The Biphasic Role of the Hypoxia-Inducible Factor Prolyl-4-Hydroxylase, PHD2, in Modulating Tumor-Forming Potential

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Abstract

Hypoxia is a common feature of solid tumors. The cellular response to hypoxic stress is controlled by a family of prolyl hydroxylases (PHD) and the transcription factor hypoxia-inducible factor 1 (HIF1). To investigate the relationship between PHD and HIF1 activity and cellular transformation, we characterized the expression levels of PHD isoforms across a lineage of cell strains with varying transformed characteristics. We found that PHD2 is the primary functional isoform in these cells and its levels are inversely correlated to tumor-forming potential. When PHD2 levels were altered with RNA interference in nontumorigenic fibroblasts, we found that small decreases can lead to malignant transformation, whereas severe decreases do not. Consistent with these results, direct inhibition of PHD2 was also shown to influence tumor-forming potential. Furthermore, we found that overexpression of PHD2 in malignant fibroblasts leads to loss of the tumorigenic phenotype. These changes correlated with HIF1α activity, glycolytic rates, vascular endothelial growth factor expression, and the ability to grow under hypoxic stress. These findings support a biphasic model for the relationship between PHD2 activity and malignant transformation. (Mol Cancer Res 2008;6(5):829–42)

Introduction

Many solid tumors frequently exhibit areas of hypoxia because they have a high rate of cellular proliferation and form aberrant blood vessels (1, 2). Since tumor hypoxia was first identified, it has been well documented that decreased oxygen tension has a strong effect on tumor progression in a variety of ways. Most prominently, hypoxia induces glycolysis and angiogenesis, which are important changes for tumor growth and clonal expansion (3-5). In addition, hypoxia promotes the stepwise progression along a benign to malignant pathway by selecting cells that have acquired transformed characteristics and have lost tumor suppressor function (6-8). Finally, the transcription factors that regulate the cellular response to hypoxia are important for tumor growth and progression (9). Characterizing the role of hypoxia signaling in tumor development, therefore, is important for understanding the basic causes of cellular transformation and tumor growth.

The cellular responses to hypoxia are primarily regulated by the transcription factor hypoxia-inducible factor 1 (HIF1; refs. 10, 11). HIF1 is a heterodimer of HIF1α and HIF1β also called the aryl hydrocarbon receptor nuclear translocator (ARNT), both of which belong to the superfamily of basic helix-loop-helix Per-ARNT-Sim proteins. HIF1β is a constitutive nuclear protein and interacts with other transcriptional factors, such as the aryl hydrocarbon receptor. HIF1α is specific to the cellular response to hypoxia and is constantly synthesized and, under normoxia, is rapidly degraded by the ubiquitin-proteosomal pathway (12). The degradation of HIF1α is mediated by the product of the von Hippel-Lindau tumor suppressor gene, which specifically interacts with the oxygen-dependent degradation domain of HIF1α. The oxygen dependence of this process is regulated by a family of prolyl hydroxylase (PHD) domain–containing enzymes, designated EGL 9 homologues, and HIF1 PHDs (13, 14).

Three mammalian PHDs (PHD1-3) regulate HIF1 signaling and each has a distinct tissue distribution, pattern of subcellular localization, and substrate specificity (15, 16). For proper activity, PHDs require oxygen, iron, α-ketoglutarate, and ascorbate. The oxygen requirement suggests that PHDs are the cellular "sensors" for hypoxia (12, 17, 18). In the presence of adequate oxygen, PHDs hydroxylate HIF1α at conserved proline residues within the oxygen-dependent degradation domain. Once hydroxylated, HIF1α becomes a substrate for von Hippel-Lindau–mediated ubiquitination and degradation (13, 14). Under hypoxic conditions, PHDs are inactive and HIF1α is stabilized and translocates to the nucleus where it forms the functional transcription factor HIF1 by dimerizing with HIF1β. HIF1-mediated transcription regulates many processes involved in cellular homeostasis and transformation,

doi:10.1158/1541-7786.MCR-07-2113

including anaerobic metabolism, O_2-carrying capacity, and angiogenesis (3, 19). Alternatively, under severe hypoxic stress, HIF1 can induce a pro-death response through transcriptional activation of proapoptotic factors such as BCL family members and modulation of the p53 signaling pathway (1, 20-22). HIF1, therefore, regulates a balance between cellular adaptation, through up-regulation of survival genes, and cell death, through modulation of various pathways.

Several studies show that modulation of PHD activity has the capability to control the HIF1 transcriptional response (23-26). Recent studies indicated a link between cancer and perturbation of PHD activity and subsequent HIF1-mediated signaling (17, 18). Furthermore, it has been suggested that von Hippel-Lindau is a tumor suppressor, at least under some circumstances, because it can modulate HIF1 activity; the latter activity is dependent on a functional PHD. In view of these reports, we hypothesize that PHD activity is linked to the tumor-forming potential of a cell, and that direct manipulation of the PHD activity within a cell will alter its tumor-forming capacity.

To test this hypothesis, we made use of three human fibroblast cell strains from the MSU-1 lineage of cells (27). These cells were derived one from the other; each one has acquired a characteristic related to malignant transformation, until the last change gave rise to a cell strain that, by acquiring one more change (e.g., a *RAS* oncogene), becomes capable of forming malignant tumors in athymic mice with a short latency (28). The three cell strains used for the present study are MSU-1.0, MSU-1.1, and PH3MT. MSU-1.0 is the infinite life span precursor to MSU-1.1. MSU-1.1 acquired partial growth factor independence. The PH3MT cell strain was derived from a malignant tumor formed in an athymic mouse that was injected with MSU-1.1 cells that had been transfected with an overexpressed *hRAS* oncogene and selected for focus formation.

Using these cells, we have shown that PHD2 represents the primary functional HIF PHD within the MSU-1 lineage of cells and that PHD2 activity decreases as the cell exhibits more transformed characteristics. Moderate decreases in PHD2 activity resulting from the use of RNA interference in the nontumorigenic cell strain, MSU-1.1, resulted in malignant transformation. Interestingly, MSU-1.1 cells with a more severe loss of PHD2 activity were unable to form tumors. Consistent with these results, chemical inhibition of PHD2 activity in transformed cells decreases the cell tumor-forming potential and the overexpression of PHD2 in malignant fibroblasts lead to inhibition of tumor growth. These results suggest a biphasic relationship between the cell tumor-forming potential and PHD activity and highlights potential difficulties when targeting this signaling cascade for therapeutics.

Results

Increasing HIF1 Protein Levels and Activity and Decreasing PHD2 Levels in the MSU-1 Cell Lineage as the Cells Become More Transformed

High levels of HIF1α protein and HIF1 signaling are hallmarks of solid tumors and cells derived from such tumors (2, 7, 29). To determine if this relationship exists in the cell strains from the MSU-1 lineage, we compared the level of

HIF1α protein in three key cell strains from the lineage using Western blot analysis. We found that the HIF1α mRNA levels were consistent across the cell strains, both in the presence and the absence of oxygen (Fig. 1A). HIF1α protein was undetectable under normoxia; however, its levels were substantially increased following exposure to hypoxia and this increase was more pronounced in the cells that had acquired the characteristics of malignant cells (Fig. 1B). A similar pattern was not observed for HIF2α (Fig. 1B). The levels of *vascular endothelial growth factor* (*VEGF*) mRNA, a classic HIF1 target gene, were also significantly increased in a manner that correlated to HIF1α levels under hypoxia (Fig. 1C). Although HIF1α protein was undetectable under normoxia, the tumor-derived PH3MT cells displayed higher basal levels of VEGF mRNA than the two nontumorigenic cell strains (Fig. 1C). These results show that the levels of HIF1α and HIF1 activity are higher in a tumor-derived cell strain than in the non-tumorigenic precursor cells.

Because PHDs are the primary regulators of HIF1α stability, we analyzed the levels of these hydroxylases in the MSU-1 cell lineage. Comparative analysis of PHD mRNA levels revealed that PHD2 is the predominant isoform in these cells, comprising >75% of the total PHD mRNA in the cells (Table 1). Western blot analysis showed that PHD2 protein levels under normoxia were inversely correlated with the increasing transformation of the MSU-1 cell strains (Fig. 1D). The apparent discrepancy between mRNA and protein levels is predominantly due to an artifact of the Western blotting procedure; however, difference in mRNA or protein stability or translation rates cannot be ruled out. These results show that PHD2 is the predominant isoform within the MSU-1 cell strains and its levels are inversely correlated with the transformed characteristics and HIF1 activity of these cells.

To determine if the inverse correlation was specific to the MSU series of fibroblast, human cell lines of varying degrees of tumorigenicity were analyzed for PHD2 protein levels. These cell lines, derived from breast tissue, also display varying severity of transformation. The fibrocystic breast cell line, MCF10A, is incapable of forming tumors in athymic mice and displays the highest levels of PHD2. The tumor cell lines that have little or no invasive activity, MCF7 and T47D, display a reduced level of PHD2 compared with the MCF10A cell line. Finally, the highly invasive tumor cell lines, MDA-MB-231, have the lowest level of PHD2 protein (Fig. 1E). These results suggest that modulation of PHD2 levels correlates with changes in the ability of the cell to form tumors and that it is not specific to the MSU series of cell strains.

HIF1 Activity Can Be Regulated by PHD2 in MSU-1 Cell Strains

The above results show a correlation between PHD2 levels and transformation potential of a cell. To directly assess the ability of PHD2 to alter cell transformation potential, the three PHDs were manipulated in the MSU series of cells using RNA interference and overexpression. The role of PHD2 in HIF signaling in these cell strains was determined using a series of transient transfection with an HRE-driven luciferase reporter construct. The specific silencing of the PHD isoforms was confirmed by Western blot analysis using MSU 1.1 cells
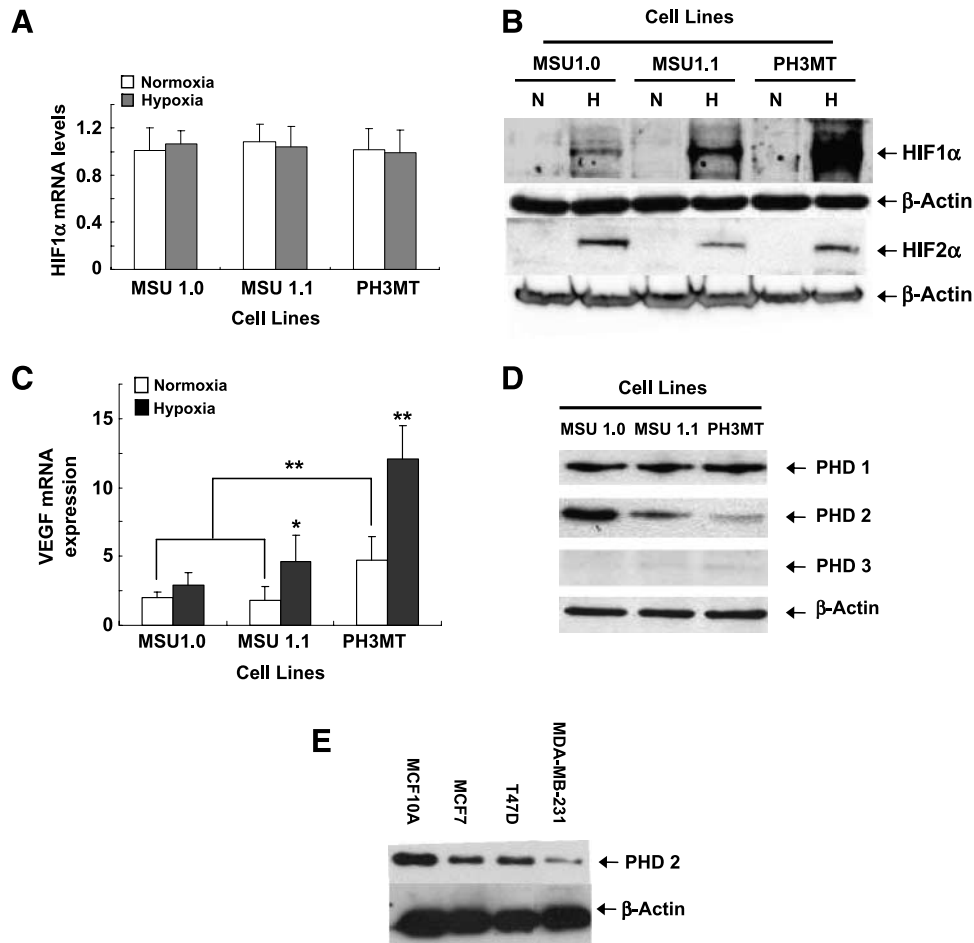
FIGURE 1. Expression levels of HIF1α and HIF1 activity in MSU-1 lineage of cells. **A.** HIF1α mRNA levels in three MSU-1 lineage of cells were determined using qRT-PCR. Cells were exposed to normoxia (20% O_2, *white column*) or hypoxia (1% O_2, *black column*) for 16 h ($n = 6$). **B.** HIF1α and HIF2α protein levels were determined in MSU-1 lineage of cells by Western blot analysis. Cells were exposed to normoxia (N, 20% O_2) or hypoxia (H, 1% O_2) for 6 h, and nuclear proteins were prepared and analyzed for HIF1α protein levels. To verify equal loading, the blots were stripped and reprobed with a β-actin antibody. **C.** VEGF mRNA levels were determined in each of the MSU-1 lineage of cells using qRT-PCR. Cells were exposed to normoxia (20% O_2, *white column*) and hypoxia (1% O_2, *black column*) for 16 h ($n = 10$, *$P < 0.05$, **$P < 0.01$). **D.** The protein levels of the PHD isoforms were determined under normoxic (20% O_2) conditions in the MSU cell lines by Western blot analysis with PHD1-3–specific polyclonal antibodies or a β-actin–specific antibody. **E.** PHD2 protein levels were assessed under normoxic (20% O_2) conditions in four different cell lines derived from human breast tissue, each with varying degrees of tumor-forming potential, by Western blot analysis with a PHD2-specific antibody. To verify equal loading, the blot was stripped and reprobed with a β-actin antibody.

transfected with each independent small hairpin RNA (shRNA) construct (Fig. 2A). In the presence of a PHD2-specific shRNA construct, there was an increase in luciferase activity under normoxic conditions (20% O_2) in the MSU-1.1 cells that was not observed for the PHD1 or PHD3 shRNAs (Fig. 2B). There was no significant change in the presence of hypoxia for any of the shRNA constructs. This suggests that the HRE-mediated transcription was maximally stimulated. In a similar set of experiments, PHD1-3 were overexpressed in PH3MT cells to determine if increases in endogenous levels of the various PHDs could inhibit hypoxia-induced transcription (Fig. 2C). The results mirrored those of the shRNA data in that only PHD2 was capable of inhibiting HRE-driven luciferase activity under hypoxia. The luciferase activity in the PHD1- and PHD3-transfected cells was similar to that found using the empty expression vector or the mock-transfected controls. These results are evidence that PHD2 is the major functional isoform in regulating HIF1 activity in the MSU-1 cell strains and that direct modulation of PHD2 activity can alter the ability of the cell to respond to hypoxic stress.

Table 1. The mRNA Levels of Each PHD Isoform Were Analyzed under Normoxia (20% O_2) in the MSU Cell Lines Using qRT-PCR

Cell Lines	PHD1	PHD2	PHD3
MSU-1.0	25.1%	74.8%	0.1%
MSU-1.1	16.6%	88.1%	0.3%
PH3MT	12%	87.7%	0.3%

NOTE: Each *HPRT* normalized value is expressed as a percent of total PHD message (i.e., the sum of normalized mRNA level for each PHD isoform) within each cell type ($n = 12$).
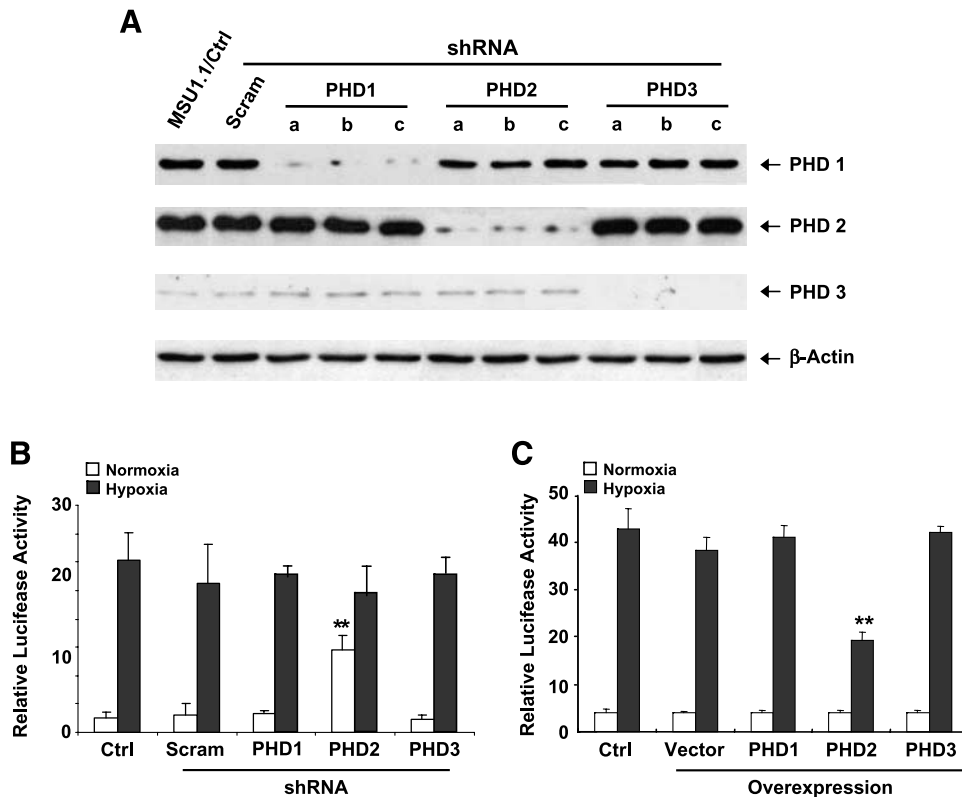
FIGURE 2. The effects of modulating PHD levels on HIF1-hypoxia signaling in MSU-1 lineage of cells. **A.** Specific silencing of PHD isoforms using shRNA. MSU-1.1 cells were mock transfected (*MSU-1.1/Ctrl*) or transfected with a scrambled shRNA cassette (*Scram*) or one of three independent shRNA cassettes targeting each PHD isoform (*PHD1-3*). Total protein was isolated and analyzed by Western blot with specific antibodies for PHD or β-actin. **B.** MSU-1.1 cells were transiently transfected with no shRNA cassette (*Ctrl*), a scrambled shRNA (*Scram*), or shRNA cassettes targeting a specific PHD isoform (*PHD1-3*), together with an HRE-driven luciferase reporter construct and a β-gal expression vector for normalization. After transfection, cells were exposed to normoxia (20% O_2, *white column*) or hypoxia (1% O_2, *black column*) for 16 h and analyzed for luciferase activity ($n = 10$, ** $P < 0.01$). **C.** PH3MT cells were transiently transfected with nothing (*Ctrl*), an empty expression vector (*Vector*), or an expression vector for the PHDs (*PHD1-3*) expression vectors as described for MSU-1.1 in B ($n = 10$; **$P < 0.01$).

Decreases in PHD2 Levels Alter the Cell Tumor-Forming Potential

Our data show that the MSU-1 cell strains are a suitable system to directly test the link between PHD levels and tumorigenesis and prompted us to examine whether the loss of PHD2 could bestow tumor-forming ability upon a nontumorigenic cell strain. To answer this question, a series of stable cell strains that have decreased levels of PHD2 was created. Nontumorigenic MSU-1.1 cells were infected with the lentiviral vector, pVCwPBam, encoding three distinct shRNA targeting PHD2 or scrambled shRNA. Initially, cell strains (shPHD2-a, shPHD2-b, and shPHD2-c) were assessed for PHD2 levels using Western blot analysis. Among those, four clonal cell strains were chosen from each shPHD2 strain that exhibited decreased PHD2 levels compared with the parental MSU-1.1 cells and scrambled shRNA-expressing controls (Fig. 3A). The strains showed differences in PHD2 levels, with shPD2-a strains 7 and 21, shPHD2-b strains 5 and 6, and shPHD2-c strains 40 and 41 having moderate reduction in PHD2, which is similar to the levels of PHD2 in PH3MT, the malignantly transformed MSU-1 lineage of cells. In contrast, shPHD2-a strains 2 and 5, shPHD2-b strains 1 and 10, and shPHD2 strains 27 and 30 showed an almost complete loss of

PHD2 expression. The levels of PHD1 and PHD3 were unaffected in any of the shPHD2-infected cell strains (Fig. 3A). To determine if the decreased PHD2 levels within the four strains had a functional consequence, we characterized HIF1α protein levels (Fig. 3B). There was substantial HIF1α protein in strains shPHD2-a 2 and 5 under normoxic conditions, compared with that of the parental or scrambled shRNA cell strains. The level of HIF1α protein was moderately up-regulated in strains shPHD2-a 7 and 21 under normoxia. All of the shPHD2 strains displayed hypoxia-induced HIF1α stabilization (Fig. 3B).

To examine whether our shPHD2 cell strains had acquired transformed characteristics, their ability to form colonies in an anchorage-independent manner (soft-agar assay) was determined. The strains with the least PHD2 (i.e., shPHD2-a 2 and 5, shPHD2-b 1 and 10, and shPHD2-c 27 and 30) were capable of forming colonies only marginally better than the scrambled shRNA strain and the parental MSU-1.1 cells; however, they did not perform as well as the positive control, the RAS-transformed A210 cells (Fig. 3C). The strains with a moderate reduction in PHD2 (i.e., shPHD2-a 7 and 21, shPHD2-b 5 and 6, and shPHD2-c 40 and 41) exhibited strong anchorage-independent growth, forming colonies larger than the positive

controls (Fig. 3C). Additional shPHD2 strains with moderate or severe decreases in PHD2 levels also displayed similar results (data not shown). These results indicate that a small loss in PHD2 expression can aggressively promote the ability of a cell to grow in an anchorage-independent manner; however, further loss of PHD2 does not significantly change the cell anchorage-independent growth phenotype.

To determine if the shPHD2 strains were capable of forming tumors, five BALB/c athymic mice (5 weeks of age) were injected at two sites per mouse for each shPHD2-a cell strains (shPHD2-a 2, 5, 7, and 21) or the scram shRNA cell strain, as a control (Fig. 4A). As expected, 5 months after injection, the scram shRNA-expressing cells did not show any tumor growth, like the parental cell strain, MSU-1.1 (30). In contrast, two shPHD2-a strains, 21 and 7, yielded high-grade fibrosarcomas at all 10 injection sites in weeks 3 and 5, respectively (Fig. 4A). The shPHD2-a strains 2 and 5, which exhibited the lowest levels of PHD2, were negative for tumor-forming ability even after 5 months (Fig. 4A). These results show that moderate decreases in PHD2 activity leads to malignant transformation, whereas further loss of PHD2 activity produces cells that do not form tumors and suggests that a biphasic role exists for PHD2
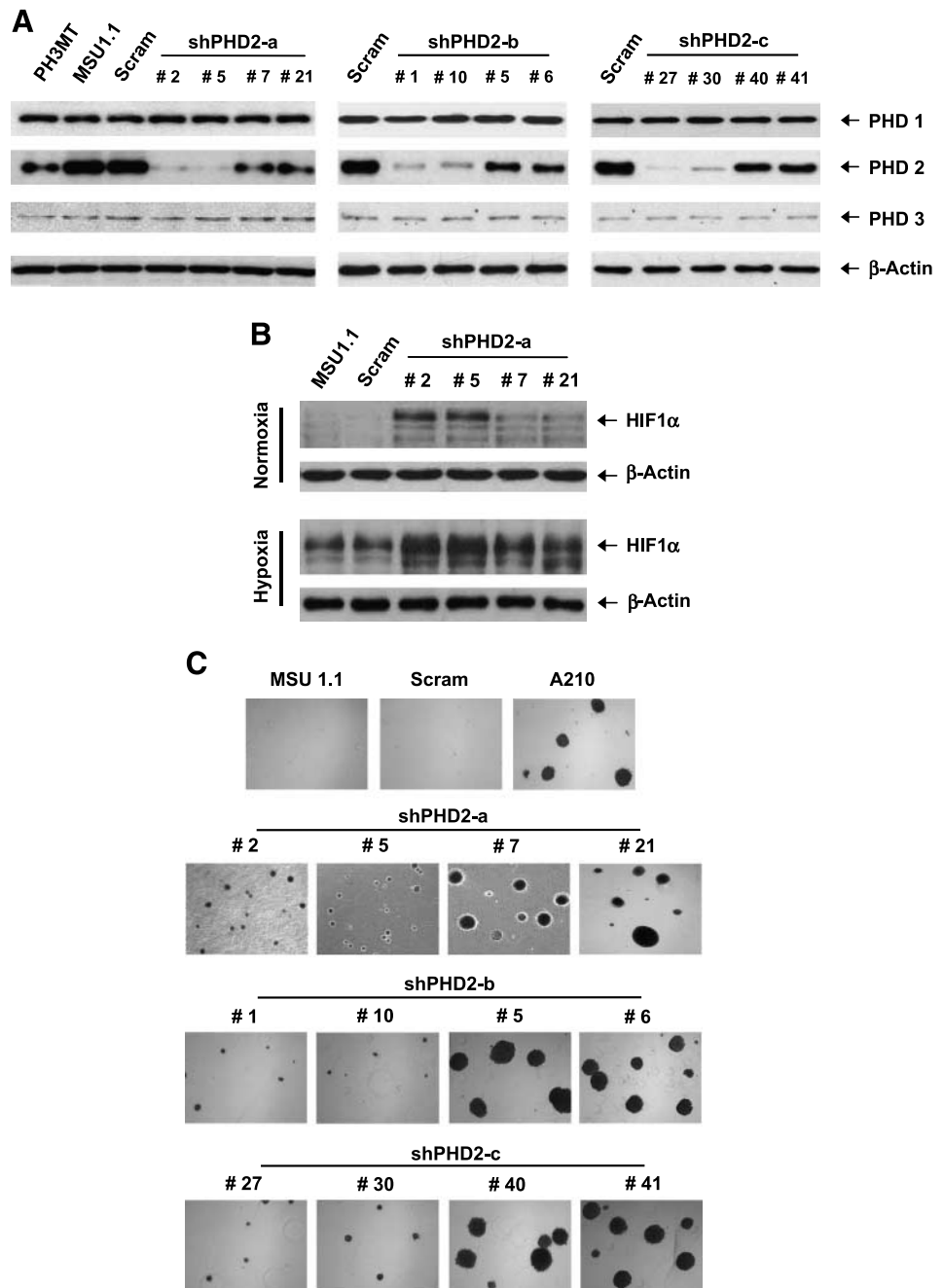

FIGURE 3. Characterization of shPHD2-infected MSU 1.1 strains. **A.** MSU-1.1 cells were infected with three independent lentiviral constructs that express distinct shRNA cassette targeting PHD2. The levels of PHD were characterized in newly created shPHD2-a strains (strains 2, 5, 7, and 21), shPHD2-b strains (strains 1, 10, 5, and 6), and shPHD2-c strains (strains 27, 30, 40, and 41) by Western blot analysis using isoform-specific PHD antibodies or a β-actin–specific antibody. The parental cell line (*MSU-1.1*) and a scrambled shRNA cell strain (*Scram*) were included as controls. **B.** HIF1α protein levels were analyzed in shPHD2-a strains exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 6 h by Western blot analysis. To verify equal loading, the blot was stripped and reprobed with a β-actin antibody. **C.** shPHD2 strains (shPHD2-a 2, 5, 7, and 21; shPHD2-b 1, 10, 5, and 6; and shPHD2-c 27, 30, 40, and 41) and scrambled shRNA clone (Scram) were assessed for anchorage-independent growth by forming colonies in agarose. Parental MSU-1.1 cells and A210, hRAS-transformed cells were included as a negative and positive control, respectively ($n = 10$).
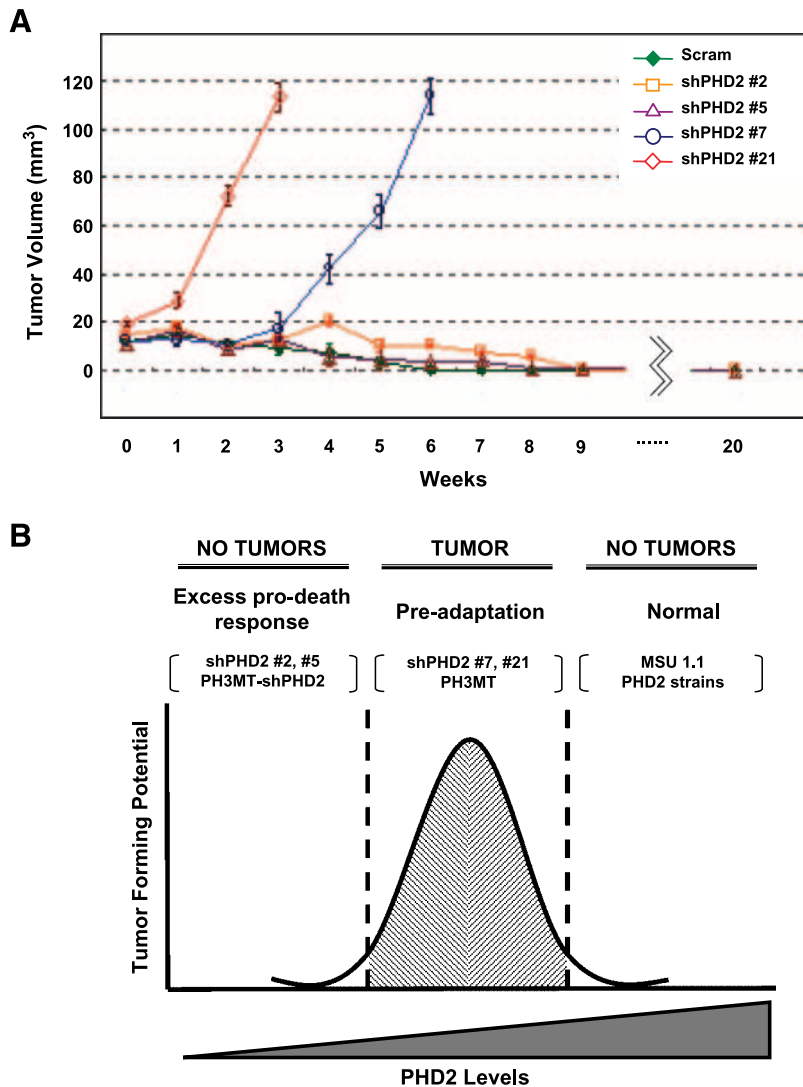
FIGURE 4. **A.** shPHD2-a strains 2, 5, 7, and 21, and scrambled shRNA clone (*Scram*) were injected into athymic mice and tumor growth was monitored weekly for 5 mo (*n* = 10). **B.** A graphical representation of the relationship between PHD2 levels and tumor-forming potential.

in tumor formation (Fig. 4B). At normal levels of PHD2 (e.g., MSU-1.1), the hypoxic response is regulated properly and no tumors are formed. With a slight decrease in PHD2 activity (e.g., shPHD2-a strains 7 and 21 and PH3MT cells), the cells gain an advantage and become malignantly transformed. As PHD2 activity is further decreased (e.g., shPHD2-a cell strains 2 and 5), the pro-death response might become the dominant signal and the increased adaptation would be overwhelmed. The dual nature of this response is presumably due to the ability of PHD2 to alter the cellular balance between hypoxic-induced adaptation and pro-death responses.

Biphasic Role of PHD in Balancing HIF1-Mediated Adaptation and Cell Death

The proposed model presented in Fig. 4B suggests that PH3MT and shPHD2-a strains 7 and 21 (i.e., small decrease in PHD2 compared with nontransformed cells) have a growth advantage, such as a higher rate of glycolysis and angiogenesis, possibly via HIF1-mediated preadaptation. HIF1 also regulates cell death through transcriptional activation of proapoptotic

factors (21, 22). The model also suggests that shPHD2-a strains 2 and 5 (i.e., severe loss of PHD2) have higher expression of pro-death genes, such as *Bcl-2/adenovirus E1B 19 kDa-interacting protein 3* (*BNIP3*), and a decreased viability under hypoxic stress.

To characterize the level of glycolysis and angiogenesis, the clones were analyzed for the expression of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), and VEGF. To determine the potential cell death signals within the cell strains, BNIP3, a known HIF1-regulated BCL2 family member, was also measured. PH3MT and shPHD2-a strains 7 and 21 showed a higher basal expression of GAPDH and LDH when compared with the parental MSU-1.1 cells or scrambled control (Fig. 5A). In contrast, shPHD2-a strains 2 and 5 had higher BNIP3 expression levels than the other clones tested (Fig. 5A). Interestingly, VEGF basal expression was increased in the four PHD2 shRNA cell strains compared with the parental strain (Fig. 5A). To determine if these expression patterns had functional significance, GAPDH and LDH enzyme assays were done. These assays confirmed the

mRNA data and showed that shPHD2-a strains 7 and 21 have a higher rate of glycolytic activity. This activity was similar to that of the malignantly transformed PH3MT cell strain (Fig. 5B). Cell growth assays were also done on each of the cell

strains in the presence and absence of hypoxia. The malignant strains shPHD2-a 7 and 21 and PH3MT had increased growth characteristics under hypoxic stress compared with the parental MSU-1.1 and scrambled control (Fig. 5C). The shPHD2-a
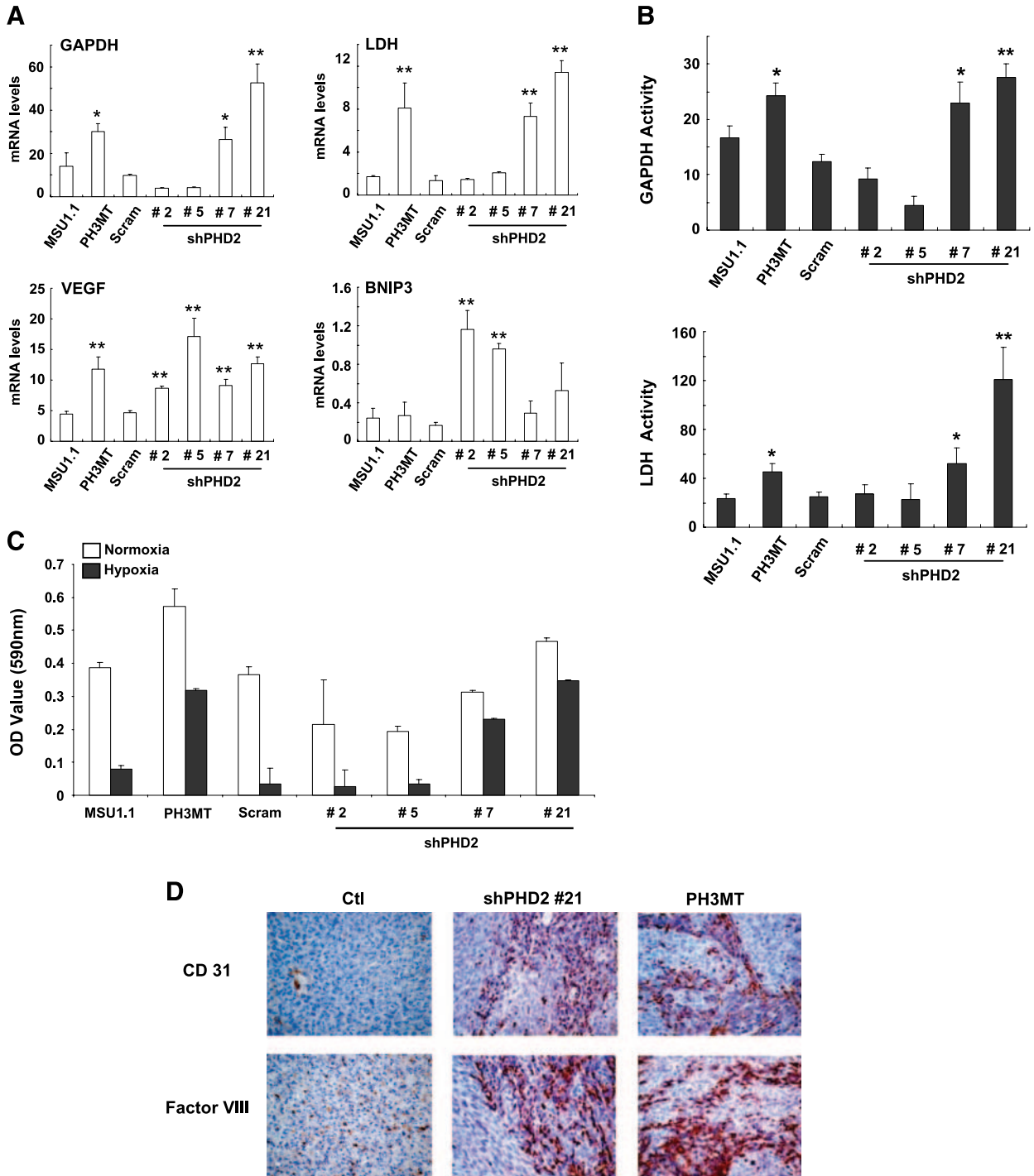

FIGURE 5. Cellular responses in shPHD2 strains. **A.** mRNA levels of GAPDH, LDH, BNIP3, and VEGF were determined in MSU-1.1; PH3MT; scrambled shRNA-infected strain (*Scram*); and shPHD2-a strains 2, 5, 7, and 21 using qRT-PCR ($n = 6$, *$P < 0.05$, **$P < 0.01$). **B.** GAPDH and LDH activities were determined in MSU-1.1, PH3MT, scrambled shRNA–infected strain (*Scram*) and shPHD2-a strains 2, 5, 7, and 21. Kinetic activity was normalized to protein concentration ($n = 8$, *$P < 0.05$, **$P < 0.01$). **C.** Each cell strain was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following exposure to normoxia (20% O_2, *white column*) or hypoxia (1% O_2, *black column*) for 3 d ($n = 4$). **D.** CD31 (PECAM) and factor VIII immunostaining was used to visualize vascularization in formalin-fixed tumor derived from shPHD2 strain 21, PH3MT, and a benign growth derived from an early MSU lineage (*Ctl*).

2 and 5 strains exhibited increased doubling time and inability to grow under hypoxic stress (Fig. 5C). Finally, the functional consequence of increased VEGF expression in shPHD2-a clone 21 was confirmed by immunostaining of the tumors derived from these clones with the endothelial-specific antibodies for CD31 (PECAM) and factor VIII. The tumors resulting from cells with moderately decreased PHD2, PH3MT, and shPHD2 21 showed a range of vascularization (Fig. 5D). These data support the biphasic model and are evidence that small decreases in PHD2 activity can lead to malignant transformation, increased glycolysis, and vascularization, whereas severe loss of PHD2 activity can inhibit cell viability and tumor-forming potential through stimulation of cell death pathways.

The Inhibition of PHD Activity Reverses the Transformed Characteristics of Cells

To further support the biphasic model and provide evidence that the results described in Figs. 3-5 were directly related to PHD activity, we inhibited PHD2 in malignantly transformed PH3MT and nontransformed MSU-1.1 cells using dimethyloxalyl glycine (DMOG). If the model is correct, mild inhibition of PHD in the MSU-1.1 should increase transformed characteristics in MSU-1.1 cells and higher concentrations of DMOG in both cell types should reverse their tumor-forming potential. DMOG was capable of inhibiting PHDs in a dose-dependent manner as evidenced by the increasing stability of HIF1α, with a maximal effect observed at 1 mmol/L (Fig. 6A). To determine if the inhibition of PHD activity alters the tumor-forming potential of PH3MT and MSU-1.1. cells, we analyzed the ability of these cells to form colonies in an anchorage-independent manner in the presence of DMOG (Fig. 6B). As predicted by the model, low concentrations of DMOG (i.e., <0.7 mmol/L) could force a percentage of the MSU-1.1 cells to form small colonies in soft agar. Higher concentrations of DMOG (i.e., 0.7 mmol/L or greater) led to a complete inhibition of the ability to form colonies in soft agar in both cell types. These results support the biphasic model and suggest that DMOG is capable of modulating the anchorage-independent growth of the transformed cells. One possible explanation for this inhibition may be due to growth inhibition or cellular senescence at higher concentrations. Presumably, this is caused by high basal expression of pro-death genes, such as *BNIP3*, as described for shPHD2-a strains 2 and 5 (Fig. 5A). Indeed, inhibition of PHD2 activity by DMOG in tumorigenic MSU 1.1 and PH3MT cells led to a significant increase in BNIP3 expression (Fig. 6C). These results suggest that malignantly transformed cells (e.g., PH3MT) can lose their tumor-forming potential in the presence of PHD inhibitors and a pro-death response is involved in this phenotypic alteration. Moreover, a nontransformed cell (e.g., MSU-1.1) can gain some transformed characteristics following exposure to low doses of DMOG.

Effects of Alterations in PHD2 Levels in the Transformed Cell Strain, PH3MT

The model also predicts that altering the PHD2 levels in malignantly transformed cells would alter the cell tumor-forming potential. To test this hypothesis, we altered the PHD2 activity in PH3MT cells. First, PH3MT cells were infected with the shPHD2 cassettes described above, and strains were selected. We were unable to expand any of the 25 cell strains, suggesting these PHD2 shRNA-expressing cells were prone to premature cell death (data not shown). This observation is supported by the previous results in which DMOG treatment led to inhibition of anchorage-independent growth in malignantly transformed cells with a corresponding increase in pro-cell death factor, BNIP3, expression. Each of these results is evidence that decreasing PHD2 activity within tumorigenic cells leads to a loss in tumor-forming potential.

The model proposed in Fig. 4B also suggests that increasing PHD2 levels within these same cells will lead to a similar decrease in tumor-forming potential. To test this, PH3MT cells were infected with the retroviral vector, pZome-1N, encoding the cDNA for PHD2. pZome-1N produces a tagged PHD2 protein and its expression can be distinguished from endogenous PHD2 based on its molecular size. In addition, a cDNA for green fluorescent protein (GFP) was also inserted into separate pZome-1N and was used to create a control cell strain. PHD2 cell strains were screened for overexpression of PHD2 using Western blot analysis and four of these (PHD2 strain 6, 8, 11, and 22) were selected for further analysis (Fig. 7A). Tagged PHD2 and GFP are visualized on the β-actin Western blot due to the protein A motif within the tag. We next examined whether overexpression of PHD2 affects hypoxia-induced HIF1α stabilization (Fig. 7B). As expected, HIF1α was undetectable under normoxia and its levels were increased by hypoxia in control, PH3MT, and GFP strains 3 and 4. However, the PHD2-overexpressing cell strains (PHD2 strains 6, 8, 11, and 22) showed reduced hypoxia-induced HIF1α accumulation. These results are in agreement with previous published reports showing that increased PHD expression can inhibit HIF1α accumulation and HIF1 activity under hypoxic stress (31-33). In addition, overexpression of PHD2 had functional consequences on HIF1-mediated up-regulations of GAPDH, LDH, and VEGF. The hypoxia-induced expression of these genes was diminished in all PHD2 strains (PHD2 strains 6, 8, 11, and 22) when compared with hypoxia-treated control cells, PH3MT, and GFP strains 3 and 4 (Fig. 7C). GAPDH and LDH enzyme assays confirmed the mRNA data and showed that PHD2 strains 6, 8, 11, and 21 lost their hypoxia-induced glycolytic activity (Fig. 7D).

To determine if PHD2 overexpression can alter the transformed phenotype of a cell, each of the PHD2 cell strains was analyzed for its ability to form colonies in soft agar (Fig. 8A). The tumorigenic parental PH3MT and control GFP strains 3 and 4 exhibited large colonies when grown in agar, whereas the PHD2 clonal cell strains 6, 8, 11, and 22 lost this ability to grow in an anchorage-independent manner. To determine whether overexpression of PHD2 leads to decreases in tumor-forming potential, PHD2 strains 6, 8, 11, and 22 were examined for their ability to form tumors in athymic mice (Fig. 8B). Parental PH3MT and GFP strains 3 and 4 were used as a control and yielded tumors within 6 weeks (Fig. 8B). In contrast, all of the PHD2 strains (strains 6, 8, 11, and 22) were negative for tumor formation after 5 months (Fig. 8B). These results show that the increase of the PHD2 activity in malignantly transformed cells can inhibit the cell-transformed phenotype and support our biphasic model relating PHD2 with tumor-forming potential.
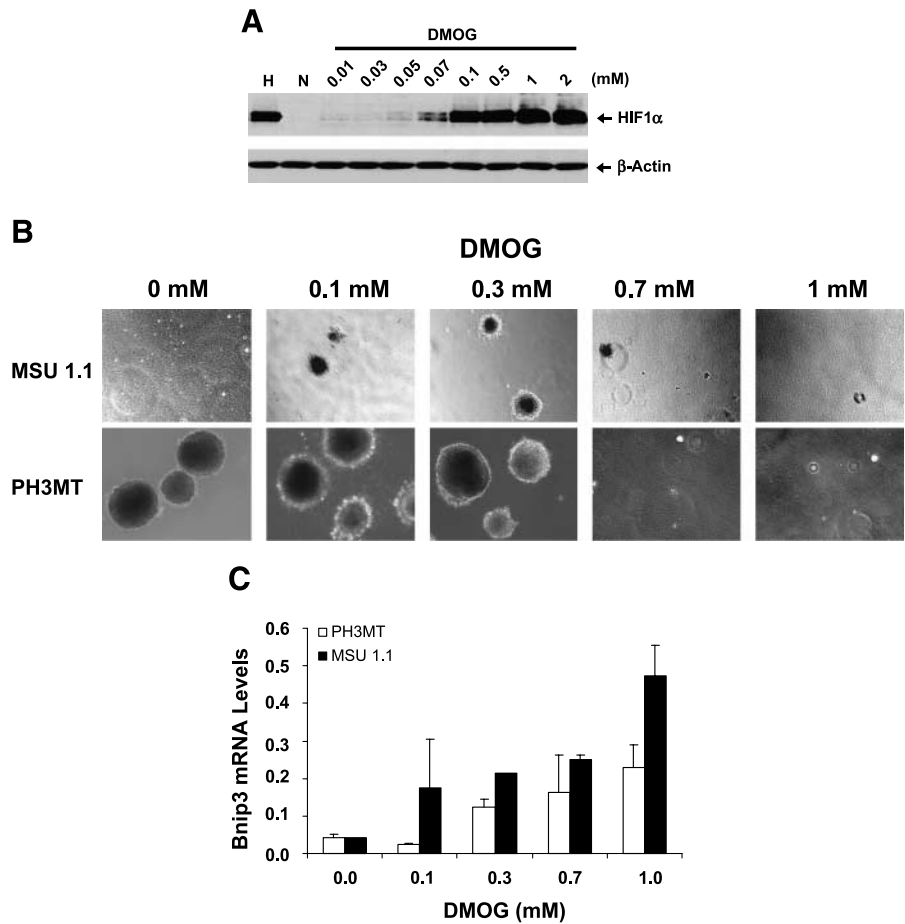
FIGURE 6. Inhibition of PHD2 activity in PH3MT and MSU-1.1 cells. **A.** HIF1α protein levels were determined in PH3MT cells treated with various concentrations of DMOG by Western blot analysis. HIF1α levels in PH3MT cells exposed to normoxia (20% O_2) or hypoxia (1% O_2) were also analyzed as a control, and β-actin antibody was used as a loading control. **B.** PH3MT and MSU 1.1 cells were assessed for anchorage-independent growth using soft agar assay, in the presence or the absence of varying concentrations of DMOG ($n = 10$). **C.** BNIP3 mRNA levels were determined in MSU-1.1 (*black columns*) and PH3MT (*white columns*) cells in the presence or the absence of varying concentrations of DMOG ($n = 6$, * $P < 0.05$).

Discussion

The results presented show that decreases in PHD2 correlate with increasing tumor-forming potential and invasiveness in engineered fibroblasts and human cancer cell lines. Moreover, direct manipulation of PHD2 levels can change the tumor-forming potential of a cell. This change in tumor-forming potential is accompanied by alterations in the adaptive response (i.e., VEGF, GAPDH, and LDH expression and activity) and the cell death response (i.e., BNIP3 expression and changes in viability under hypoxic stress). Finally, the results also suggest that these changes in tumor-forming potential are most likely due to PHD2-mediated changes in the HIF1α/hypoxia signaling cascade. Although no direct evidence was presented, there are several lines of evidence to support a role for HIF1α in these PHD2-induced changes in cellular transformation. First, the changes in endogenous levels of HIF1α in the engineered MSU cells correlate with the PHD2 levels. Second, manipulation of PHD2 in the MSU cells leads to changes in HIF1α levels and activity. Finally, the change in PHD2 levels leads to corresponding changes in a battery of known HIF1α target genes, including *GAPDH* and *VEGF*.

PHD/HIF1-regulated genes are involved in many cellular processes, including cell proliferation, angiogenesis, metabolism, migration, and others, which are required for adaptive survival of tumor cells (3, 4, 6, 19). Alternatively, severe

hypoxia exposure can lead to cell death through HIF1-mediated up-regulation of pro-cell death factors or p53-dependent processes (21, 34-36). PHD-mediated hypoxia signaling, therefore, regulates a delicate balance between life and death through cellular adaptation and a programmed death response. Survival of a cancer cell is dependent on its ability to maintain cell growth and/or decrease its programmed death response once it is exposed to the hypoxic microenvironment of a tumor. The correlation between PHD2 levels and tumor-forming potential suggests that these hydroxylases might be involved in altering this balance (Figs. 1-4). Presumably, small decreases in PHD activity would promote an adaptive response without increasing pro-death signals (Fig. 5). In addition, the decreased hydroxylase activity and subsequent increase in HIF1-mediated signaling can explain the observation that tumors and corresponding cell strains have an increase in hypoxia signaling, even in the presence of normal oxygen concentrations. The direct link between decreased PHD activity, increased HIF1 signaling, and increased glycolytic activity might also explain the Warburg effect (37). Previous reports have shown that HIF1 is necessary for the Warburg effect, and a cellular decrease in PHD activity would explain the increased tumor dependence on aerobic glycolysis (6, 18, 38, 39). It is possible that one step in the transformation process is the sustained decrease in PHD activity through genetic or
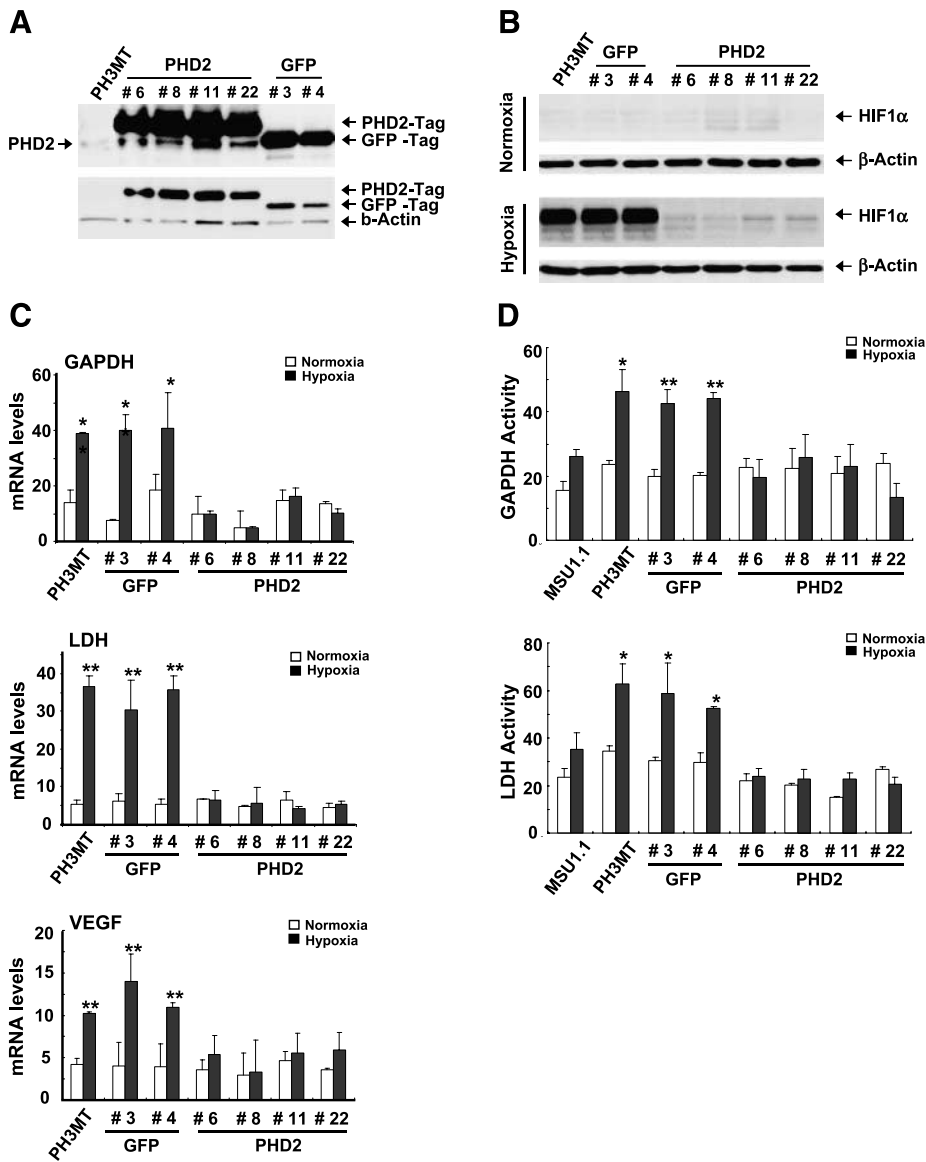
FIGURE 7. Overexpression of PHD2 in PH3MT cells. **A.** PH3MT cells were infected with a retroviral construct that expresses PHD2 cDNA. The expressions of PHD2-TAP were characterized in the parental PH3MT; PHD2 strains 6, 8, 11, and 22; and GFP cell strains 3 and 4 by Western blot analysis using a PHD2-specific antibody or β-actin antibody. The PHD2 and GFP proteins are visible due to the protein A tag. **B.** HIF1α protein levels in PHD2 strains following exposure to normoxia (20% O_2) or hypoxia (1% O_2) were analyzed by Western blotting with a HIF1α monoclonal antibody or β-actin antibody. **C.** mRNA levels of GAPDH, LDH, and VEGF were determined in PH3MT; GFP strains 3 and 4; and PHD2 strains 6, 8, 11, and 22 using qRT-PCR. Cells were exposed to normoxia (20% O_2, *white column*) or hypoxia (1% O_2, *black column*) for 16 h ($n = 8$, *$P < 0.05$, **$P < 0.01$). **D.** GAPDH and LDH activities were determined in MSU-1.1; PH3MT; GFP strains 3 and 4; and PHD2 strains 6, 8, 11, and 22. Cells were exposed to normoxia (20% O_2, *white column*) or hypoxia (1% O_2, *black column*) for 16 h, and enzymatic activity was normalized to protein concentration ($n = 8$, *$P < 0.05$, **$P < 0.01$).

epigenetic mechanisms. This would serve to preadapt the cells (e.g., increased glycolytic rate) to the hypoxic environment found in many, and perhaps all, tumors and give them a growth advantage upon tumor development. It is also possible that this loss of PHD activity and subsequent increased glycolytic activity causes the malignant transformation, as Warburg had proposed (37).

Interestingly, cells with a severe loss of PHD2 showed no ability to form tumors in athymic mice (Figs. 3 and 4A). These cell strains, both MSU-1.1–derived and PH3MT-derived, displayed growth abnormalities such as signs of premature cell death, increased doubling time, and an inability to grow under hypoxic stress (Fig. 4B). It is hypothesized that this is due to an uncontrolled pro-death response and that this response is driven by direct HIF1-mediated transcription of genes such as *BNIP3* and *NIX*. The almost complete loss of PHD2 in shPHD2 strains 2 and 5, and subsequent HIF1 activation, would also

presumably contribute to p53-mediated cell cycle arrest and apoptosis. Given the overwhelming pro-death response following the almost complete loss of PHD2 activity, no amount of adaptive cell signaling can support continued expansion in the tumor microenvironment.

These two groups of cell strains, mild decrease and severe decrease in PHD2 levels, led to the proposed biphasic model presented in Fig. 4B. This model predicts that transformed cells are within the phase of the curve that supports tumor formation and movement in either direction (more or less PHD activity) will alter the cell tumor-forming potential. The tumorigenic PH3MT cells and modulation of PHD2 levels in these cell strains strongly support our model. First, the PH3MT cells have a decreased level of PHDs when compared with the MSU-1.0 or MSU-1.1 (Fig. 2B). Second, PH3MT cells that express the PHD2 shRNA cassette (movement left along the abscissa; Fig. 4B) stop growing after colony selection and cannot be

expanded. Third, tumorigenic cells (PH3MT, shPHD2-a 7 and 21) lost their transformed characteristics when PHD activity was drastically decreased by a hydroxylase inhibitor (Fig. 6). Fourth, overexpression of PHD2 (movement right along the abscissa; Fig. 4B) in the PH3MT cells inhibits the ability of the cell to grow in an anchorage-independent manner and form tumor in athymic mice, suggesting they have lost a transformed phenotype (Fig. 8). Finally, a recent report has shown that overexpression of PHD1 in colon cancer cells decreases tumor growth (26). Taken together, these results provide evidence for the biphasic model of PHD activity and tumor-forming potential and suggest the clinical importance of characterizing the factors that establish the boundaries dictating the separate areas within this model.

The biphasic model will have profound consequences on chemotherapeutic agents that target PHDs and the hypoxia signaling cascade for cancer therapy. Cells that have amassed the cellular mutations necessary to progress toward complete transformation (e.g., MSU-1.1 cells) might become fully tumorigenic when exposed to a drug that only partially blocks this cascade, whereas other cells at a different transformation stage (e.g., PH3MT cells) will undergo cell death. Alternatively, new therapies that completely shut down PHD activity will have detrimental consequences on normal cellular homeostasis

by promoting an uncontrolled pro-death response. Ideally, any drug targeting this pathway should be specific to precancerous and hypoxic cells, thus increasing the chance that decreases in PHD activity within the cell will push these cells toward cell death. The data presented here implies that cells will have a biphasic response to such agents and if this is the case, it will create difficulties when targeting these enzymes for cancer therapeutics.

Materials and Methods

Cell Culture, DMOG Treatment, and Western Blot Analysis

MSU-1 cell strains were maintained in αMEM (Mediatech, Inc.), and human embryonic kidney cells (HEK293) and phoenix-ampho cells were cultured in DMEM (Mediatech) supplemented with 10% FCS (HyClone), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Invitrogen). Cells were grown in a 37°C incubator with 5% CO_2 (Precision). DMOG (Sigma) was dissolved in 1× PBS before use in these studies.

Preparation of total and nuclear proteins and Western blot analysis were done as described previously (22, 40). The following antibodies were used: rabbit polyclonal anti-PHD1,
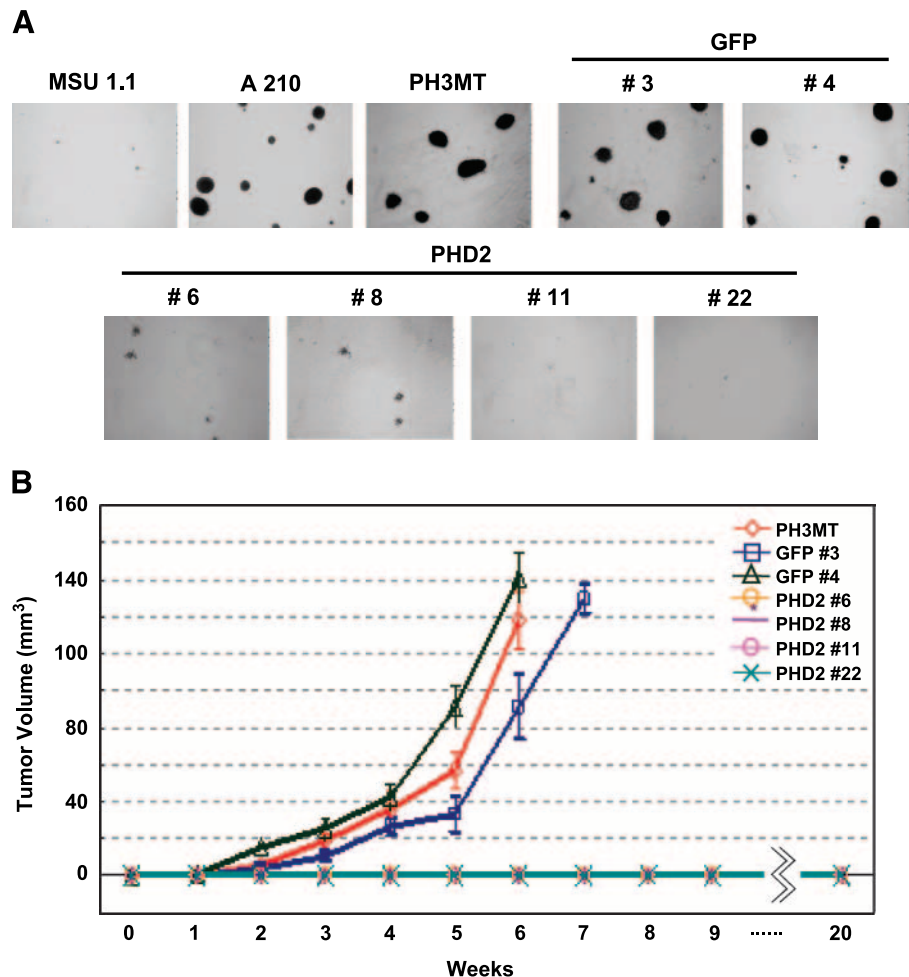

FIGURE 8. Characterization of tumor-forming potential in PHD2 strains. **A.** PHD2 strains 6, 8, 11, and 22 and GFP strains 3 and 4 were assessed for anchorage-independent growth by forming colonies in soft agar. Parental PH3MT cells and A210 and MSU-1.1 cells were used as positive controls and negative control, respectively (n = 10). **B.** PHD2 strains 6, 8, 11, and 22 and GFP strains 3 and 4 were injected into athymic mice and tumor growth was monitored weekly for 5 mo (n = 10).

anti-PHD2, and anti-PHD3; mouse monoclonal anti-HIF1α and anti-HIF2α (Novus); rabbit polyclonal anti–β-actin (a generous gift from Dr. John Wang, Michigan State University, East Lansing, MI), and goat anti-rabbit and mouse (Sigma).

Quantitative Real-time PCR Analysis

For gene expression analysis, cells were exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 16 h. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined by UV spectrometry and analyzed for integrity using spectrometry and denaturing gel electrophoresis. One microgram total RNA was reverse-transcribed using Super-Script First-Stranded Synthesis System (Invitrogen) according to the manufacturer's protocol and primed with oligo(dT)$_{18}$ primers. Gene expression was measured by quantitative real-time PCR (qRT-PCR), based on Sybr-Green methodology (Applied Biosystems) as previously described (41). Analysis was done using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers used for qRT-PCR were as follows: *VEGF*, 5′-TCCTCACACCATTGAAACCA-3′ and 5′-GATCCTGCCCTGTCTCTCTG3′; *PHD1*, 5′-CAGGATGG-GAGTGGAGAGTT-3′ and 5′-AGTGGTAGAGGTGGC-TGTGG-3′; *PHD2*, 5′-GAGCTGTGCGGGAAGATG-3′ and 5′-GCACACGAGCTTGTGCTTCT-3′; *PHD3*, 5′-AGCTTCC-TCCTGTCCCTCAT-3′ and 5′-ACGTGGCGAACATAACC-TGT-3′; *HIF1α*, 5′-ACAAGTCACCACAGGACAG-3′ and 5′-AGGGAGAAAATCAAGTCG-3′; *GAPDH*, 5′-CAGCCT-CAAGATCATCAGCA-3′ and 5′-GTCTTCTGGGTGGCAGT-GAT-3′; *LDH*, 5′-AGGCCCGTTTGAAGAAGAGT-3′ and 5′-TGCACAACCTCCACCTAGAA-3′; *BNIP3*, 5′-GCTGGAACACGTACCATCCT-3′ and 5′-ATCTGCC-CATCTTCTTGTGG-3′. The level of each gene was normalized to the expression level of the *hypoxanthine phosphoribosyl-transferase* (*HPRT*) gene and each primer set was determined to be specific by BLAST and dissociation curve analysis.

Design of shRNA and shRNA-Lentiviral Constructs

The sequences of shRNA targeting PHD1, PHD2, or PHD3 (three independent sequences per isoform) were designed using the Ambion web-based design tool;[9] *PHD1-a*, TCA-GAACTGGGACGTTAAG; *PHD1-b*, GACTATATCGTGCC-CTGCATG; *PHD1-c*, CGCAGGAAGGCCATGGTGGCG; *PHD2-a*, TAAAGACTGGGATGCCAAG; *PHD2-b*, GACGA-AAGCCATGGTTGCTTG; *PHD2-c*, CTTCAGATTCGG-TCGGTAAAG; *PHD3-a*, TCTGCCCTCACTGAAGACT; *PHD3-b*, GTCTAAGGCAATGGTGGCTTG; *PHD3-c*, CAGGTTATGTTCGCCACGTGG. Control scrambled shRNA, TGCGTCTTGTTCATCTCCT, was also designed using a web-based tool.[10] The sequence of each shRNA was analyzed by BLAST to ensure specificity for each target. shRNA constructs were generated using a two-step PCR approach. Briefly, the first-round PCR generated an amplicon of the U6 promoter with the sense strand of the shRNA cassette and the loop. The second round of PCR added the antisense strand of shRNA

cassette. The shRNA cassettes that had been generated were then cloned into the pGEM-T-Easy vector (Promega). Once shRNA cassettes were verified as functional, they were subcloned into the lentiviral vector, pVCwPBam vector (a generous gift from Dr. David Looney, University of California San Diego, San Diego, CA). A lentiviral vector, pVCwPBam, was used for the expression of shRNA cassette and it contains a puromycin-resistant sequence for the creation of stable cells.

Luciferase Assay

Each cell strain was transiently transfected using Lipofect-amine 2000 via the manufacturer's instructions (Invitrogen). DNA used in the transfections was diluted in Opti-MEM medium (Invitrogen) and consisted of an HRE-driven luciferase reporter (42) and shRNA-pGEMT-easy plasmids described above. A β-galactosidase expression vector was also used to control for transfection efficiency. Separate transfections were done using a similar protocol with expression plasmid for PHD1, PHD2, or PHD3 (a generous gift of Dr. Steven McKnight, University of Texas, Southwestern, Dallas, TX; ref. 13). Each transfection was adjusted to contain the same total concentration of transfected DNA using an empty expression vector. Following transfection, the cells were incubated for 16 h and then exposed to normoxia (20% O_2) or hypoxia (1% O_2) for 18 h. Cells were lysed and analyzed for luciferase activity using Luciferase Assay System (Promega) according to the manufacturer's protocol.

Viral Packaging, Infection, and Selection

The three independent shPHDs and scrambled shRNA-pVCwPBam vectors were cotransfected into HEK293 cells with the packaging plasmids, pC34N and pVSV-G, as described above (a generous gift from Dr. David Looney, University of California San Diego, San Diego, CA). Following a 24-h incubation, the medium was replaced with fresh growth medium and the cells were incubated for an additional 24 h. Viral particles were purified by centrifugation ($650 \times g$ for 5 min), followed by filtering with 0.45-μm membrane filter (Millipore). MSU-1.1 cells (1×10^5) were plated 24 h before infection on 60-mm tissue culture dishes. Purified virus containing polybrene (5 μg/mL, Sigma) was added to the cells and incubated for 24 h. Medium was replaced and the cells were cultured for 48 h. Infected cells were then selected with puromycin (0.4 μg/mL, USBiologicals) and individual clones were isolated.

The cDNAs for PHD2 and GFP were inserted into the retroviral vector pZOME-1N and were packaged using Phoenix-ampho cells (a generous gift from Garry Nolan, Stanford University, Palo Alto, CA; ref. 43). Purified viral particles were used to infect PH3MT cells, and infected cells were selected and individual clones were isolated as described above.

Anchorage Independence and Tumor Formation Assays

Assessment of the ability of the cell to grow in an anchorage-independent manner was done as previously described (44). Cells were fed weekly for 3 wk and fixed in glutaraldehyde (2.5%). When necessary, DMOG was added to feed medium at the desired concentrations.

Athymic BALB/c mice, 5 wk of age, were implanted s.c. in the rear flank with 1-cm^3 absorbable gelatin sponges (Pharmacia/Upjohn Company) to serve as a matrix for cell injection. One week after implantation, 10^7 cells suspended in 0.2 mL MEM were injected into each sponge. The mice were monitored weekly for tumor growth and the size of tumor was measured. When the tumor reached 1 cm in diameter, the mice were euthanized and the tumors were removed. Tumors were then fixed with neutral buffered formalin for histologic examination. All mice were euthanized 5 mo after injection, even if tumors were not observed.

GAPDH, LDH, and 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide Assays

Cells were lysed with 100 μL of 1% Triton X-100 (Sigma) and cell debris was removed by centrifugation ($3,500 \times g$ for 5 min). Supernatants (2.5 μL) were transferred into 96-well plate, and 200 μL of GAPDH reagent [100 mmol/L $C_7H_{16}NOSNa$ (pH 8.6), 1 mmol/L NAD^+, 1.5 mmol/L DL-glyceraldehyde 3-phosphate (Sigma), and 20 mmol/L NaH_2PO_4, 6 mmol/L cysteine (Fisher Scientific)] or LDH reagent [50 mmol/L K_2HPO_4, 200 μmol/L $NADH.Na_2$ (Sigma) and 6.5 mmol/L pyruvate (Invitrogen)] were added to each well for GAPDH or LDH assays, respectively. NADH kinetics was measured by absorbance at 340 nm for 5 min at 37°C with a 12-s reading interval. Values were normalized to protein concentration of sample. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done as previously described (22).

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest with regard to this publication.

Acknowledgments

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The Biphasic Role of the Hypoxia-Inducible Factor Prolyl-4-Hydroxylase, PHD2, in Modulating Tumor-Forming Potential

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