of transcription factors, which are key regulators of oxygen homeostasis and are composed of an oxygen-labile  $\alpha$  subunit and a constitutive beta subunit. HIF posi-

tively contributes to the growth of solid tumors (Kondo et al., 2002; Ryan et al., 1998; Ryan et al., 2000), and its overexpression in a wide variety of tumor types correlates with poor prognosis, resistance to radiotherapy and chemotherapy, and increased patient mortality (Harris, 2002; Semenza, 2002; Unruh et al., 2003; Zhong et al., 1999). Given the importance of HIF in multiple facets of tumor progression, mutations that enhance HIF activity might be expected to be involved in a variety of human cancers. So far, no genetic mutations in any of the HIF subunits affecting the stability or activity of these proteins have been identified in human tumors. However, elevated HIF levels in cancers can result from mutations of key regulators of HIF stability. Mutation of the von Hippel-Lindau gene (*VHL*), an upstream negative regulator of HIF, accounts for a subset of tumors with elevated HIF levels (Ohh et al., 2000). *VHL* is a tumor suppressor gene, and *VHL* disease is characterized by a distinct subset of highly vascular tumor (Kaelin, 2007).

## SIGNIFICANCE

Virtually all solid tumors require a vascular supply to provide nutrients and oxygen and to eliminate wastes. A tumor can exploit the existing vessels of the invaded tissue and can mobilize bone marrow-derived cells to induce neovascularization. Although PHD2 was originally identified as a negative regulator of the protein stability of the hypoxia-inducible factor (HIF)- $\alpha$  transcription factors, we demonstrate that PHD2 has additional HIF-independent functions. PHD2 levels are altered in a subset of human tumors, suggesting that it has a role in tumor progression. A key role of PHD2 in mediating the angiogenic switch has been identified, regulating both angiogenesis and the recruitment of vascular modulatory cells. These studies provide evidence for an important role of PHD2 in controlling tumor growth.



### Figure 1. PHD2 Levels Decrease in Human Cancers

(A) Variation of PHD2 levels in the NCI-60 panel of cell lines immunoblotted for PHD1, PHD2, PHD3, HIF-1 $\alpha$ , HIF-2 $\alpha$ , and  $\alpha$ -tubulin.

(B) Immunohistochemical staining for PHD2 in colon adenocarcinoma (bottom panels) compared to corresponding normal adjacent colon tissue (top panels) from several different patients. Scale bar, 500  $\mu$ m.

(C) Oncomine database analysis to examine PHD2 mRNA expression levels in adjacent, nontumoral colon versus colon carcinoma ( $p < 0.0005$ ).

(D) HCT116 cells with shRNA designed to specifically target PHD2. Cells were treated with DFO (100  $\mu$ M; 4 hr) or proteasome inhibitor (MG132: 10  $\mu$ M; 4 hr) and harvested to examine protein levels of PHD2, HIF-1 $\alpha$ , and  $\alpha$ -tubulin.

## RESULTS

### PHD2 Levels Decrease in Human Cancers

The important role of PHD2 in regulating HIF led us to investigate how normoxic PHD2, HIF-1, and HIF-2 protein levels relate to one another. The abundance of PHD proteins directly correlates with the amount of PHD activity (Appelhoff et al., 2004). Western blot analysis of the NCI60 panel, a set of 60 cell lines derived from nine distinct tissues, revealed that PHD2 levels are highly variable and do not necessarily inversely correlate with HIF levels as expected (Figure 1A). Nor did HIF levels always correlate with PHD1

or PHD3 levels. Tumors of the same tissue of origin also display disparate levels of PHD2. For example, the MCF7 breast cancer cell line has very little PHD2, compared with the BT-549 line. In some cases, a reduced level of PHD2 correlates with an increase in HIF-1 $\alpha$  levels (e.g., HS 578T) or HIF-2 $\alpha$  (e.g., SK-MEL-5). HIF levels are also sometimes elevated despite the presence of PHD2, as seen in 786-O cells, in which the loss of VHL results in HIF stabilization.

The reduction of PHD2 in a number of cancer cell lines suggests the possibility that PHD2 loss contributes to tumorigenesis and represents a pathway for HIF activation in a subset of tumors. Our analysis of published human cancer gene expression data sets revealed that PHD2 expression is significantly decreased in tumors, compared with normal tissue (Figure S2A) (Ramawamy et al., 2001). In particular, PHD2 mRNA is specifically lower in colon carcinoma compared to normal colon tissue (Figure 1C) (Graudens et al., 2006). To further investigate the role of PHD2 in human tumors, we stained colorectal tumors and matched, noninvolved adjacent colon tissue (Figure 1B). Four of the ten patient samples had decreased PHD2 in colon tumors relative to normal tissue. Together these data suggest that disruption of PHD2 could contribute substantially to tumorigenesis in human cancers.

PHD2 is one of three prolyl hydroxylases originally identified as negative regulators of HIF. Proline hydroxylation serves to regulate the stability of the  $\alpha$  subunits of HIF, a critical step in the cellular response to low oxygen, but additional roles for these genes remain elusive (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). PHD2 (EGLN1, HPH-2) has been proposed to be the key oxygen sensor because transiently silencing PHD2 results in normoxic stabilization of HIF-1 $\alpha$  (Berra et al., 2003). In contrast, silencing PHD1 or PHD3 has little effect on HIF levels. Furthermore, *PHD2* is an essential gene because *Phd2* knockout mice die between embryonic day 12.5 and 14.5, whereas *Phd1* and *Phd3* knockout mice appear normal (Takeda et al., 2006). Conditional inactivation of *Phd2* increased serum erythropoietin levels and, consequently, red blood cell production, leading to polycythemia and congestive heart failure (Minamishima et al., 2008). Specific knockout of *PHD2* in endothelial cells affects cell proliferation, whereas heterozygosity of *PHD2* in normal mouse stroma plays a role in vessel maturation (Mazzone et al., 2009; Takeda and Fong, 2007). Overall, these results suggest a key physiological role for PHD2. We hypothesized that deregulation of PHD2 might contribute to a number of other tumor types that have high levels of HIF. In this study, we examined the role of PHD2 in regulating tumorigenesis (see Figure S1 available with this article online).

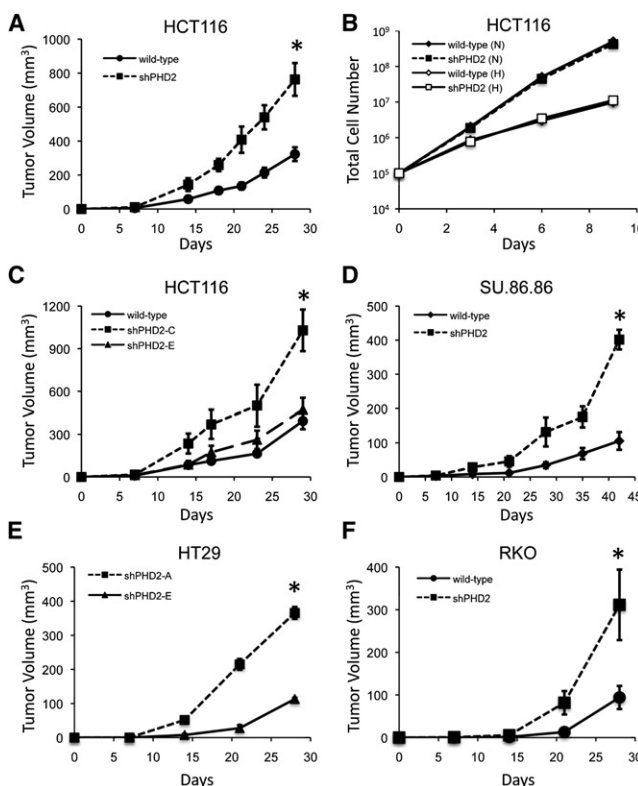
### Loss of PHD2 Increases Tumor Growth

To directly compare the growth of tumors with and without PHD2, we designed short hairpin RNA (shRNA) constructs complementary to different regions of PHD2 to target PHD2 mRNA for degradation. Five constructs targeting five unique sequences of PHD2 were stably expressed in HCT116 cells, a colon carcinoma cell line that had moderate levels of PHD2 and an intact HIF response (Figures 1A and 1D). Constructs A and C, which were used in subsequent experiments, completely abrogated PHD2 protein and confirmed previous findings that PHD2 silencing is sufficient to stabilize HIF-1 $\alpha$  protein in normoxic conditions (Berra et al., 2003). Knockdown of PHD2 was nearly as effective as inhibiting the prolyl hydroxylases with desferrioxamine (DFO) but not as strong as the proteasome inhibitor MG132 (Figure 1D). Construct E, which did not alter PHD2 or HIF-1 $\alpha$  protein levels, served as an shRNA control. In HIF reporter assays, PHD2 knockdown resulted in a 7-fold increase in HIF activity under normoxic conditions, which was further augmented by hypoxia, likely due to inhibition of PHD1, PHD3, or factor inhibiting HIF (FIH) (Figure S2B) (Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001). Western blots demonstrated that the shRNA knockdown of PHD2 is specific and has no effect on the other HIF hydroxylases (Figure S2C). These data demonstrate an effective and specific disruption of PHD2 and subsequent stabilization of HIF-1 $\alpha$ .

We next assessed the consequences of PHD2 disruption on tumor growth. Implanting PHD2 knockdown cells (construct A) into the flanks of SCID mice revealed that PHD2 loss confers a significant growth advantage over control tumors (Figure 2A). The effect on in vivo tumor growth is not due to intrinsic differences in proliferation rates because PHD2 knockdown and control cells had identical in vitro growth rates under normoxic and hypoxic conditions (Figure 2B). To further confirm the specificity of the PHD2 shRNA, cells expressing a control shRNA that does not affect PHD2 expression (construct E) or a second shRNA targeting a different region of PHD2 (construct C) were also implanted into SCID mice (Figure 2C). Tumors expressing the ineffective shRNA construct E exhibit wild-type tumor growth rates, whereas tumors expressing the effective construct C have a significant growth advantage. The efficiency of PHD2 knockdown is therefore directly correlated to the rate of tumor growth, demonstrating that the effects are specific to PHD2 silencing. The effect of shRNA on tumor growth is not unique to HCT116 cells because stable knockdown of PHD2 in a pancreatic carcinoma cell line (SU.86.86) and in two additional colorectal carcinoma cell lines (HT29 and RKO) also amplified tumor growth (Figures 2D, 2E, and 2F; Figures S2D and S4A). Thus, loss of PHD2 increases tumor growth, compared with control cells with wild-type PHD2, indicating a role of PHD2 in tumorigenesis.

### HIF-Independent Tumor Suppressor Function of PHD2

As HIF-1 $\alpha$  is the only functionally characterized target of PHD2 to date, we next determined whether the effects of PHD2 on tumorigenesis depended on HIF by employing HIF-deficient cells. Stable expression of an shRNA to HIF-1 $\alpha$  in HCT116 cells prevented HIF-1 $\alpha$  protein accumulation in response to either hypoxia or DFO (Figure S3A). Simultaneous knockdown of PHD2 and HIF-1 $\alpha$  enabled us to examine HIF-dependent and HIF-independent effects by PHD2 disruption. Silencing HIF-1 $\alpha$  in HCT116



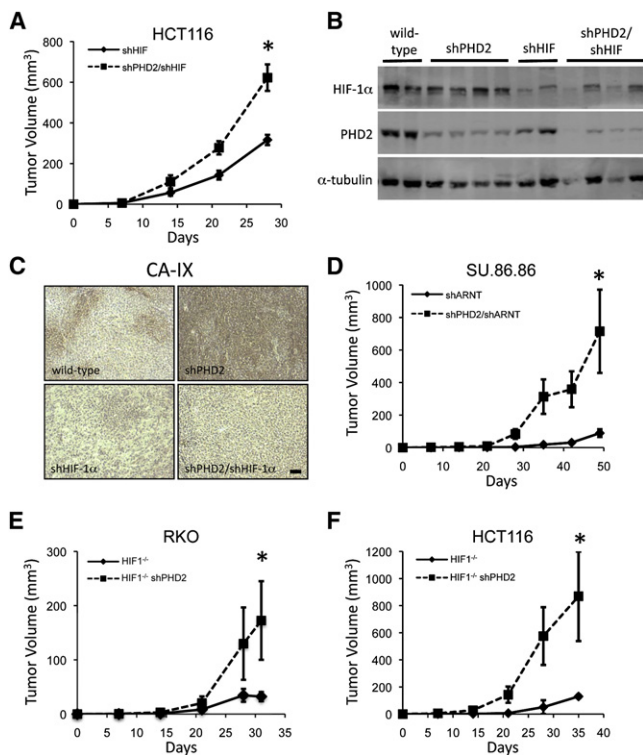
**Figure 2. Loss of PHD2 Increases Tumor Growth**

(A) In vivo growth of wild-type or PHD2 knockdown (Construct A) HCT116 xenograft tumors (\* $p < 0.00001$ ). (B) In vitro growth curve of HCT116 cells with or without stable knockdown to PHD2 under either normoxic (N, 21% O<sub>2</sub>) or hypoxic (H, 2% O<sub>2</sub>) conditions. (C) Growth of wild-type or PHD2 knockdown (construct C or E) HCT116 tumors in mice (\* $p < 0.001$ ). (D) Growth of wild-type SU.86.86 or cells expressing PHD2 shRNA in SCID mice (\* $p < 0.000005$ ). (E) In vivo growth of HT29 cells expressing shRNA to PHD2 (Construct A) or control (construct E) (\* $p < 0.000005$ ). (F) Growth of wild-type RKO cells or cells expressing shRNA to PHD2 in SCID mice (\* $p < 0.05$ ). All error bars represent ± SEM.

cells is sufficient to eliminate all HIF activity in HIF-reporter assays (Figure S3B). Since the HIF reporter used does not distinguish between HIF-1 and HIF-2, these data indicate that HIF-2 $\alpha$  is nonfunctional in these cells. Western blot analysis further demonstrated a lack of HIF-2 $\alpha$  protein induction under hypoxia in these cells (Figure S3C). In vitro growth under normal oxygenated conditions or hypoxia was unaffected by the different shRNA constructs (Figure S3D). These data demonstrate that HIF knockdown is effective in these cell lines, enabling us to examine whether the effects of PHD2 disruption on tumor growth are reliant on HIF.

In contrast to the expectation that PHD2 would affect tumor growth through its regulation of HIF, we found that the improved tumor growth did not depend on the presence of HIF. Cells with both PHD2 and HIF-1 $\alpha$  disrupted exhibited increased tumor growth, compared with cells that only had HIF-1 $\alpha$  silenced (Figure 3A). These results indicate that PHD2 has HIF-independent functions that contribute to tumor growth. Western blot





**Figure 3. HIF-Independent Tumor Suppressor Function of PHD2**

(A) Tumor growth of HCT116 cells expressing shRNA to *HIF-1 $\alpha$*  alone or in combination with shRNA to PHD2 (\**p* < 0.05). (B) PHD2 and *HIF-1 $\alpha$*  silencing is maintained in vivo. Protein extracts from tumors in Figure 3A were immunoblotted to examine levels of *HIF-1 $\alpha$* , PHD2, and  $\alpha$ -tubulin. (C) Tumor sections of the given genotype were stained for CA-IX expression. Scale bar, 200  $\mu$ m. (D) Tumor growth of SU.86.86 cells with shRNA to PHD2 or shRNA to PHD2 and *HIF-1 $\beta$*  (\**p* < 0.05). (E) In vivo growth of RKO cells deficient in *HIF-1 $\alpha$*  or *HIF-1 $\alpha$* -deficient cells expressing PHD2 shRNA (\**p* < 0.05). (F) In vivo growth of *HIF-1 $\alpha$* -deficient HCT116 cells or those expressing PHD2 shRNA (*p* < 0.05). All error bars represent  $\pm$  SEM.

analysis demonstrated that PHD2 and *HIF-1 $\alpha$*  knockdown was maintained in the corresponding tumors (Figure 3B). Moreover, PHD2 levels in vivo were uniformly high in control tumors but dramatically reduced in the tumors expressing shRNA to PHD2, as determined by immunostaining tumor sections (Figure S3E). Staining of PHD2-deficient tumor sections revealed high levels of CAIX, a specific HIF target, indicating that shRNA to PHD2 was sufficient to result in functional HIF in vivo, whereas wild-type tumors displayed a classic hypoxic staining pattern of CAIX (Figure 3C). Expression of *HIF-1 $\alpha$*  shRNA in PHD2 knockdown tumors completely abrogated CAIX staining, demonstrating that *HIF-1 $\alpha$*  silencing was maintained in vivo. It has been suggested that PHD2 itself is a HIF target (D'Angelo et al., 2003). However, PHD2 staining in wild-type tumors was homogeneous and did not exhibit a hypoxic pattern, revealing that PHD2 protein is unaffected by hypoxia in this cell type. Thus, while PHD2 knockdown results in up-regulation of HIF activity, this activity is not necessary for the effects of PHD2 on tumorigenesis.

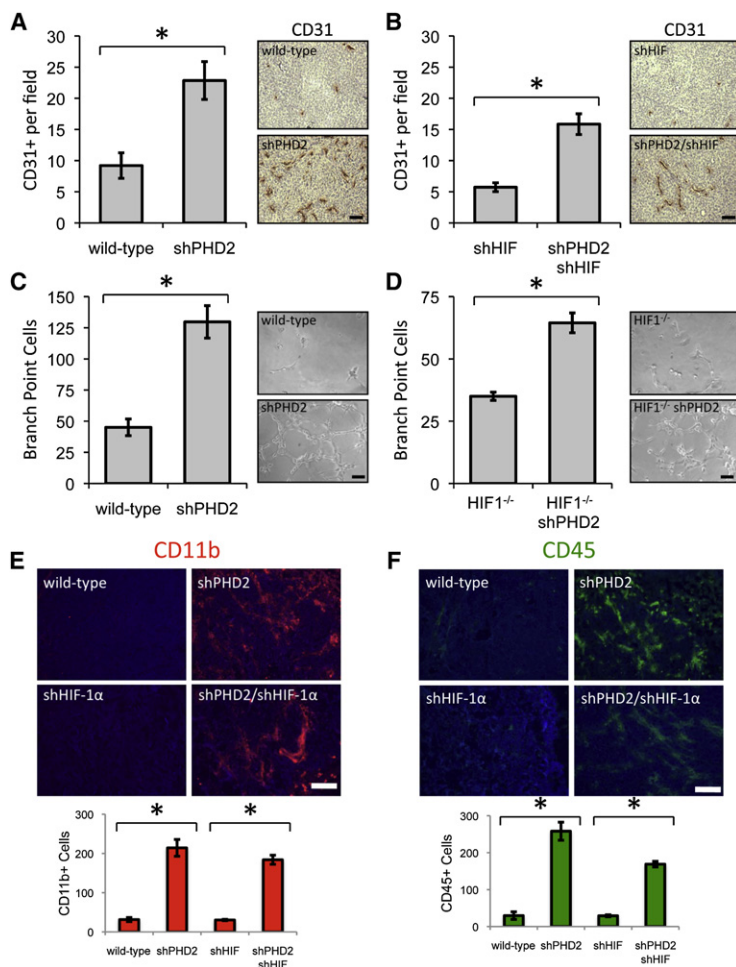
To further evaluate whether HIF is necessary for the enhanced tumor growth seen in PHD2-silenced cells, we next examined the dependence on HIF in three additional models—SU.86.86 cells, and RKO and HCT116 cells in which the *HIF-1 $\alpha$*  gene has been deleted by homologous recombination (Figure S4A) (Dang et al., 2006). As SU.86.86 cells possess both *HIF-1 $\alpha$*  and *HIF-2 $\alpha$* , we inhibited both HIF family members through expression of an shRNA to ARNT (*HIF-1 $\beta$* ), the constitutive binding partner of *HIF- $\alpha$*  (Figure S4B). Simultaneous knockdown of ARNT and PHD2 increases tumor growth over tumors expressing ARNT shRNA alone (Figure 3D). Silencing PHD2 in both *HIF-1 $\alpha$*  null HCT116 and RKO cell lines also increases tumor growth (Figures 3E and 3F; Figures S2D and S4A). Taken together, these data indicate that PHD2 functions as a tumor suppressor and that this activity does not require HIF in multiple settings.

### PHD2 Regulates Angiogenesis and Recruitment of BMDCs

To determine how PHD2 loss increases tumor growth, we performed immunohistochemical analyses on tumor sections. Examining sections from HCT116 and SU.86.86 tumors for the endothelial cell marker CD31 staining revealed that tumors expressing PHD2 shRNA have a significant increase in blood vessels (Figures 4A; Figure S4C). Furthermore, in cells expressing shRNA to *HIF-1 $\alpha$*  or ARNT, PHD2 silencing also increased blood vessels, indicating that PHD2 can affect angiogenesis, even in the absence of *HIF-1 $\alpha$*  (Figure 4B; Figure S4D). The increase in tumor volume could be attributed to an increase in proliferation of PHD2 knockdown tumors as determined by Ki-67 staining (Figures S5A and S5B). TUNEL staining of these tumors, however, is unaffected by PHD2 loss (Figure S5C). Together these data indicate that PHD2 normally functions to restrict the tumor vasculature, suggesting a mechanism by which the loss of PHD2 enhances tumor growth.

There are two main contributors to the tumor vasculature—angiogenesis and vasculogenesis. To further delineate the effect of PHD2 loss on the tumor vasculature, we employed a short-term in vitro assay to measure angiogenesis. This assay assesses the propensity of primary endothelial cells to sprout, forming three-dimensional structures resembling capillaries, in response to appropriate cues. Conditioned media from HCT116 cells with silenced PHD2 under normoxic conditions significantly increases the number of cells in capillary branch points, a surrogate marker for elevated angiogenesis (Figure 4C). Similarly, conditioned medium from cells expressing PHD2 knockdown construct C also increases tube formation, whereas conditioned medium from cells expressing control construct E is unable to increase in vitro angiogenesis (Figures S5D and S5E). Conditioned medium from *HIF-1 $\alpha$* -deficient HCT116 cells with silenced PHD2 increases tube formation, compared with that from cells with *HIF-1 $\alpha$* -deleted alone (Figure 4D). Silencing ARNT by siRNA in HT29 cells with PHD2 shRNA also demonstrated a HIF-independent effect of PHD2 on angiogenesis (Figures S5F and S5G). These results indicate that PHD2, regardless of the presence or absence of HIF, influences angiogenesis in multiple cancer cell types.

Vasculogenesis requires the de novo production of endothelial cells and is dependent on BMDCs, in part to provide the appropriate cues to recruit progenitor cells and other vasculatory



**Figure 4. PHD2 Regulates Angiogenesis and Recruitment of Bone Marrow-Derived Cells**

(A) CD31 staining in sections from HCT116 tumors expressing PHD2 shRNA compared to wild-type tumors (\* $p < 0.01$ ). Scale bar, 500  $\mu$ m. (B) CD31 staining in sections from HCT116 tumors expressing shRNA to both PHD2 and HIF-1 $\alpha$  compared to those expressing HIF-1 $\alpha$  shRNA alone (\* $p < 0.0005$ ). Scale bar, 500  $\mu$ m. (C) In vitro tube formation of endothelial cells in conditioned media from wild-type HCT116 cells or cells expressing PHD2 (\* $p < 0.00005$ ). Scale bar, 1 mm. (D) In vitro tube formation of endothelial cells in conditioned media from HIF-1 $\alpha$ -deficient HCT116 or HIF-1 $\alpha$ -deficient HCT116 cells expressing PHD2 shRNA (\* $p < 0.00005$ ). Scale bar, 1 mm. (E) Staining of HCT116 tumors for bone marrow-derived myelomonocytic cells with CD11b (red) and nuclei with DAPI (blue) (\* $p < 0.005$ ). Scale bar, 100  $\mu$ m. (F) HCT116 tumor staining for CD45 (green) and nuclei with DAPI (blue) (\* $p < 0.005$ ). Scale bar, 100  $\mu$ m. All error bars represent  $\pm$  SEM.

(Figures 5B and 5D). This result raises the possibility that, unlike PHD2 regulation of HIF, the HIF-independent control of angiogenesis does not depend on the hydroxylase activity PHD2.

To assess this possibility pharmacologically, HIF-1 $\alpha$ -deficient cells were treated with dimethylxaloylglycine (DMOG), a pan-hydroxylase inhibitor. Conditioned media from DMOG-treated HCT116 or RKO cells fails to increase angiogenesis (Figures 5C and 5D). Furthermore, DMOG-treated HIF-1 $\alpha$ -deficient cells with shRNA targeting PHD2 do not exhibit a further increase in tube formation (Figures 5C and 5D). These data also suggest that the hydroxylase activity is not mediating this function of PHD2.

On the basis of these findings, we next examined whether PHD2 lacking hydroxylase activity was able to rescue the tube formation capability of PHD2-silenced cells. We generated shRNA-resistant PHD2 constructs by introducing silent mutations in the region targeted by the shRNA. Transfection of this shRNA-resistant construct allowed overexpression of wild-type PHD2, which rescued the wild-type levels of angiogenesis, further confirming specificity that the shRNA is targeting PHD2. Importantly, an shRNA-resistant PHD2 construct with mutations at two critical residues, HD313 and D315, in the hydroxylase catalytic site (McDonough et al., 2006) also rescued wild-type levels of angiogenesis (Figures 5E and 5F). These data, along with the pharmacological and hypoxia findings, indicate that the hydroxylase function of PHD2 is not necessary for its angiogenic suppressor activity.

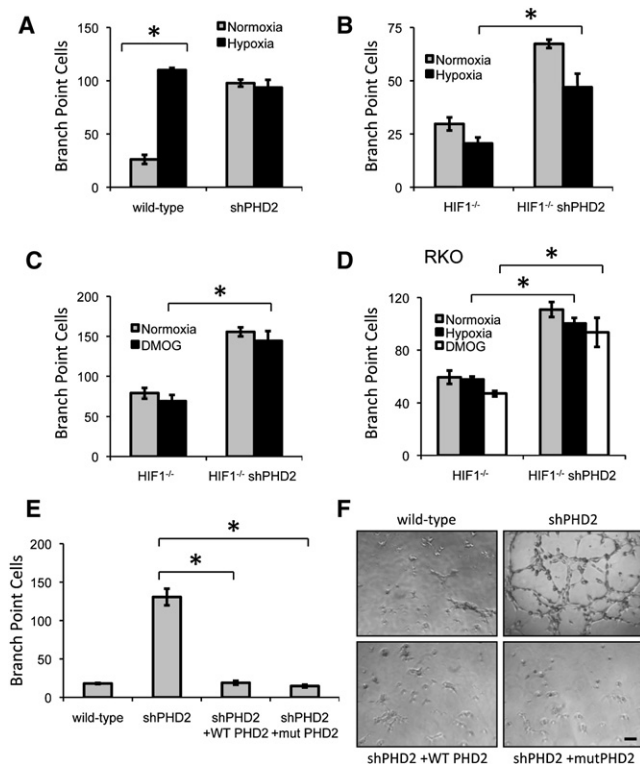
modulatory cells (De Palma et al., 2003; Shojaei et al., 2007; Yang et al., 2004). Thus, to determine whether recruitment of BMDCs also contributes to the augmented tumor growth, we stained tumors for the myeloid-monocyte lineage marker CD11b and the leukocyte lineage marker CD45. HCT116 tumors with reduced PHD2 display a 6–7-fold increase in infiltrating BMDCs, regardless of the presence or absence of HIF, compared with control tumors (Figures 4E and 4F). Both CD11b and CD45 are increased in tumors lacking PHD2, compared with control tumors, suggesting that PHD2 regulates the recruitment of BMDCs, which contribute to vasculogenesis.

#### Hydroxylase Function of PHD2 Is Not Necessary for Regulating Angiogenesis

We next investigated the role of hypoxia in the regulation of angiogenesis by PHD2. As expected, conditioned medium from hypoxia-treated HCT116 cells induces tube formation, demonstrating that hypoxia can increase angiogenesis through HIF-dependent mechanisms (Figure 5A). Hypoxic treatment of cells expressing PHD2 shRNA does not further increase tube formation (Figure 5A). Interestingly, although knockdown of PHD2 enhances tube formation induced by conditioned medium from HIF-1 $\alpha$ -deficient HCT116 or RKO cells, this effect is not mimicked by hypoxia, a treatment that reduces PHD2 hydroxylase activity

#### NF- $\kappa$ B Regulates PHD2-Mediated Angiogenesis

Having identified a role of PHD2 in regulating tumor vasculature independent of its role in regulating HIF, we investigated whether another transcription factor also regulated by PHD2. Cummins et al. (2006) demonstrated that transiently silencing PHD1 or PHD2 increased NF- $\kappa$ B activity. In agreement, we also find that shRNA to PHD2 is sufficient to increase NF- $\kappa$ B reporter expression (Figure 6A). Prolyl hydroxylase inhibition or hypoxia treatment of cells with an intact HIF pathway is able to increase



**Figure 5. Hydroxylase Function of PHD2 Is Not Necessary for Regulating Angiogenesis**

(A) The ability of conditioned media from wild-type or PHD2 shRNA expressing HCT116 cells with or without hypoxia condition (2% O<sub>2</sub>, 16 hr) in inducing in vitro tube formation (\*p < 0.001).

(B) The ability of conditioned media from HIF1<sup>-/-</sup> HCT116 cells or HIF1<sup>-/-</sup> HCT116 cells expressing PHD2 shRNA under normoxia or hypoxia (2% O<sub>2</sub>, 16 hr) in inducing in vitro tube formation (\*p < 0.01).

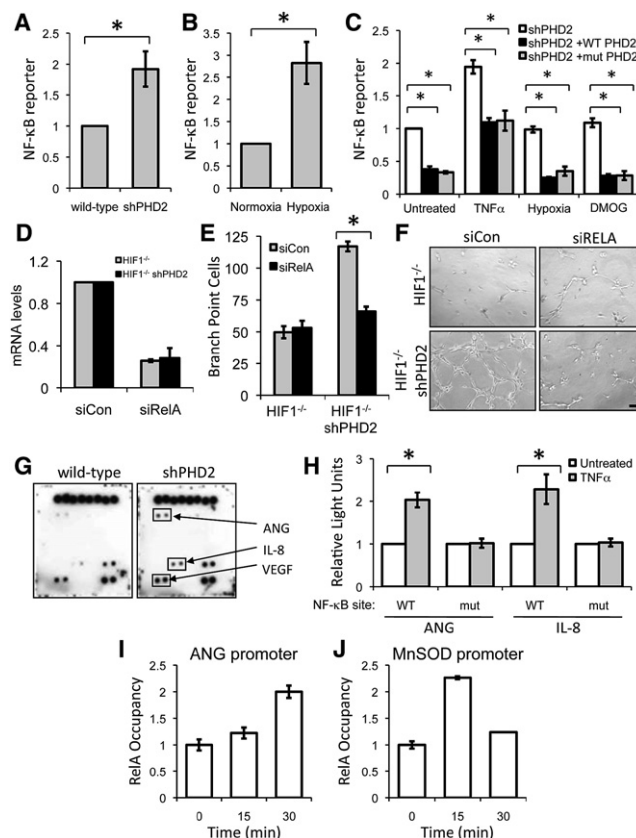
(C) Conditioned media from HCT116 HIF1<sup>-/-</sup> cells or cells expressing shRNA to PHD2 treated with DMOG (1 mM, 16 hr) was subjected to in vitro tube formation (\*p < 0.001).

(D) In vitro tube formation of conditioned media from RKO HIF1<sup>-/-</sup> cells or cells expressing shRNA to PHD2 treated with hypoxia (2% O<sub>2</sub>) or DMOG (1 mM) for 16 hr (\*p < 0.01).

(E) HCT116 cells expressing PHD2 shRNA were transfected with either wild-type PHD2 or hydroxylase mutant PHD2 (H313A H315D). Conditioned media from these cells were collected and subjected to a tube formation assay (\*p < 0.001). All error bars represent ± SEM.

(F) Representative photographs of primary endothelial cells seeded onto Matrigel in conditioned media from HCT116 cells stably expressing shRNA to PHD2 and transfected with either wild-type PHD2 or a hydroxylase mutant PHD2. Scale bar, 2 mm.

NF-κB (Cummins et al., 2006) (Figure 6B). To more directly examine the requirement for the hydroxylase function of PHD2 in regulating NF-κB in an HIF-independent manner, we restored PHD2 levels in PHD2-silenced cells by transfecting either wild-type or hydroxylase mutant PHD2 into HIF-1α-deficient HCT116 cells. Both wild-type and mutant PHD2 decreased NF-κB activity and limited the induction of NF-κB (Figure 6C). Stimulation of NF-κB by TNF-α is similarly impaired when wild-type or hydroxylase mutant PHD2 is expressed (Figure 6C). Subjecting these HIF-deficient cells to hypoxia or pan-hydroxylase inhibition does not increase NF-κB activity, further confirming that, in addition



**Figure 6. NF-κB Regulates PHD2-Mediated Angiogenesis**

(A) NF-κB reporter assay of wild-type or HCT116 cells expressing PHD2 shRNA (\*p < 0.05).

(B) NF-κB is hypoxia-responsive. HCT116 cells were transfected with a NF-κB luciferase reporter and treated with hypoxia (2% O<sub>2</sub>, 16 hr) (\*p < 0.005).

(C) Wild-type or hydroxylase mutant PHD2 were overexpressed along with a NF-κB luciferase reporter construct in HCT116 HIF-1<sup>-/-</sup> cells stably expressing shRNA to PHD2. Following transfection, these cells were not treated or treated with TNF-α (20 ng/ml; 6 hr), hypoxia (2% O<sub>2</sub>; 16 hr), or DMOG (1 mM; 16 hr) (\*p < 0.05).

(D) Quantitative real-time PCR to demonstrate knockdown of RELA in HCT116 cells with HIF-1α knocked out.

(E) Conditioned media from HCT116 HIF-1<sup>-/-</sup> cells expressing PHD2 shRNA transfected with pool of nontargeting siRNA or siRNA targeting RELA were subjected to a tube formation assay (\*p < 0.0005).

(F) Primary endothelial cells were seeded onto Matrigel in conditioned media from HIF-1α knockout cells or those expressing shRNA to PHD2 and assayed for tube formation. Scale bar, 1 mm.

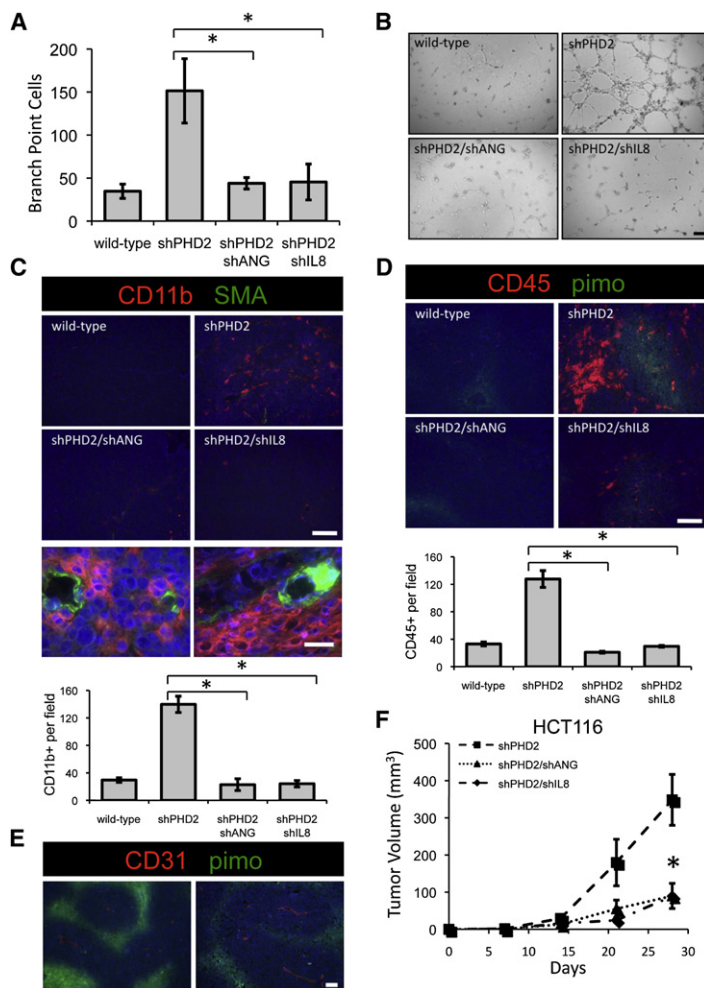
(G) Angiogenesis antibody arrays were carried out in duplicate with conditioned media from either wild-type HCT116 or cells stably expressing PHD2 shRNA.

(H) Reporter assays with ANG or IL-8 promoters along with the promoters with mutant NF-κB sites. Two days after transfection, cells were treated with TNF-α (20 ng/ml; 6–8 hr) and luciferase activity was determined (\*p < 0.005).

(I and J) RelA is recruited to the ANG promoter. HCT116 cells expressing shRNA to PHD2 were treated with TNF-α (20 ng/ml) for the given amount of time. ChIP with α-RELA antibodies was performed, and RELA occupancy is shown. MnSOD promoter is used as a positive control for recruitment of RELA to a NF-κB target promoter. IgG control ChIPs were also performed as a negative control. All error bars represent ± SEM.

tion to the previously described hypoxia-mediated regulation of NF-κB, PHD2 can also regulate NF-κB in a hypoxia-, hydroxylase-, and HIF-independent manner.





**Figure 7. PHD2 Regulation of ANG and IL-8 Mediates Tumor Vasculature**

(A) Conditioned media from HCT116 cells expressing PHD2 shRNA and shRNA targeting either ANG or IL-8 were subjected to a tube formation assay (\* $p < 0.05$ ).

(B) Tube formation with conditioned media from the given genotype. Scale bar, 2 mm.

(C) Tumor staining for CD11b myelomonocytic precursor cells (red) and pericytes with vascular smooth muscle actin (green). Scale bar, 100 microns. Bottom panels are higher magnification of PHD2-silenced tumors. Scale bar, 500 microns. Quantification of CD11b-positive cells (\* $p < 0.001$ ).

(D) Tumor staining for CD45 (red), hypoxia with pimonidazole (green), and nuclei with DAPI (blue). Scale bar, 100 microns. Quantification of CD45-positive cells (\* $p < 0.001$ ).

(E) Higher magnification images of wild-type HCT116 tumors stained for CD31 (red) and pimonidazole (green). Scale bar, 100 microns.

(F) Tumor growth of cells expressing the given shRNA in SCID mice (\* $p < 0.005$ ). All error bars represent  $\pm$  SEM.

To test whether NF- $\kappa$ B is necessary for enhanced angiogenesis, we transfected PHD2 silenced cells with siRNA to RELA, the p65 subunit of NF- $\kappa$ B, and examined the ability of conditioned media from these cells to stimulate tube formation in vitro (Figures S6A, S6B, and S6C). PHD2 silencing in *HIF-1 $\alpha$* -deficient cells is unable to enhance in vitro angiogenesis in the absence of RELA (Figures 6D, 6E, and 6F). In cells with *HIF-1 $\alpha$* , NF- $\kappa$ B is also necessary for this effect on angiogenesis (Figures S6B and S6C). Together these data support the conclusion that NF- $\kappa$ B mediates the HIF-independent tumor suppressor function of PHD2.

#### PHD2 Regulates Secreted Proangiogenic Factors

We sought to determine which secreted factors regulated by PHD2 could explain the effects on tumor vasculature. The secreted VEGF level is unaffected by the lack of PHD2 or the lack of both PHD2 and *HIF-1 $\alpha$*  and is induced by hypoxia normally (Figures S6D and S6E). This finding demonstrates that the effect of PHD2 on the tumor vasculature is distinct from both HIF and VEGF. We then probed antibody arrays with conditioned media from control HCT116 cells and cells expressing PHD2 shRNA (Figure S6F). In agreement with the ELISA results, levels of VEGF were high in the conditioned media regardless of genotype (Figure 6F). However, we determined that PHD2 consistently regulates two angiogenic factors, namely inter-

leukin-8 (IL-8) and angiogenin (ANG) (Figure 6E) (Fett et al., 1985; Koch et al., 1992). Because we were also searching for a cytokine regulated by PHD2 in an HIF-independent manner, we probed arrays with conditioned media from HCT116 *HIF-1* knockout cells and the corresponding cells with PHD2 knockdown (Figure S6G). Again, we identified that PHD2 knockdown increases secretion of IL-8 and ANG, regardless of HIF. Silencing of RELA in PHD2 shRNA cells reduced secreted ANG and IL-8 by about half in agreement with other reports that ANG and IL-8 can regulate angiogenesis and are regulated by NF- $\kappa$ B (Figure S6H) (Garkavtsev et al., 2004; Karashima et al., 2003; Wu et al., 1997).

To directly confirm the regulation of ANG and IL-8 by NF- $\kappa$ B, we performed reporter assays, using the respective promoters of ANG and IL-8 (Figure 6H). Stimulation of NF- $\kappa$ B with TNF- $\alpha$  increases activity of both ANG and IL-8 promoters, demonstrating that both are NF- $\kappa$ B responsive genes. Mutation of the NF- $\kappa$ B sites in their respective promoters eliminated NF- $\kappa$ B responsiveness. As IL-8 is already a known NF- $\kappa$ B target (Karashima et al., 2003), we sought to further corroborate NF- $\kappa$ B regulation of ANG. Chromatin immunoprecipitation with RELA antibodies demonstrates that RELA is recruited to the promoter of ANG (Figure 6I). The *MnSOD* promoter, another NF- $\kappa$ B target, was used as a positive control (Figure 6J). These data further validate ANG and IL-8 as NF- $\kappa$ B target genes.

#### PHD2 Regulation of ANG and IL-8 Mediates Tumor Vasculature

We next examined whether ANG and IL-8 mediated the angiogenic phenotype conferred by PHD2 loss by silencing ANG or IL-8 in HCT116 with PHD2 knockdown (Figures S7A, S7B, S7E, and S7F). Knockdown of either ANG or IL-8 decreased the ability of the PHD2 knockdown cells to induce capillary branching, indicating that PHD2-mediated angiogenesis is directed in part by ANG and IL-8 (Figures 7A and 7B; Figures S7C and S7D). Staining HCT116 tumors for CD11b and CD45 revealed that mobilization of BMDs by PHD2 silencing requires ANG and IL-8 (Figures 7C and 7D). CD11b-positive cells were

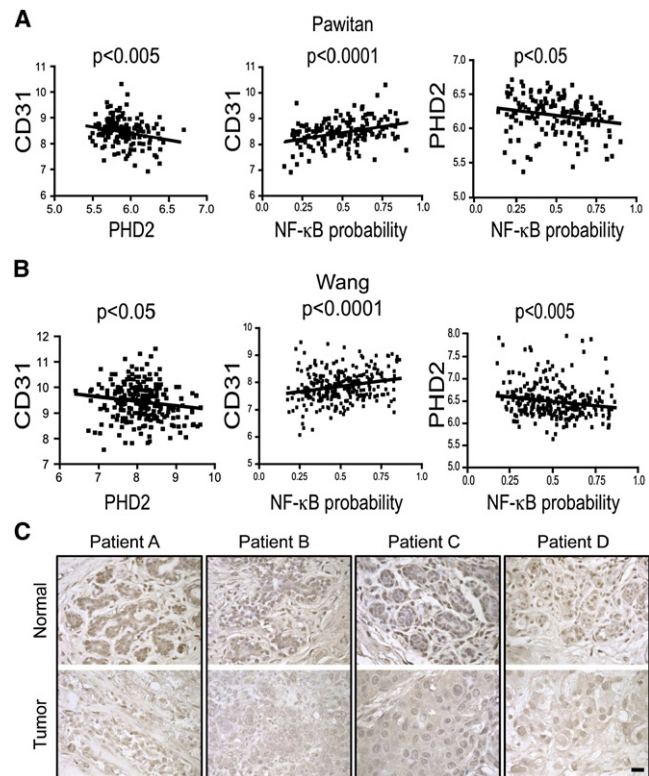
associated with mature blood vessels as determined by staining pericytes with smooth muscle actin (Figure 7C, lower panel). The functional maturity was further verified by staining sections with CD31 and the hypoxia marker pimonidazole, which showed that CD31-positive blood vessels are in nonhypoxic regions (Figure 7E). We found a distinct, nonoverlapping staining pattern between hypoxia and CD45, indicating that BMDCs are found in the better perfused areas of the tumor with access to the vasculature, further supporting the idea of a HIF/hypoxia-independent effect of PHD2 in recruiting BMDCs to a tumor (Figure 7D). To verify that this is a specific recruitment of BMDCs and not a general infiltration of macrophages, we stained these tumors for F4/80, a pan-macrophage marker, finding equivalent numbers of macrophages in all tumors, regardless of genotype and despite increased blood vessels in PHD2-silenced tumors (Figure S7G). Thus, in tumors that have reduced PHD2, mobilization of BMDCs may contribute to the vasculogenic tumor phenotype. To evaluate the effect of BMDC mobilization on tumor growth, we implanted ANG- and IL-8-deficient HCT116 cells in mice and found that disruption of ANG or IL-8 impaired the enhanced tumor growth induced by PHD2 loss (Figure 7F). These data indicate that PHD2 loss increases tumor growth through ANG- and IL8-mediated angiogenesis and recruitment of BMDCs.

#### Decreased PHD2 Expression Correlates with Increases in NF- $\kappa$ B Activity and Tumor Angiogenesis in Human Cancers

To further evaluate the relationship between PHD2 and the tumor vasculature in human cancers, we extracted data on PHD2 mRNA expression level from two published studies of breast cancer composed of different patient populations. One study examined 286 patients with lymph node-negative breast cancers who did not receive adjuvant therapy, whereas the other examined 159 population-derived breast cancer patients (Pawitan et al., 2005; Wang et al., 2005). Despite these differences in patients' characteristics, we noted a statistically significant negative association between the expression level of PHD2 and that of the endothelial cell marker gene CD31 in both data sets, further supporting the *in vivo* relevance of the ability of PHD2 to inhibit tumor vasculature in human tumors (Figures 8A and 8B left panels).

We next assessed the extent to which NF- $\kappa$ B activation is related to both PHD2 expression and markers of angiogenesis. We used binary logistic regression to estimate the probability of NF- $\kappa$ B activation as modeled on TNF- $\alpha$  stimulation training sets to generate NF- $\kappa$ B signatures (Bild et al., 2006; Mori et al., 2008). These signatures were used to approximate the likelihood of NF- $\kappa$ B activation in the two breast cancer data sets. We found a significant positive association between NF- $\kappa$ B activation and CD31 expression (Figures 8A and 8B, middle panels) as well as a significant negative correlation between NF- $\kappa$ B activation and PHD2 levels in both data sets (Figures 8A and 8B, right panels). These observations are consistent with the hypothesis that PHD2 represses the expression of CD31 through the inhibition of NF- $\kappa$ B.

We then examined PHD2 protein levels in clinical breast cancer samples by performing immunohistochemistry on breast tumors along with the corresponding normal tissue from the



**Figure 8. Decreased PHD2 Expression Correlates with Increases in NF- $\kappa$ B Activity and Tumor Angiogenesis in Human Cancers**

(A and B) Left panels: Significant negative correlation between the expression level of PHD2 (x axis) and CD31 (y axis) in the breast cancer samples profiled in two expression studies of breast cancers. Middle panels: Significant positive correlation between NF- $\kappa$ B pathway activity (x axis) and expression of CD31 (y axis). Right panels: Significant negative correlation between the expression level of PHD2 (x axis) and NF- $\kappa$ B activity (y axis).

(C) Immunohistochemical staining for PHD2 in invasive breast carcinomas (bottom panels) compared to matched, uninvolved, adjacent breast tissue (top panels) from several different patients. Scale bar, 100  $\mu$ m.

same patient (Figure 8C). Of the 11 patient samples, six had higher levels of PHD2 in the normal tissue than in the matched tumor tissue, indicating that the loss of PHD2 occurs in clinically relevant situations and implicating PHD2 in tumorigenesis. PHD2 regulates tumor vasculature through both angiogenesis and neovascularization. Taken together our data suggests that PHD2 is at the intersection of multiple, complementary pathways. PHD2 controls both HIF-dependent and NF- $\kappa$ B-dependent pathways mediating both local sprouting and neovascularization and, consequently, regulates tumor growth (Figure S1).

#### DISCUSSION

Induction of angiogenesis through local sprouting and neovascularization is essential to the rapid growth of solid tumors. The identity and regulation of the factors responsible for these processes are areas of active investigation as these signaling pathways could provide valuable avenues for therapeutic intervention. HIF and VEGF have garnered considerable attention for their roles in mediating tumor angiogenesis. Recently, interest



has grown in the regulators of HIF and in alternative mechanisms of pathological angiogenesis (Fischer et al., 2007; Shojaei et al., 2007). In this report, we demonstrate that PHD2 functions as a tumor suppressor by limiting ANG and IL-8, which play roles in both angiogenesis and BMDC recruitment contributing to vasculogenesis. Despite the role of PHD2 in regulating HIF stability, the tumor suppressor function of PHD2 observed here does not solely depend on the presence of HIF. Eliminating HIF did not reduce tumor growth in the PHD2-silenced background in multiple cancer types. PHD2 knockdown elevated normoxic NF- $\kappa$ B activity and, consequently, increased IL-8 and ANG, both of which contribute to enhance tumor blood supply and thus tumor growth. Moreover, analysis of clinical data reveals a reduction of PHD2 in human cancers, which is also correlated with an increase in tumor vasculature. Together, these findings highlight the importance of HIF- and VEGF-independent pathways in regulating tumor angiogenesis.

BMDCs are increasingly appreciated to be important contributors to the expansion of tumor vasculature (Gao et al., 2008; Lyden et al., 2001; Shojaei et al., 2007; Yang et al., 2004). However, the mechanisms by which tumors recruit these cells are not yet fully understood. A few factors have been implicated, such as the Id proteins, MMP-9, and Bv8, but the entire repertoire of signals responsible for the recruitment is largely unknown. Our current data provide evidence that the release of IL-8 and ANG is enhanced in tumors with reduced PHD2 and that these factors are important for both recruitment of BMDCs and for local angiogenesis. The precise mechanisms by which PHD2 restricts release of IL-8 and ANG and how vasculogenesis is mediated by BMDC recruitment in response to IL-8 and ANG remains to be determined. We demonstrate that NF- $\kappa$ B is required for enhanced angiogenesis upon PHD2 silencing. NF- $\kappa$ B, in turn, regulates ANG and IL-8. Intriguingly, inhibition of either ANG or IL-8 appears to be sufficient to abolish the effect of losing PHD2, suggesting potential clinical applications. In particular, inhibiting either ANG or IL-8 may be potential avenues for treatment of tumors that have become resistant to anti-VEGF therapies. Our findings demonstrate that a biological consequence of NF- $\kappa$ B regulation by PHD2 is to impair tumor growth through disruption of the blood supply, suggesting that this pathway may be important in human cancers.

How does PHD2 come to be down-regulated in some tumors? One possibility is that PHD2 itself might be mutated in a way that reduces its activity, expression, or stability. A recent report describes a patient with a PHD2 mutation who presents with both polycythemia and multiple tumors (Ladroue et al., 2008). In addition, PHD2 was found to be mutated at a high rate in endometrial cell lines (Kato et al., 2006). Another possibility is that methylation of the PHD2 promoter could result in epigenetic silencing of PHD2. Nevertheless, dysregulation of PHD2 has important consequences, contributing to tumorigenesis in multiple ways. Our analysis indicates that PHD2 levels are significantly reduced in certain clinical cases, such as colon and breast tumors. Although this reduction in PHD2 may contribute to tumor growth through enhanced stability of HIF, our work and the work of others indicate that PHD2 also has additional HIF-independent regulatory functions. For example, in agreement with the large variability of PHD2 protein levels in the NCI60 panel, simultaneous protein expression of HIF-1 and PHD2 has been found in

a subset of head and neck tumors (Jokilehto et al., 2006). Our data suggest that PHD2 can regulate HIF and NF- $\kappa$ B in a complementary, additive fashion with both pathways likely contributing to tumor growth. Both the HIF-dependent hypoxia response and the HIF-independent function of PHD2 can influence NF- $\kappa$ B and IL-8. It therefore remains to be seen in which contexts one PHD2 function can prevail over the other and whether HIF-dependent or HIF-independent pathways come to dominate tumorigenesis. One possibility is that these pathways cooperate to provide redundant pathways for activating NF- $\kappa$ B and promoting angiogenesis. Alternatively, HIF-dependent mechanisms may be predominant in hypoxic regions, while disruption of PHD2 may allow for HIF-independent mechanisms that promote angiogenesis in nonhypoxic regions. Future studies will elucidate whether PHD2 regulation of HIF is important for tumor initiation while regulation of NF- $\kappa$ B is important for tumor progression or dissemination.

As the search for HIF inhibitors is an active area of cancer biology, this study suggests that PHD2 also plays a critical role in tumor growth. However, HIF inhibition may not be sufficient to impede tumor growth especially in tumors in which PHD2 activity is lost. PHD2 has at least two tumor suppressor activities: 1) negatively regulating HIF through hydroxylation and 2) negatively regulating NF- $\kappa$ B in an HIF-independent manner, both of which in turn modulate tumor vasculature through angiogenesis and vasculogenesis. Further studies on the mechanism of PHD2 loss will be essential to gain new insights into which tumors may benefit clinically from anti-angiogenic therapies. Identifying other PHD2 targets and binding partners, as well as delineating how they influence tumorigenesis, will be a burgeoning field of study and will be critical in furthering our understanding of tumor progression.

## EXPERIMENTAL PROCEDURES

### Cells

Cell pellets from the NCI60 panel of cell lines were provided by Dr. Giovanni Melillo (NCI-Frederick). HCT116 and RKO cells were cultured in DMEM, SU.86.86 cells were cultured in RPMI 1640, and HT29 cells were cultured in McCoy's 5a media. HIF-1 knockout cells were a kind gift from Duyen and Long Dang (University of Michigan) and were cultured in McCoy's 5a media. All cell lines were maintained in 10% FCS. Primary human umbilical vein endothelial cells (HUVECs) were grown in complete Endothelial Growth Media (Clonetics). Transfection of plasmid DNA or RNA oligos were performed with Lipofectamine-Plus Reagent (Invitrogen) or DharmaFECT Reagent 1 (Dharmacon), according to manufacturer's directions.

### Reagents

MG132 was purchased from EMD, DFO was purchased from Sigma, DMOG was purchased from Biomol, ECMatrix was purchased from Millipore, TNF- $\alpha$  was purchased from R&D, and pimonidazole was purchased from NPI. ON-TARGETplus SMART pools against ANG, ARNT, IL-8, and RELA and non-targeting control (siCon2) were obtained from Dharmacon. Human angiogenesis antibody arrays were from Panomics. Immunoblotting was performed as described elsewhere (Chan et al., 2005). Antibodies used are as follows: from Becton Dickinson, HIF-1 $\alpha$  (clone 54), HIF-1 $\beta$  (clone 29), CD31/PECAM (clone WM59), and CD11b (clone M1/70); from Novus Biologicals, HIF-2 $\alpha$  (clone ep190b), PHD1 (NB100-310), PHD2 (NB100-137), PHD3 (NB100-139), and FIH (NB100-428); from Santa Cruz, CAIX (Clone H); from Research Diagnostics, clone DM1A; and from AbD Serotec, CD45 (clone YW62.3). An Invivo<sub>2</sub> hypoxia chamber (Ruskin Inc.) was used for hypoxia treatment. Colon and breast tissue microarrays were purchased from US Biomax. All human tissues for the tissue microarrays were obtained with informed consent and under

IRB- and HIPAA-approved protocols according to US federal laws and are exempt from consideration by the Stanford Administrative Panel on Human Subjects in Medical Research.

#### shRNA Constructs

Oligos for specific degradation of target RNA were designed using the Ambion siRNA Target Finder and cloned into RNAi-Ready pSiren RetroQ (BD Biosciences). All experiments refer to individual constructs and not a mixture of constructs.

#### Plasmids

Fusion PCR was used to make the catalytic hydroxylase mutations in PHD2 (H313A and D315A), which were confirmed by sequencing. A 1.3 kilobase sequence upstream of the transcriptional start site of ANG was cloned into pGL3-promoter. A NF- $\kappa$ B binding site was identified by TRES (<http://bioportal.bic.nus.edu.sg/tres/>).

#### Reporter Assays

Luciferase activity was determined by Dual-Glo Luciferase assay reagent (Promega) measured in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Firefly luciferase was normalized to constitutive Renilla luciferase.

#### In Vivo Experiments and Immunohistochemistry

All animal experiments were approved by Stanford's Administrative Panel on Laboratory Animal Care (APLAC) and in accordance with institutional and national guidelines. Two to five million cells were implanted subcutaneously into the flanks of CB17/SCID mice (4–6-week-old males supplied by the Stanford University Research Animal Facility or Charles River Laboratories). Tumors were measured with calipers. Volume was calculated by the following formula: width<sup>2</sup> × length × 0.5. Five-micron sections were cut for immunohistochemistry. Sections were counterstained with hematoxylin and eosin or nuclei were stained with DAPI. Five random fields were counted per section to determine the amount of a given stain.

#### In Vitro Growth Curve

One hundred thousand cells were plated on 60 mm dishes. Three days later, cells were trypsinized and counted on a hemacytometer, and 100,000 cells were replated.

#### In Vitro Angiogenesis Assay

Fifty thousand cells of a given genotype were plated in 1 ml of Endothelial Basal Media with 2% FCS. Conditioned media was collected two days later. Five to ten thousand HUVECs were plated in 100  $\mu$ l of conditioned media on Matrigel. Cells were incubated for 4 to 6 hr at 37°C and were visualized by light microscopy. The amount of angiogenesis was quantified by counting the number of cells in branch point capillaries ( $\geq 3$  cells per branch) in five random fields per replicate.

#### Real-Time Quantitative RT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen) as per manufacturer's directions. Total RNA (1.5  $\mu$ g) was reversed transcribed with random hexamer primers and MMLV-RT. Power SYBR Green PCR reactions were performed in triplicate for each sample and analyzed using the ABI Prism 7900HT sequence detection system. Data were normalized to *TBP* levels.

#### Chromatin Immunoprecipitation

Cells were treated with TNF- $\alpha$  (20 ng/ml) for the indicated time and cross-linked for 10 min with 1% formaldehyde. Cross-linking was stopped by the addition of 0.125 M glycine. Chromatin was sonicated to approximately 300 bp using a Bioruptor (Diagenode). Purified chromatin was incubated with an  $\alpha$ -RELA antibody (Biomol International). Chromatin was then de-cross-linked, and ChIP-associated sequences were detected by quantitative real-time PCR.

#### Gene Expression Analysis

PHD2 expression data were analyzed by Oncomine ([www.oncomine.org](http://www.oncomine.org)). Data from two human clinical cancer studies were used (Graudens et al., 2006; Ramaswamy et al., 2001). The Ramaswamy study included 90 normal

samples and 190 cancer samples. The Graudens study included 12 normal colon samples and 18 colorectal carcinomas. The expression levels of all probe sets for PHD2 and CD31 were extracted from the expression studies of indicated breast cancer samples after robust multi-array normalization (Pawitan et al., 2005; Wang et al., 2005). The binary regression analysis was based on TNF- $\alpha$ -treated cells relative to control as training sets. This gene signature was then used to estimate the pathway activities of NF- $\kappa$ B for each breast cancer sample in both data sets relative to the expression values of PHD2 and CD31.

#### Statistical Analysis

Student's *t* test or one-way ANOVA with post-hoc Tukey-Kramer tests were used to determine significance. All error bars represent the standard error of the mean.

#### SUPPLEMENTAL DATA

Supplemental Data includes Supplemental Experimental Procedures, including primer sequences and shRNA sequences, and seven figures and can be found with this article online at [http://www.cell.com/cancer-cell/supplemental/S1535-6108\(09\)00144-5](http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00144-5).

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#### REFERENCES

- Appelhoff, R.J., Tian, Y.M., Raval, R.R., Turley, H., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., and Gleadle, J.M. (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J. Biol. Chem.* 279, 38458–38465.
- Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1 $\alpha$  in normoxia. *EMBO J.* 22, 4082–4090.
- Bild, A.H., Yao, G., Chang, J.T., Wang, Q., Potti, A., Chasse, D., Joshi, M.B., Harpole, D., Lancaster, J.M., Berchuck, A., et al. (2006). Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 439, 353–357.
- Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* 6, 389–395.
- Chan, D.A., Sutphin, P.D., Yen, S.E., and Giaccia, A.J. (2005). Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1  $\alpha$ . *Mol. Cell. Biol.* 25, 6415–6426.
- Cummins, E.P., Berra, E., Comerford, K.M., Ginouves, A., Fitzgerald, K.T., Seeballuck, F., Godson, C., Nielsen, J.E., Moynagh, P., Pouyssegur, J., and Taylor, C.T. (2006). Prolyl hydroxylase-1 negatively regulates I $\kappa$ B kinase-beta, giving insight into hypoxia-induced NF $\kappa$ B activity. *Proc. Natl. Acad. Sci. USA* 103, 18154–18159.
- D'Angelo, G., Duplan, E., Boyer, N., Vigne, P., and Frelin, C. (2003). Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. *J. Biol. Chem.* 278, 38183–38187.
- Dang, D.T., Chen, F., Gardner, L.B., Cummins, J.M., Rago, C., Bunz, F., Kantsevov, S.V., and Dang, L.H. (2006). Hypoxia-inducible factor-1 $\alpha$  promotes

- nonhypoxia-mediated proliferation in colon cancer cells and xenografts. *Cancer Res.* 66, 1684–1693.
- De Palma, M., Venneri, M.A., Roca, C., and Naldini, L. (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat. Med.* 9, 789–795.
- Fett, J.W., Strydom, D.J., Lobb, R.R., Alderman, E.M., Bethune, J.L., Riordan, J.F., and Vallee, B.L. (1985). Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry* 24, 5480–5486.
- Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., Chorianopoulos, E., Liesenborghs, L., Koch, M., De Mol, M., et al. (2007). Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* 131, 463–475.
- Gao, D., Nolan, D.J., Mellick, A.S., Bambino, K., McDonnell, K., and Mittal, V. (2008). Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science* 319, 195–198.
- Garkavtsev, I., Kozin, S.V., Chernova, O., Xu, L., Winkler, F., Brown, E., Barnett, G.H., and Jain, R.K. (2004). The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. *Nature* 428, 328–332.
- Graudens, E., Boulanger, V., Mollard, C., Mariage-Samson, R., Barlet, X., Gremy, G., Couillault, C., Lajemi, M., Piatier-Tonneau, D., Zaborski, P., et al. (2006). Deciphering cellular states of innate tumor drug responses. *Genome Biol.* 7, R19.
- Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100, 57–70.
- Harris, A.L. (2002). Hypoxia—a key regulatory factor in tumour growth. *Nat. Rev. Cancer* 2, 38–47.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIF1 $\alpha$  targeted for VHL-mediated destruction by proline hydroxylation: implications for O<sub>2</sub> sensing. *Science* 292, 464–468.
- Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Targeting of HIF-1 $\alpha$  to the von Hippel-Lindau ubiquitylation complex by O<sub>2</sub>-regulated prolyl hydroxylation. *Science* 292, 468–472.
- Jokilehto, T., Rantanen, K., Luukkaa, M., Heikkinen, P., Grenman, R., Minn, H., Kronqvist, P., and Jaakkola, P.M. (2006). Overexpression and nuclear translocation of hypoxia-inducible factor prolyl hydroxylase PHD2 in head and neck squamous cell carcinoma is associated with tumor aggressiveness. *Clin. Cancer Res.* 12, 1080–1087.
- Kaelin, W.G. (2007). Von hippel-lindau disease. *Annu. Rev. Pathol.* 2, 145–173.
- Karashima, T., Sweeney, P., Kamat, A., Huang, S., Kim, S.J., Bar-Eli, M., McConkey, D.J., and Dinney, C.P. (2003). Nuclear factor-kappaB mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8. *Clin. Cancer Res.* 9, 2786–2797.
- Kato, H., Inoue, T., Asanoma, K., Nishimura, C., Matsuda, T., and Wake, N. (2006). Induction of human endometrial cancer cell senescence through modulation of HIF-1 $\alpha$  activity by EGLN1. *Int. J. Cancer* 118, 1144–1153.
- Koch, A.E., Poverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Eliner, V.M., Eliner, S.G., and Strieter, R.M. (1992). Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 258, 1798–1801.
- Kondo, K., Kico, J., Nakamura, E., Lechpammer, M., and Kaelin, W.G., Jr. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. *Cancer Cell* 1, 237–246.
- Ladroue, C., Carcenac, R., Leporrier, M., Gad, S., Le Hello, C., Galateau-Salle, F., Feunteun, J., Pouyssegur, J., Richard, S., and Gardie, B. (2008). PHD2 mutation and congenital erythrocytosis with paraganglioma. *N. Engl. J. Med.* 359, 2685–2692.
- Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruck, R.K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* 16, 1466–1471.
- Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., and Whitelaw, M.L. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295, 858–861.
- Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L., et al. (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* 7, 1194–1201.
- Mahon, P.C., Hirota, K., and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1 $\alpha$  and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* 15, 2675–2686.
- Mazzone, M., Dettori, D., Leite de Oliveira, R., Loges, S., Schmidt, T., Jonckx, B., Tian, Y.M., Lanahan, A.A., Pollard, P., Ruiz de Almodovar, C., et al. (2009). Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 136, 839–851.
- McDonough, M.A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Lienard, B.M., Zondlo, J., Oldham, N.J., Clifton, I.J., Lewis, J., et al. (2006). Cellular oxygen sensing: crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proc. Natl. Acad. Sci. USA* 103, 9814–9819.
- Minamishima, Y.A., Moslehi, J., Bardeesy, N., Cullen, D., Bronson, R.T., and Kaelin, W.G., Jr. (2008). Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure. *Blood* 111, 3236–3244.
- Mori, S., Rempel, R.E., Chang, J.T., Yao, G., Lagoo, A.S., Potti, A., Bild, A., and Nevins, J.R. (2008). Utilization of pathway signatures to reveal distinct types of B lymphoma in the Emicro-myc model and human diffuse large B-cell lymphoma. *Cancer Res.* 68, 8525–8534.
- Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V., and Kaelin, W.G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat. Cell Biol.* 2, 423–427.
- Pawitan, Y., Bjohle, J., Amler, L., Borg, A.L., Egyhazi, S., Hall, P., Han, X., Holmberg, L., Huang, F., Klaar, S., et al. (2005). Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. *Breast Cancer Res.* 7, R953–R964.
- Ramaswamy, S., Tamayo, P., Rifkin, R., Mukherjee, S., Yeang, C.H., Angelo, M., Ladd, C., Reich, M., Latulippe, E., Mesirov, J.P., et al. (2001). Multiclass cancer diagnosis using tumor gene expression signatures. *Proc. Natl. Acad. Sci. USA* 98, 15149–15154.
- Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1  $\alpha$  is required for solid tumor formation and embryonic vascularization. *EMBO J.* 17, 3005–3015.
- Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., and Johnson, R.S. (2000). Hypoxia-inducible factor-1 $\alpha$  is a positive factor in solid tumor growth. *Cancer Res.* 60, 4010–4015.
- Semenza, G.L. (2002). Involvement of hypoxia-inducible factor 1 in human cancer. *Intern. Med.* 41, 79–83.
- Shojaei, F., Wu, X., Zhong, C., Yu, L., Liang, X.H., Yao, J., Blanchard, D., Bais, C., Peale, F.V., van Bruggen, N., et al. (2007). Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450, 825–831.
- Takeda, K., and Fong, G.H. (2007). Prolyl hydroxylase domain 2 protein suppresses hypoxia-induced endothelial cell proliferation. *Hypertension* 49, 178–184.
- Takeda, K., Ho, V.C., Takeda, H., Duan, L.J., Nagy, A., and Fong, G.H. (2006). Placental but not heart defects are associated with elevated hypoxia-inducible factor  $\alpha$  levels in mice lacking prolyl hydroxylase domain protein 2. *Mol. Cell. Biol.* 26, 8336–8346.
- Unruh, A., Ressel, A., Mohamed, H.G., Johnson, R.S., Nadrowitz, R., Richter, E., Katschinski, D.M., and Wenger, R.H. (2003). The hypoxia-inducible factor-1  $\alpha$  is a negative factor for tumor therapy. *Oncogene* 22, 3213–3220.
- Wang, Y., Klijn, J.G., Zhang, Y., Sieuwerts, A.M., Look, M.P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M.E., Yu, J., et al. (2005). Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 365, 671–679.



- Wu, G.D., Lai, E.J., Huang, N., and Wen, X. (1997). Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to overlapping elements within the interleukin-8 promoter. The role of Oct-1 as a transcriptional repressor. *J. Biol. Chem.* 272, 2396–2403.
- Yang, L., DeBusk, L.M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., Matrisian, L.M., Carbone, D.P., and Lin, P.C. (2004). Expansion of myeloid immune suppressor Gr<sup>+</sup>CD11b<sup>+</sup> cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 6, 409–421.
- Yu, F., White, S.B., Zhao, Q., and Lee, F.S. (2001). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc. Natl. Acad. Sci. USA* 98, 9630–9635.
- Zhong, H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L., and Simons, J.W. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.* 59, 5830–5835.