Aberrant Promoter CpG Methylation Is a Mechanism for Impaired PHD3 Expression in a Diverse Set of Malignant Cells

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Abstract

Background: The prolyl-hydroxylase domain family of enzymes (PHD1-3) plays an important role in the cellular response to hypoxia by negatively regulating HIF-α proteins. Disruption of this process can lead to up-regulation of factors that promote tumorigenesis. We observed decreased basal expression of PHD3 in prostate cancer tissue and tumor cell lines representing diverse tissues of origin. Furthermore, some cancer lines displayed a failure of PHD3 mRNA induction when introduced to a hypoxic environment. This study explores the mechanism by which malignancies neither basally express PHD3 nor induce PHD3 under hypoxic conditions.

Methodology/Principal Findings: Using bisulfite sequencing and methylated DNA enrichment procedures, we identified human PHD3 promoter hypermethylation in prostate, breast, melanoma and renal carcinoma cell lines. In contrast, non-transformed human prostate and breast epithelial cell lines contained PHD3 CpG islands that were unmethylated and responded normally to hypoxia by upregulating PHD3 mRNA. Only treatment of cells lines containing PHD3 promoter hypermethylation with the demethylating drug 5-aza-2'-deoxycytidine significantly increased the expression of PHD3.

Conclusions/Significance: We conclude that expression of PHD3 is silenced by aberrant CpG methylation of the PHD3 promoter in a subset of human carcinoma cell lines of diverse origin and that this aberrant cytosine methylation status is the mechanism by which these cancer cell lines fail to upregulate PHD3 mRNA. We further show that a loss of PHD3 expression does not correlate with an increase in HIF-1α protein levels or an increase in the transcriptional activity of HIF, suggesting that loss of PHD3 may convey a selective advantage in some cancers by affecting pathway(s) other than HIF.

Introduction

The cellular response to reduced oxygen availability (hypoxia) is controlled by a class of proteins called hypoxia-inducible factors (HIF-α). There are 3 known isoforms of HIF-1α: HIF-1α, HIF-2α and HIF-3α. HIF-1α and HIF-2α are transcription factors. HIF-3α appears to lack transcriptional activity and may play a role in negative regulation of the HIF pathway [1]. Thus, from here on, when referring to HIF-α, we are referring to only HIF1 and HIF2. Transcriptionally active HIF1 and 2 are heterodimers composed of the HIF-α subunit and aryl hydrocarbon nuclear translocator receptor [ARNT/HIF-β]. HIF-1α activates the transcription of EPO, VEGF, heme oxygenase-1 and several other critical intracellular responses to hypoxia including enzymes of the glycolytic pathway [2,3]. While less is known about HIF-2α transcriptional targets, HIF-2α appears to play a lesser role in the glycolytic response with more emphasis on EPO and VEGF transcription [4].

HIF-α mRNA levels are generally stable in cells. It is not until after translation that HIF-α is tightly controlled. During periods of normal physiological oxygen concentration, HIF-α subunits are kept at low levels by constant proteolytic degradation. First, a hydroxylase reaction is catalyzed by a family of prolyl hydroxylase domain-containing proteins (PHD/EGLN/HPH) which utilize iron, oxygen and 2-oxoglutarate as co-factors to enzymatically catalyze hydroxylase on the oxygen-dependent
degradation domain (ODD) of the HIFα-subunit [5]. Hydroxylated proline residues on HIF-α are recognized by Von Hippel-Lindau (VHL) protein, an E3 ubiquitin ligase that ubiquitinates the HIF-α subunit, targeting it to the proteasome [6]. Under hypoxic conditions, HIF prolyl hydroxylase activity is decreased and HIF-1α protein accumulates. HIF-α subunits translocate to the nucleus and dimerize with the constitutively expressed ARNT subunit [7,8]. This heterodimer acts to turn on transcription of genes involved in oxygen homeostasis and glucose metabolism [2].

Three main isoforms of HIF prolyl-hydroxylase domain containing proteins, PHD1-3, have been identified [9]. These isoforms have been reported to have different specificities for HIF-1α and HIF-2α [10], and also differ in their subcellular localization. It has been shown that PHD1 is exclusively present in the cytoplasm, PHD2 is mainly located in the nucleus and PHD3 is evenly distributed in both cytoplasm and nucleus [11]. PHD2 and PHD3, however, are considered to be the major isoforms that contribute to HIF-1 and -2α degradation in cells [12,13]. In normoxia, PHD2 is the primary enzyme that hydroxylates HIF-1α [14], whereas PHD3 has been reported to play an important role in HIF-2α hydroxylation and also in retaining cellular hydroxylase capacity in a hypoxic environment [10,15].

In normal cells, PHD3 mRNA and protein are expressed at low levels during normoxia, but are significantly induced upon exposure to hypoxia. In contrast, PHD3 expression in a significant number of cancer cell types has been shown to be low or absent not only during normoxia, but also under hypoxic conditions [10,16]. To date, no mechanism has been discovered to explain this defect in hypoxic inducibility. Interestingly, Hatzimichael et al. have recently demonstrated that the promoter of PHD3 is methylated in certain primary B-cell dyscrasias [17]. We had observed a decrease in PHD3 mRNA expression in human breast and prostate carcinoma cell lines, with an absence of PHD3 upregulation in response to hypoxia. Therefore, we were interested to determine whether PHD3 promoter methylation was responsible for this aberrant expression pattern. In this study, we show that the promoter region of PHD3 is methylated in representative human prostate carcinoma, melanoma, renal carcinoma and breast cancer cell lines. Furthermore, we show that neither HIF-1α protein levels nor hypoxic response through an HRE-luciferase reporter vector are compromised in PHD3 methylated compared to non-methylated cell lines. These results indicate that PHD3 promoter methylation is utilized by malignancies derived from diverse human cell types. Furthermore, these data suggest that loss of PHD3 expression may not affect the transcriptional response through the HIF pathway, leaving open the possibility that PHD3 silencing in tumors is selected through the loss of specific interactions with other cellular pathways.

Methods

Cell culture

Normal human prostate epithelial cells (NPrEC) were purchased from Clonetics, Lonza Inc. (Walkersville, MD) and were grown on the recommended PrEGM media supplied by Clonetics, Lonza Inc. The hTERT-HME1 cells were cultured in mammary epithelial basal medium MEGM (Lonza Inc.) at 37°C and 5% CO₂ according to the manufacturer’s instructions (Lonza Inc.). The DU 145, 22RV.1, PC-3, MDA-MB-435 (MB-435), and MCF7 cell lines were obtained from ATCC (Manassas, VA). MCF7, DU145 and MDA-MB-435 cells were cultured in Eagle’s Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS). PC-3 cells were cultured in F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM Na Pyruvate, and supplemented with 100 U/ml pen/strep. All cell lines were routinely maintained at 37°C in a humidified atmosphere with 5% CO₂. Fresh media was replaced every three days while routine subculture was performed by washing with 1X PBS and detaching cells with TrypLE Express.

Semi-quantitative RT-PCR

Total RNA was extracted from individual cell lines using RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified using a NanoDrop 1000. To assess PHD3 and GAPDH expression, 500 ng of total RNA was used for reverse transcription using a OneStep RT-PCR Kit (Qiagen). The PHD3 forward primer is 5'-GGGGATAC-TACGTCAGGGAG-3' and the reverse primer is 5'-AGTCTT-CAGTGAGGGCAGATTC-3'. GAPDH expression was assessed using GAPDH-specific primers. PCR conditions for PHD3 and GAPDH were the same except that 28 cycles of PCR were performed for PHD3 analysis and 23 cycles were performed for GAPDH. The parameters used were: 95°C for 5 minutes followed by the stated number of cycles of 94°C for 1 minute; 56°C for 1 minute, and 72°C for 1 minute, ending with a final extension at 72°C for 7 minutes. The amplified products were electrophoresed on a 1% agarose gel and stained with ethidium bromide to visualize the bands.

Quantitative real time RT-PCR

Total RNA was isolated from cells using Trizol, followed by DNase treatment and NaOAc precipitation. The reverse transcription was carried out with High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PHD3 TaqMan primer-probe was utilized from Applied Biosystems (Hs00222966_m1). The quantitative real-time PCR was set up as follows: 10 ng of RNA was used as template for each real-time PCR reaction (10 ng reaction volume); primer pairs at 0.3 μM for GAPDH with Syber Green Master Mix (Applied Biosystems). For PHD3, TaqMan universal master mix was used. The DNA polymerase was activated by heat at 95°C for 10 min followed by 40 cycles, denaturing at 95°C for 15 s, annealing and elongating at 60°C for 1 min. Data were collected with ABI PRISM 7000 sequence detection system. Data were analyzed using the ΔΔCt method.

Western Blot analysis

Cells were immediately washed with ice cold phosphate-buffered saline (pH 7.4). Cells were lysed on the plate in 200 μl RIPA cell-lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na Deoxycholate, 1% TX-100) plus 1 mM NaF, 10 mM NaVO₄, 10 mM PMSF, and 1/100 protease inhibitor cocktail (Sigma), immediately boiled for 2 minutes and then sonicated. SDS-polyacrylamide gels (7%, PHD3; 15% HIF1, HIF2) were used for protein electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes and treated with anti HIF-1α (Abcam, Cambridge, MA) 1:500 overnight at 4°C. Anti PHD3, NB100-139 and anti HIF-2α antibodies (Novus Biologicals, Littleton, CO) were used at 1:500 and 1:200 respectively overnight at 4°C. Equal protein loading was confirmed on all immunoblots using human actin antibody (Sigma, St. Louis, MO) at a dilution 1:2000. Goat anti-rabbit IgG (BD Transduction Laboratories, San Diego, CA) was used as a secondary antibody against all primary antibodies. Bands were visualized by chemiluminescence with ECL plus reagent (Pierce, Rockford, IL) on a Typhoon FLA 7000.

Sodium bisulfite sequencing

Genomic DNA was extracted with the use of the DNeasy Tissue Kit (Qiagen, Valencia, CA), and sodium bisulfite conversion was...
performed with the use of the EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA). A pair of primers was designed to amplify the PHD3 promoter of both bisulfite modified methylated and unmethylated DNA but not unmethylated DNA. Nested PCR amplification on converted DNA used the following primers: outside forward: 5'-GTGTGGGATTGTTTTT-TAG-3' (SB1); outside reverse: 5’-CCAAATCAACCCTCA-TATATAT-C-3’ (SB2); and nested inner primers (SB3) and (SB4) whose sequences and locations are described in detail below.

The resulting PCR products were gel-extracted with the use of the Qiagen Gel Extraction Kit, or gel digestion with β-agarase followed by EtOH precipitation, and cloned with the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was extracted with the use of the QiaPrep Spin Plasmid Miniprep Kit (Qiagen). Sequencing was performed by the sequencing core facility maintained by the University of Iowa and results were tabulated for methylation status of each of the 58 CpGs contained in the amplicons from each cell line.

5-Aza-dC treatment

Cells were counted, and seeded (day 0) at approximately 750,000 cells/100 mm dish. Fresh 5-Aza-dC (5 mM) was added to the dish on days 1, 3, and 5 while a control flask was left untreated. On day 3, 5-Aza-dC treated cells were split into two 60 mm dishes in media supplemented with 5 mM 5-Aza-dC. On day 6, one of each of the 60 mm dishes was placed in a hypoxia chamber and plated under 1% O2, 94% N2 and 5% CO2 at 37°C. On day 7, all cells were harvested with 500 μl Trizol for RNA extraction.

Chromatin Accessibility

Chromatin accessibility measurements were conducted as previously described by Rose et al. [18]. The primers CA1 and CA2 were located within the region of the PHD3 gene queried for DNA methylation (primer sequences and locations described in detail below). After nuclei extraction and a 5 minute DNase I digestion the DNA was extracted and real-time PCR was conducted on an ABI 7000 Sequence Detection System. The accessibility index for each amplified area was then determined by the following formula (accessibility index = 2^(-[Ct Uncut) - [Ct Dnase treated])). GAPDH chromatin accessibility was also determined as a positive control for a constitutively expressed gene to control for equivalent DNase digestion between the cell lines examined.

HRE-Luciferase assay

Cell lines ~85% confluent in 60 mm dishes were transfected with an HRE-luciferase reporter vector [19] (2.5 μg and Renilla luciferase (1.5 μg) according to Lipofectamine 2000 transfection reagent protocol. Transfection media was removed after 6 hours and replaced with fresh medium. Cells were then plated under 94% N2, 5% CO2, 1% O2 gas mixture in a hypoxia chamber, or normoxia for 24 hours and then lysed according to the Dual luciferase reporter assay system (Promega, Madison, WI) protocol. Luminescence was measured 3 times per sample using a Tecan SpectraFluor Plus lumimeter.

Adenoviral Transduction

The adenoviral PHD3 construct was a generous gift from Dr. Robert Freeman from Rochester University (unpublished). Briefly, the human PHD3 coding sequence was engineered into the pDC315 vector and contains an N-terminal FLAG tag. PC3 cells were grown to ~85% confluency and then transduced with 20, 40 or 60 MOI of Ad-PHD3. Approximately 36 hours following transduction, cells were lysed with RIPA buffer and western blotted according to the procedures outlined above.

Clinical Samples

Clinical prostate tumor samples were received as frozen blocks in OCT. Sections were cut and ground with mortal and pestle. DNA and RNA were extracted in with Qiagen DNeasy Tissue kit and Trizol respectively.

Methylated DNA Enrichment

Genomic DNA was harvested from cells and tissue using a Qiagen DNeasy Tissue kit. 2 μg of DNA in 120 μl of 10 mM Tris pH 8.0 was sonicated into fragments of approximately 150 bp using a Covaris S2. Fragmentation was done according to the Covaris protocol. 1 μg (60 μl) of sheared input gDNA was used according to the protocol supplied by the MethyImiiner kit (Invitrogen). Binding reactions between beads containing methyl-CpG binding domains and sheared genomic DNA were performed at 4°C overnight. Bound DNA was eluted using progressively increasing NaCl concentrations. Eluates were precipitated using NaOAc and EtOH precipitation and resuspended in 60 μl 10 mM Tris pH 8.0. Real time PCR was performed using 1 μl resuspended DNA, SYBR Green master mix and 100 nM Fwd Primer: 5’-GAGCTCCACGACCCTTTC-3’ and Rev Primer: 5’-GCAAGTGTTGGCTTCCCAT-3’ in a 10 μl reaction volume. The kit was validated using samples from human tumor cell lines with known PHD3 CpG island methylation status as determined by bisulfite sequencing (Figure S1).

Statistical Analysis

Significant differences between groups of data were determined using a t-test for all bar graphs or ANOVA for box plot powered by SigmaPlot 11.0 software. n = 3 was used for each data set unless otherwise noted.

Results

PHD3 mRNA is aberrantly silenced in human melanoma, prostate and breast carcinoma cell lines

A panel of human carcinoma cell lines was screened for PHD3 mRNA expression. This panel consisted of three prostate cancer cell lines (DU 145, 22RV.1 and PC-3), two breast cancer cell lines (MCF7, HS578T), one melanoma (MDA-MB-435), a non-transformed prostate epithelial cell line (NPrEC) and a non-transformed, immortalized breast cell line (hTERT-HME1). We found that PHD3 mRNA was expressed at different levels varying from abundant to almost undetectable levels as determined by conventional RT-PCR (Figure 1a). The prostate cancer cell lines showed decreased PHD3 mRNA expression compared to the normal prostate epithelial cells. A comparison of three prostate cancer cell lines showed that PHD3 is expressed in DU 145 and 22 RV.1, whereas in PC-3, PHD3 mRNA is nearly undetectable. The melanoma cell line, MDA-MB-435 was also found to have very low PHD3 mRNA expression. Among the mammary cell lines, HS578T had much lower PHD3 mRNA than the HME1 mammary epithelial cells and MCF7 cells expressed far more PHD3 mRNA than its normal HME1 mammary epithelial cell counterpart. To confirm and extend the results shown in Figure 1a, quantitative real time RT-PCR analysis of PHD3 mRNA expression were conducted and the results are shown in Figure 1b. Similarities in expression were found with both the methods used, and PHD3 mRNA expression was nearly undetectable in PC-3, MDA-MB-435 and HS578T cell lines.
Re-expression of PHD3 with DNA methyltransferase inhibitor 5-Aza-dC

The near absence of PHD3 mRNA expression in a subset of cell lines suggested an epigenetic mechanism might be responsible for their silencing. Unlike genetic mutations that accumulate in cancer, epigenetic modifications are reversible [20]. We hypothesized that if DNA methylation of the PHD3 gene was responsible for its reduced expression, a DNA methyltransferase inhibitor such as 5-Aza-2'-deoxycytidine (5-Aza-dC) should induce its expression. This is in line with 5-Aza-dC's purported ability to re-activate genes previously silenced by DNA methylation in cancer cells [21].

We chose 2 cell lines displaying the most marked decrease in PHD3 expression, MB-435 and PC-3, and 2 cell lines displaying moderate to high basal PHD3 expression, MCF7 and DU 145 for treatment with 5-Aza-dC. When MB-435 and PC-3 cells were treated with 5-Aza-dC, there were significant increases in the PHD3 mRNA expression compared to their respective untreated controls [Figure 2]. Furthermore, MB-435 cells became responsive to PHD3 mRNA upregulation by hypoxia to a significant degree following hypoxic exposure. Cells that already expressed PHD3 at moderate levels did not respond to 5-Aza-dC by significantly upregulating PHD3. Although this finding suggests that CpG methylation is involved in silencing, further direct queries of epigenetic alterations at this locus were necessary to more deeply address this question.

The PHD3 CpG island is aberrantly CpG methylated and displays decreased chromatin accessibility in human carcinoma cells

To determine whether PHD3 gene methylation is present at the CpG island in cell lines that have reduced PHD3 mRNA expression and respond to 5-Aza-dC by upregulating PHD3 mRNA, we utilized sodium bisulfite sequencing to identify methylated CpG sites. Figure 3A, illustrates the CpG island in the 5'-end of the PHD3 gene. The 58 CpG sites in the region analyzed are represented by vertical lines. A putative hypoxia response element (HRE) in the promoter region of the gene is also indicated. We found that these CpGs were highly methylated in
the PHD3 negative cell lines PC-3, MB-435, and HS578T (Figure 3B). These represent examples of human melanoma, prostate, and mammary carcinoma cells respectively. We also noted that many cell lines appeared to be heterogeneous with respect to PHD3 promoter methylation status. Within certain cell lines, some clones display high levels of methylation, whereas other have very few to no methylated CpGs. This small population of unmethylated or hemimethylated cells within a cell line may explain our ability to detect very low levels of PHD3 mRNA in cell lines displaying largely methylated PHD3 CpG islands. Furthermore, areas of CpG methylation in some methylation positive cell lines overlap with a putative HRE in the PHD3 promoter region, which could hinder the ability of PHD3 to be induced upon hypoxic stimuli. In contrast to the positive methylation status in PHD3 negative cells, the CpGs in the PHD3 CpG island were largely unmethylated in the PHD3 positive cells, NPrEC, DU 145, 22RV.1, HME1, and MCF7.

DNA methylation is typically associated with other alterations to chromatin structure that participate in cell-type specific gene expression patterns. Aberrant cytosine methylation in the 5'-regulatory regions of genes is typically associated with deacetylated histones, and thus a state of DNA that is generally inaccessible to transcription factors and other enzymes that act on DNA, such as polymerase II. This is a mechanism of gene silencing often exploited by cancer cells [22]. In a chromatin accessibility assay, we found the promoter region of PHD3 in PC-3 cells was resistant to cutting by DNase I when compared to the MCF7 PHD3 promoter, whereas there was little change in GAPDH promoter accessibility between the two cell lines (Figure S2). This evidence further supports the hypothesis that PHD3 promoter methylation and heterochromatin formation are part of the mechanism for reduced expression of PHD3 in these human breast and prostate cancer cell lines.

PHD3 expression is not induced upon exposure to hypoxia in cell lines containing PHD3 promoter methylation

Unlike PHD1, both PHD2 and PHD3 genes contain hypoxia response elements, and can be induced by hypoxia by the HIF-1 and HIF-2 transcription factor complex. In the case of PHD3, mRNA and protein expression can be relatively low during normoxic conditions, with marked increases upon hypoxic insult [10]. Therefore, we tested PC-3, DU 145, MB-435 and MCF7 cell lines for their ability to upregulate PHD3 following 24 hours of hypoxia (1% O2) (Figure 4A). We found cell lines that contained PHD3 promoter methylation (PC-3, MB-435) failed to appreciably upregulate PHD3 mRNA under these conditions. However, we did note a very small upregulation of PHD3 mRNA in PC-3 cells. This can likely be attributed to the heterogeneity of PHD3 promoter methylation between specific clones in this cell line (see Figure 3B). In contrast, PHD3 mRNA was much more prone to upregulation in the unmethylated cell line MCF7. Upregulation of PHD3 in DU 145 cells varied by experiment, and averaged as a non-significant trend toward hypoxic upregulation.

Our discovery of PHD3 promoter methylation in melanoma, breast and prostate cancer cell lines prompted us to ask whether cell lines from other malignant tissues contained methylation at the PHD3 locus. Therefore, we further performed real-time PCR and bisulfite sequencing on a panel of 3 human renal cell carcinoma cell lines (A-498, ACHN and 769-P), and on cDNA prepared from normal kidney tissue (Figure 4B). We found that ACHN and 769-P cells express nearly undetectable levels of PHD3 mRNA, whereas A-498 expresses levels comparable to normal tissue. Of these cell lines, the PHD3 positive A-498 displayed an unmethylated PHD3 promoter whereas 769-P cells displayed an aberrantly methylated PHD3 promoter. Interestingly though, we did not detect any CpG methylation at the promoter of PHD3 negative
ACHN cells, suggesting an alternative mechanism for silencing in this cell line.

PHD3 promoter methylation status does not correlate with hypoxia induced HIF-1α protein accumulation or HIF transcriptional activity

The presence of PHD3 promoter methylation in such a broad range of epithelial malignancies suggests that it may be a selective advantage for tumor survival. One hypothesis is that PHD3 silencing by promoter methylation may allow for an increased HIF transcriptional response during hypoxic conditions. In order to determine whether PHD3 promoter methylation specifically affects the hypoxia response pathway, we performed western blots on cell lysates from MCF7, PC-3, MB-435 and DU 145 cell lines to compare the HIF protein levels and the HIF transcriptional response to hypoxia (Figure 5a). Following 24 hours of hypoxia, HIF-1α protein was upregulated in all the cell lines regardless of PHD3 expression status. We also observed that DU 145 cells appear not to express detectable levels of PHD3 protein. We are unsure whether this is due to limits of detection by our PHD3 antibody, or if DU 145 cells downregulate PHD3 expression by a posttranslational mechanism. Our antibody appears to be specific to PHD3 as transduction of an adenoviral-PHD3 expression vector into PC3 cells produces a band at an identical molecular weight as the band seen in MCF7 cells (Figure S3). Interestingly though, MCF7 cells, which do express basal levels of PHD3 mRNA and protein, displayed the largest induction of HIF-1α protein. Thus, HIF-1α protein levels, in general, did not show any correlation with presence or absence of PHD3. We also found HIF-2α to be expressed under normoxic conditions in MCF7. Moreover, MB-435 cells, which express the lowest levels of PHD3 mRNA out of all the cell lines tested appear not to express HIF-2α at an appreciable level. Thus, loss of PHD3 does not appear to be significantly correlated with an accumulation of HIF-1α or HIF-2α levels in these cell lines.

To further investigate the effect of PHD3 promoter methylation on the transcriptional response of the hypoxia response pathway,
we transfected MCF7, PC-3, MB-435 and DU 145 cancer cell lines with an HRE-luciferase reporter construct [19]. Following 24 hours of hypoxia, luciferase activity was measured and plotted relative to luciferase activity in normoxic cells (Figure 5B). There was no correlation between PHD3 promoter methylation status and hypoxic induction of luciferase. The results of this experiment resembled the pattern of HIF-1α protein accumulation seen in Figure 5A. MCF7 cells showed the largest induction luciferase activity, whereas the other cell lines, which do not express detectable levels of PHD3 protein, were comparable to one another. Although not absolute, these data are highly suggestive that HIF protein stabilization and transcriptional activity is largely independent of PHD3 expression.

**Figure 4. The methylated PHD3 gene in melanoma, prostate, breast and renal carcinoma cell lines is refractory to induction by hypoxia.** A) Melanoma, prostate and breast carcinoma cell lines were treated with hypoxia (1% O2) or normoxia (21% O2) for 24 hours. Total RNA was extracted and converted to cDNA by reverse transcription. Quantitative real-time reverse transcription-PCR analysis of PHD3 was performed with normalization to GAPDH gene expression. Relative quantitation was determined by the DDCt method. ND = not detectable. Error bars = SEM. n = 3. B) Renal clear cell carcinoma cell lines were either untreated or treated with hypoxia as in A). The right panel depicts relative PHD3 mRNA levels compared to mRNA extracted from normal renal tissue. The left panel depicts the methylation status at each of 58 CpG dinucleotides present in the PHD3 CpG island of the representative renal carcinoma cell lines. Error bars = SEM. n = 3. doi:10.1371/journal.pone.0014617.g004

**PHD3 promoter methylation is absent in primary human prostate adenocarcinomas**

Recent data published by Hatzimichael et al. suggested that acquisition of PHD3 promoter methylation may be a relatively common event in certain plasma cell neoplasias [17]. Therefore, we asked whether primary human prostate neoplasias contained methylation at the PHD3 CpG island. We extracted DNA and RNA from frozen sections of 10 prostate cancer specimens containing a minimum of 70% malignant tissue with a Gleason score ranging from 7–9 as well as 3 benign prostate specimens. Real-time PCR of extracted RNA showed that all 10 tumors contained decreased PHD3 mRNA expression compared to 3 benign tissue specimens (Figure 6A and B).
Probing for methylated CpGs in select tumor specimens using the MethylMiner kit indicated the possible presence of methylated CpGs at the PHD3 CpG island in tumor sample 1 (Figure S4), which also contained the lowest PHD3 mRNA levels. However, further bisulfite sequencing of 8 clones from tumor sample 1 and tumor sample 4 did not detect any methylated CpGs (data not shown). This could be due to better sensitivity to population average methylation states as compared to the single molecule at a time approach of bisulfite sequencing.
Perturbations in the cellular responses to hypoxia are well known to play a role in the malignant process. Familial mutations in \textit{VHL}, a negative regulator of the HIF-\(\alpha\) proteins, results in vascular tumors of the brain, spinal cord and retina, as well as appearance of renal clear-cell carcinomas [23]. PHD proteins play a role upstream of VHL regulation; they hydroxylate HIF-\(\alpha\) proteins, creating a binding site for VHL [24,25,26]. Thus, it is feasible that deregulation of PHD activity or expression could also contribute to the malignant process. In fact, an absence of PHD3 upregulation following hypoxia has been observed in multiple human cell lines from tumors of the breast, prostate and brain [10,16]. A recent clinical study of breast tumors containing \textit{BRCA} mutations supports the hypothesis that PHD3 plays an important role in malignancy. This study found a positive correlation between decreased PHD3 expression and a basal phenotype, which is considered a higher grade and more aggressive tumor [27].

**Figure 6. PHD3 mRNA expression is downregulated in multiple primary human prostate cancer specimens.** A) Total mRNA was isolated from frozen sections of primary human prostate cancer specimens with Gleason scores ranging from 7–9. Quantitative real-time PCR was performed using PHD3 specific TaqMan primer-probe. Relative PHD3 mRNA expression for each tumor sample is represented as (average \(\Delta Ct\) of \(n = 3\) benign prostate tissue samples) – (\(\Delta Ct\) tumor sample). Samples were normalized to GAPDH. Dotted lines represent +/- 1 SD for benign tissue PHD3 mRNA expression. B) Box plot depicting PHD3 mRNA expression from samples shown in A. p value is based on ANOVA between 3 benign samples and 10 malignant samples.

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Here, we report aberrantly silenced basal mRNA expression of PHD3 in breast, prostate, melanoma and renal cell carcinoma cell lines, and the absence of PHD3 mRNA induction upon hypoxic stimulus. PHD3 expression could be recapitulated in some PHD3 negative cell lines after treatment with 5-Aza-dC, a DNA methyltransferase inhibitor, implicating DNA methylation as a mechanism for the decreased expression of PHD3 mRNA in these cell lines. PHD3 promoter methylation was verified by sequence analysis of PCR products cloned from bisulfite-treated genomic DNA. We found that among the human cancer cell lines investigated, PC-3, MB-435, HS578T, and 769-P cell lines have hypermethylated PHD3 CpG islands. Furthermore, the PHD3 promoter region was more resistant to DNase I in PC-3 cells (hypermethylated PHD3 promoter) compared to MCF7 cells (hypomethylated PHD3 promoter). The methylation of the PHD3 promoter in these carcinoma cell lines appears to be aberrant since insignificant DNA methylation was found in the non-transformed cell counterparts of prostate and mammary epithelial cell lines NPrEC and HME1 respectively. The apparently aberrant PHD3 promoter methylation status in these cell lines is the likely mechanism, at least in part, for PHD3 transcriptional suppression because DNA methylation is typically associated with a condensed heterochromatin state, and is known to inhibit transcription factor binding to promoter regions of genes [29].

Although all members of the PHD family have the ability to hydroxylate both HIF-1 and HIF-2α, the specificities appear to differ slightly. PHD2 has been reported to play a more pronounced role in the regulation of HIF-1α, whereas PHD3 more strongly affects HIF-2α stability [10]. Therefore, it would seem likely that loss of PHD3 expression by promoter methylation would convey a cellular advantage mediated through increased HIF-1α and/or HIF-2α stability during hypoxia. This could lead to increased expression of VEGF and erythropoietin with subsequent vascular recruitment. Tumors of the breast, skin, kidney and prostate, being solid tumors, would certainly benefit to increased expression of VEGF and erythropoietin with these findings to suggest that PHD3 silencing by CpG methylation recently reported in plasma and B-cell neoplasia by Hatzimichael et al. [17]. These interesting prospects related to identification of the downstream targets of PHD3 signaling will undoubtedly become a focus of future investigations.

Our inability to detect PHD3 promoter DNA methylation in primary human prostate tumors was surprising, however our results are supported by a recent study by Huang et al. [38], who screened 168 invasive breast carcinomas and did not find evidence of PHD3 DNA methylation using melting curve analysis of bisulfite converted DNA. We cannot rule out the possibility that a low level of semi-methylated PHD3 CpG islands are present in their samples as the “intermediately methylated” controls were not truly intermediately methylated, but rather were mixtures of 100% methylated DNA with 0% methylated DNA. Nonetheless, it seems clear that a large proportion of primary epithelial tumors does not contain a high degree of PHD3 promoter methylation, and may not be the ideal specimens for detection of methylation at this locus. Given PHD3’s purported ability to negatively regulate the NFκB pathway [30,35], and the widely reported involvement of NFκB in cell migration and metastasis [39,40], future studies on PHD3 CpG island methylation in clinical samples of metastatic disease as opposed to primary tumors may yield more positive results.

Here, we are the first to report DNA methylation of the PHD3 CpG island in solid tumor cell lines derived from diverse cell types. PHD3 methylation in carcinoma cells was associated with their inability to appropriately upregulate PHD3 mRNA upon exposure to hypoxia. We are also the first to show evidence that this aberrant expression fails to correlate with an increase in HIF protein accumulation and transcriptional activity upon exposure to hypoxia in the cell lines examined. The presence of PHD3 promoter hypermethylation and PHD3 silencing in such a wide range of cancer types suggests this might be a common event that elicits a selective advantage for tumors. Our data suggest that at least in some cell lines, the nature of this advantage may extend beyond hypoxia resistance. Furthermore, the selective event may occur during or after the process of invasion/metastasis, as we and others have not found evidence of methylation in primary solid tumors [38].
Supporting Information

Figure S1 Validation of methylated DNA enrichment as a tool for detecting methylated CpG regions in human genomic DNA. A) Genomic DNA from human melanoma and breast cancer lines was enriched for methylated CpG dinucleotides at the PHD3 CpG island using the MethylMiner kit, followed by quantitative real-time PCR analysis using PHD3 CpG island-specific PCR primers. S = supernatant, representing unmethylated DNA. E1 - E5 represent elutions with 200, 650, 1100, 1550, 2000 mM NaCl respectively. Input = 1/60th total input DNA. Total amounts of eluted DNA from each fraction are represented as a fraction of input (left). Methylated CpG sequences from bisulfite-converted DNA in the corresponding cell lines are depicted for comparison (right). B) Control 100% methylated and 0% methylated oligos supplied by the MethylMiner kit were subjected to the MethylMiner protocol. S = supernatant, representing unmethylated DNA. E1 - E5 represent elutions with 200, 650, 1100, 1550, 2000 mM NaCl respectively. Identical volumes of DNA from each eluate were subjected to quantitative real-time PCR using primers supplied by the MethylMiner kit. DNA content in each fraction is represented as arbitrary units.

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Figure S2 The methylated PHD3 gene in non-expressing cells is maintained in a less accessible state than the non-methylated PHD3 gene in expressing cells. A) Nuclei from PHD3-positive MCF7 and PHD3-negative PC-3 carcinoma cell lines were isolated and enzymatically restricted with DNase I. Primers CA1 and CA2 (see Fig. 3A) were used for quantitative real-time PCR (right panels) to amplify a region also assessed for cytosine methylation. Accessibility indices (left panels) were calculated as follows: AI = 2[(Ct DNase treated) - (Ct Untreated)]. B) GAPDH accessibility indices were simultaneously assessed as a control for a constitutively expressed gene in both cell lines.

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Figure S3 PHD3 antibody specificity. PC3 cells were transduced with an increasing MOI of adenoviral-PHD3 vector. Western blot using Novus100-139 antibody co-incubated with anti-actin antibody indicated an approximately 27 kDa band in MCF7 cells that migrates at the same molecular weight as a band present in PHD3 transduced PC3 cells. Found at: doi:10.1371/journal.pone.0014617.s003 (3.00 MB TIF)

Figure S4 Methylated DNA enrichment of genomic DNA isolated from primary human prostate cancer. A) Total genomic DNA was isolated from frozen sections of 7 malignant prostate cancers and 3 benign prostate samples and subjected to the MethylMiner protocol. Tumor sample number corresponds to samples shown in figure 6A. S = supernatant, representing unmethylated DNA. E1-E4 represents elutions with 300, 550, 800, and 2000 mM NaCl. PHD3 CpG island DNA content in each fraction is represented as a fraction of total PHD3 CpG island DNA present in input.

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Author Contributions

Conceived and designed the experiments: FED. Performed the experiments: TLP MFP SV SJF. Analyzed the data: TLP MFP FED. Contributed reagents/materials/analysis tools: AJC MLT. Wrote the paper: TLP FED. Assisted with writing and editing: TLP MFP SJF. Provided funding resources for the work: SV MLT AJC FED. Isolated proteins from human tumor samples and all cell lines under normoxia and hypoxia; performed transfection studies for promoter reporter experiments; performed bisulfite sequencing and Methylminer experiments and provided key data for figures 6a (right panel) and 6b: TLP. Extracted, quantified, and analyzed RNA and DNA from all cell lines; designed primers and performed bisulfite sequencing to determine methylation patterns. Provided key data for figures 1, 3, and 4: MFP. Extracted RNA, DNA and protein from breast cancer cell lines treated with 5-aza-dC for initial RT-PCR and western blotting experiments; provided key data for figures 1, 2, 5: SV. Extracted RNA, DNA and protein from human cell lines and tumor samples, performed bisulfite and Methylminer experiments and provided key data for figures 6a (right panel) and 6b: TLP. Extracted, quantified, and analyzed RNA and DNA from all cell lines; designed primers and performed bisulfite sequencing to determine methylation patterns. Provided key data for figures 1, 3, and 4: MFP. Extracted RNA, DNA and protein from breast cancer cell lines treated with 5-aza-dC for initial RT-PCR and western blotting experiments; provided key data for parts of figures 1 and 3: AJC. Isolated proteins for western blotting; provided reagents and expertise for western blots and trained the other authors in protein techniques; provided key data for figure 6a (left panel): MLT. Provided overall guidance and direction in all phases of experimental design, implementation, data acquisition and interpretation, and manuscript writing, editing and preparation: FED.

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