Biochemical and Biophysical Research Communications 438 (2013) 71–77

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hypoxia mediated expression of stem cell markers in VHL-associated hemangioblastomas

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ARTICLE INFO

Article history:
Received 5 July 2013
Available online 18 July 2013

Keywords:
Hemangioblastomas
Hypoxia
HIF signaling
Inhibitor
Angiogenesis
Cancer

ABSTRACT

Hemangioblastomas of the retina, central nervous system, and kidney are observed in patients with mutations in the von Hippel-Lindau (VHL) tumor suppressor gene. Mutations in the VHL lead to constitutive activation of hypoxia-inducible-factor (HIF) pathway. HIF-mediated expression of pro-angiogenic genes causes extensive pathological neovascularization in hemangioblastomas. A number of studies have shown coexistence of pro-angiogenic and stem cell markers in 'tumorlet-like stromal cells' in the retinal and optic nerve hemangioblastomas, leading to suggestions that hemangioblastomas originate from developmentally arrested stem cells or embryonic progenitors. Since recent studies have shown that the HIF pathway also plays a role in the maintenance/de-differentiation of normal and cancerous stem cells, we evaluated the role of the HIF pathway in the expression of stem cell markers in *VHL*−/− renal cell carcinoma cells under normoxia or *VHL*+/+ retinal pigment epithelial cells under hypoxia. Here we show that the expression of stem cell markers in hemangioblastomas is due to activation of the HIF pathway. Further, we show that honokiol, digoxin, and doxorubicin, three recently identified HIF inhibitors from natural sources, blocks the expression of stem cell markers. Our results show the mechanism for the cytological origin of neoplastic stromal cells in hemangioblastomas, and suggest that inhibition of the HIF pathway is an attractive strategy for the treatment of hemangioblastomas.

1. Introduction

Hemangioblastomas (also known as the blood vessel tumor or VHL disease) are multisystem tumor syndromes, where mutations in the VHL protein (pVHL) lead to constitutive activation of the HIF pathway. HIF is a member of PAS family of basic helix-loop-helix heterodimeric transcription factors, consisting of α subunits (HIF-1α, -2α, and -3α) and HIF-1β/ARNT subunit. Normally, hydroxylation of the conserved proline residues (Pro402 and Pro564 in human HIF-1α) by the oxygen-dependent HIF prolyl hydroxylases/dioxygenases (PHD1–3) under normoxic conditions allows the binding of HIF-α isoforms to the wild-type (wt)-pVHL. This binding of hydroxylated HIF-α to pVHL, the substrate recognition subunit of an E3 ubiquitin ligase, allows rapid proteosomal degradation of HIF-α under normoxia [1,2]. However, in patients with mutated pVHL, even though HIF-α gets hydroxylated at the proline residues under normoxia, it is not bound by mutated pVHL, allowing it to escape proteosomal degradation. Subsequently, HIF-α translocates to the nucleus, dimerizes with the HIF-1β subunit, and recruits transcriptional co-activators. The active HIF-α/β heterodimer binds to a core DNA sequence (G/ACGTG) in the hypoxia-response-element (HRE) present in the promoters of target genes, causing overexpression of hypoxia response genes, which includes a number of pro-angiogenic factors [*e.g.* vascular endothelial growth factor (VEGF), erythropoietin (EPO), platelet-derived growth factor (PDGF), etc.]. As a result, hemangioblastomas of retina, central nervous system (CNS), and kidney are highly vascular in nature [3].

In addition to these vascular cells, hemangioblastomas are composed of 'stromal cells'. However, the cytological origin of these neoplastic stromal cells remains unknown. Number of studies have shown coexistence of pro-angiogenic and stem cell markers in tumorlet-like stromal cells in the retinal and optic nerve hemangioblastomas [4,5], leading to the suggestion that hemangioblastomas originate from developmentally arrested stem cells or embryonic progenitors [4–7]. Failure to specify the histological origin of stromal cells in hemangioblastomas has precluded the development of nonsurgical therapies for these multisystem blood vessel tumor syndromes.

All current therapies for hemangioblastomas have significant limitations and side-effects; while anti-VEGF therapies (*e.g.* Macugen, Lucentis, etc.) had minimal detectable beneficial effects [8,9].

Abbreviations: VHL, von Hippele-Lindau; HIF, hypoxia-inducible-factor; HRE, hypoxia response element; PHD, prolyl hydroxylation domain; VEGF, vascular endothelial growth factor; EPO, erythropoietin; PDGF, platelet-derived growth factor; JMJD1A, Jmj-domain containing protein 1A.

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http://dx.doi.org/10.1016/j.bbrc.2013.07.028

This lack of efficacy of anti-VEGF therapies is possibly due to over-expression of other pro-angiogenic factors (*e.g.* EPO, PDGF, etc.) in hemangioblastomas. The possible future approaches to successfully control hemangioblastomas may rely on blocking some master modulator, such as the HIF pathway. Thus, characterization of novel HIF inhibitors may have a considerable therapeutic impact on the treatment of hemangioblastomas. We have recently shown that honokiol, a biphenolic phytochemical extracted from the *Magnolia* genus (which has been used for thousands of years in the traditional Japanese and Chinese medicine) is a potent inhibitor of the HIF pathway as well as hypoxia-induced expression of histone lysine demethylases in a number of cancer and retinal pigment epithelial cell lines [10], thus providing an evidence-based scientific explanation of honokiol's therapeutic benefits. Further, screening a library of compounds that are in clinical practice/trials using a reporter assay identified cardiac glycosides (*e.g.* digoxin, ouabain, and proscillaridin A) and anthracycline chemotherapeutic agents (*e.g.* doxorubicin and daunorubicin) as potent inhibitors of the HIF pathway [11,12]. Administration of digoxin or doxorubicin in xenograft mouse models inhibited the transcription of endogenous HIF-dependent genes, increased latency, and decreased tumor growth [11,12]. Here, we investigate the role of the HIF pathway in the expression of stem cell markers in hemangioblastomas and suggest that inhibition of the HIF pathway is an attractive target for the treatment of hemangioblastomas.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were supplied by the Sigma–Aldrich Chemical Co. (Saint Louis, MO) unless otherwise stated, and were of analytical grade or higher. Honokiol was purchased from the Stanford Chemicals (Irvine, CA), while digoxin and doxorubicin were ordered from Carbosynth LLC (San Diego, CA).

2.2. Cell culture

Human retinal pigment epithelial cell lines (D407 and ARPE19) and renal cell carcinoma derived cell lines (RCC4, RCC4–T314, PRC3, and WT8) were used for these studies. RCC4 and PRC3 cells lack a functional *VHL* gene, whereas RCC4–T314 and WT8 cells have a wt-*VHL* incorporated into them. The RCC4, RCC4–T314, PRC3, and WT8 cell lines were generously provided by Dr. M. Celeste Simon (University of Pennsylvania Cancer Center), while D407 cell line was generously gifted by Dr. Richard Hunt (University of South Carolina). DMEM medium, supplemented with 10% heat inactivated FBS, 100 μg/ml of streptomycin, and 100 U/ml of penicillin (media components were purchased from Corning), was used to grow D407, ARPE19, RCC4, and T314. PRC3 and WT8 were maintained in the above mentioned media supplemented with 1 mg/ml G418. Cells were cultured in a CO_2 incubator that was maintained at 37 °C, 5% CO_2, and 90% relative humidity.

2.3. Exposure of cells to hypoxia

D407 and ARPE19 cells were exposed to hypoxic conditions in a bactron anaerobic chamber (Sheldon Manufacturing, Cornelius, OR) using a gas mix of 1% O_2, 5% CO_2, and 94% N_2 and maintained at 37 °C. The chamber was purged with the hypoxic gas mix according to the manufacturer's instructions to generate hypoxia. Cells were aseptically transferred into the chamber upon reaching ≈70% confluency. Cell lysis for RNA extraction was performed after exposure to hypoxia for 12 h in the hypoxic chamber to avoid equilibration of the cells to normoxic conditions. RCC4, T314, PRC3 and WT8 cell lines were treated in a similar way, albeit under normoxic conditions due to absence of wt-pVHL in RCC4 and PRC3, which mimic hypoxic conditions with respect to the HIF pathway.

2.4. Treatment of cells with HIF inhibitors

All the drugs (digoxin, doxorubicin, and honokiol) used for the study were prepared in DMSO. The inhibition studies were carried out by adding 1 μM of digoxin or 1 μM of doxorubicin or 20 μM of honokiol into the media in a flask containing ≈70% confluent cells. In the control samples 0.1% of DMSO, corresponding to the DMSO concentration in the cells treated with highest inhibitor concentration, was added.

2.5. RNA extraction from cells

RNA was extracted from cells as described in our earlier publications [13,14]. Briefly, cells exposed to different treatment conditions (*i.e.* normoxia or hypoxia, with or without HIF inhibitors) were lysed in TRI® reagent (Molecular Research Center, Inc., Cincinnati, OH). Aqueous phase containing RNA was separated, to which 0.7 volumes of isopropanol was added and centrifuged to precipitate RNA. The RNA pellet was washed twice with 75% ethanol and dissolved in DNase/RNase-free water. M-MLV reverse transcriptase (BioChain Institute, Inc, Hayward, CA) was used to generate cDNA from the extracted total RNA samples.

2.6. Quantitative real-time PCR analysis

The quantitative real-time PCR (qPCR) reactions (20 μl) were performed with 2.5 units of Taq polymerase (Bulldog Bio, Inc., Portsmouth, NH), 0.25 mM dNTP (Fisher Scientific, Hanover Park, IL), 0.25 × SYBR green (Invitrogen, Grand Island, NY), 80 ng cDNA, and 1.5 μM of reverse and forward primers. The qPCR reactions were performed in biological and experimental duplicates. LightCycler® 480 qPCR instrument (Roche Diagnostics Corporation, Indianapolis, IN) was used for the qPCR. Ribosomal protein L32 was used as an internal control to normalize the sample to obtain ΔC_t value. $2^{\Delta\Delta C_t}$ method was used to analyze the relative gene expression levels.

3. Results and discussion

3.1. HIF-mediated expression of pro-angiogenic genes in renal cell carcinoma and retinal pigment epithelial cell lines

The human RCC4 cell line lacks wt-pVHL and hence has a constitutively active HIF pathway. RCC4–T314 cells were derived from the RCC4 cells by stably transfecting wt-*VHL*, and thus, these cells do not activate the HIF pathway under normoxia. Under hypoxia, HIF-α isoforms are stabilized in RCC4–T314 cells leading to activation of the HIF pathway [15]; While, 786-O cells, another renal cell carcinoma cell line that is also *VHL*−/−, expresses only HIF-2α [16,17]. Unlike *VHL*−/− RCC4 cells [15], HIF-1α is not detectable in *VHL*−/− 786-O cells [17]. The loss of HIF-1α expression in some *VHL*−/− RCC cells confers an *in vivo* growth advantage [18], possible due to the role of HIF-1α in apoptosis [19]. Reintroduction of wt-*VHL* in 786-O cells leads to downregulation of HIF-2α protein levels, restores normal oxygen-dependent regulation of HIF-2α subunit and transcription of pro-angiogenic genes which, in turn, suppresses tumor formation in mouse xenografts [16,17,20]. Importantly, a HIF-2α variant, which lacks pVHL-binding sites, prevents tumor suppression by exogenously introduced wt-*VHL* in *VHL*−/− cells [18,21]. Conversely, downregulation of HIF-2α in *VHL*−/− cells, similar to the effects observed with the reintroduc-

tion of wt-*VHL* in these cells [16,20], serves to inhibit expression of pro-angiogenic genes, inhibits the proliferation of vascular endothelial cells, and to suppress tumor growth by *VHL*−/− cells [22]. Further, HIF-α inactivation abolishes the cellular response to hypoxia *in vitro*, indicating that HIF-α is the only pVHL target required for this response, and the tumor forming ability of *VHL*−/− 786-O cells is dependent on the constitutive activation of the HIF pathway. Due to this established link between VHL loss and activation of the HIF pathway, RCC4 and PRC3 (*i.e.* 786-O cells with empty vector) cell lines with their *VHL*+/+ counterparts (RCC4–T314 and WT8) are ideal cell-based system for determining the HIF-mediated transcription phenomena.

The cDNA from RCC4, RCC4–T314, PRC3, and WT8 cells were synthesized under normoxic conditions and the level of expressions of pro-angiogenic genes were compared. These experiments demonstrated a significant upregulation of a number of HIF-dependent pro-angiogenic genes in RCC4 and PRC3 cells compared to RCC4–T314 and WT8 cells (Fig. 1A and B), respectively, representing the constitutively active HIF pathway in these cells. Our studies also demonstrated that Jmj-domain containing protein 1A (JMJD1A) was one of the strongly induced histone lysine demethylases in *VHL*−/− renal cell carcinomas cell lines (Fig. 1). Recent studies in renal cell carcinomas have revealed that higher levels of JMJD1A are present around the blood vessels, suggesting its role in angiogenesis [23]. JMJD1A regulates the expression of adrenomedullin (ADM) and growth differentiation factor 15 (GDF15) by directly binding to their promoters and demethylating dimethylated histone-3 lysine-9 [24]. These genes play critical roles in HIF-dependent signaling and angiogenesis [25,26]. Therefore, we further investigated the expression of these two JMJD1A targets and observed significant induction in *VHL*−/− renal cell carcinoma cell lines compared to their *VHL*+/+ counterparts. Further, since a retinal cell line similar to RCC4 and PRC3 cells with respect to VHL inactivation is not available, we determined that treating the human retinal pigment epithelial cell lines (D407 and ARPE-19) with hypoxia induces the expression of these pro-angiogenic genes. Similar to the renal cell carcinoma cells, our results demonstrated that hypoxia indeed induced the expression of pro-angiogenic genes, JMJD1A, and its target genes in these retinal pigment epithelial cell lines (Fig. 1C and D). Overexpression of some of these markers was also confirmed at protein levels by Western blots (data not shown).

3.2. HIF-mediated expression of stem cell markers in renal cell carcinoma and retinal pigment epithelial cell lines

A number of studies have shown a coexistence of pro-angiogenic and stem cell markers in hemangioblastomas [4,5]. Therefore, it has been suggested that hemangioblastomas originate from developmentally arrested stem cells or embryonic progenitors [4–7]. However, recent studies have shown that the HIF pathway also plays a role in the expression of stem cell markers in cancer cells [27,28]. Therefore, we evaluated the extent to which these markers are induced under normoxic conditions in *VHL*−/− RCC4 and PRC3 cells compared to RCC4–T314 and WT8 cells, respectively, and under hypoxic conditions in noncancerous retinal pigment epithelial cells (D407 and ARPE19).

For these studies, markers from different pathways downstream to the HIF pathway were selected. These markers play important roles in stem cell maintenance/differentiation [29], and can be classified into: (i) Wnt pathway markers [Lymphoid enhancer-binding factor 1 (*LEF1*), Leucine-rich repeat-containing G protein-coupled receptor 5 (*LGR5*) and Transcription Factor 7 (*TCF7*)]; (ii) drug resistance proteins [Multi-drug resistance 1 (*MDR1*, also known as P-glycoprotein 1 or P-gp), Multidrug resistance-associated protein 2 (*MRP2*) and Breast cancer resistance protein (*BCRP*)]; (iii) Yamanaka factors [Octamer-binding transcription factor 4 (*OCT3/4*), Kruppel-like factor 4 (*KLF4*), c-myc, and Sex determining region Y-box 2 (*SOX2*)]; and (iv) cancer stem cell-associated markers [Aldehyde Dehydrogenase 1 (*ALDH1*),

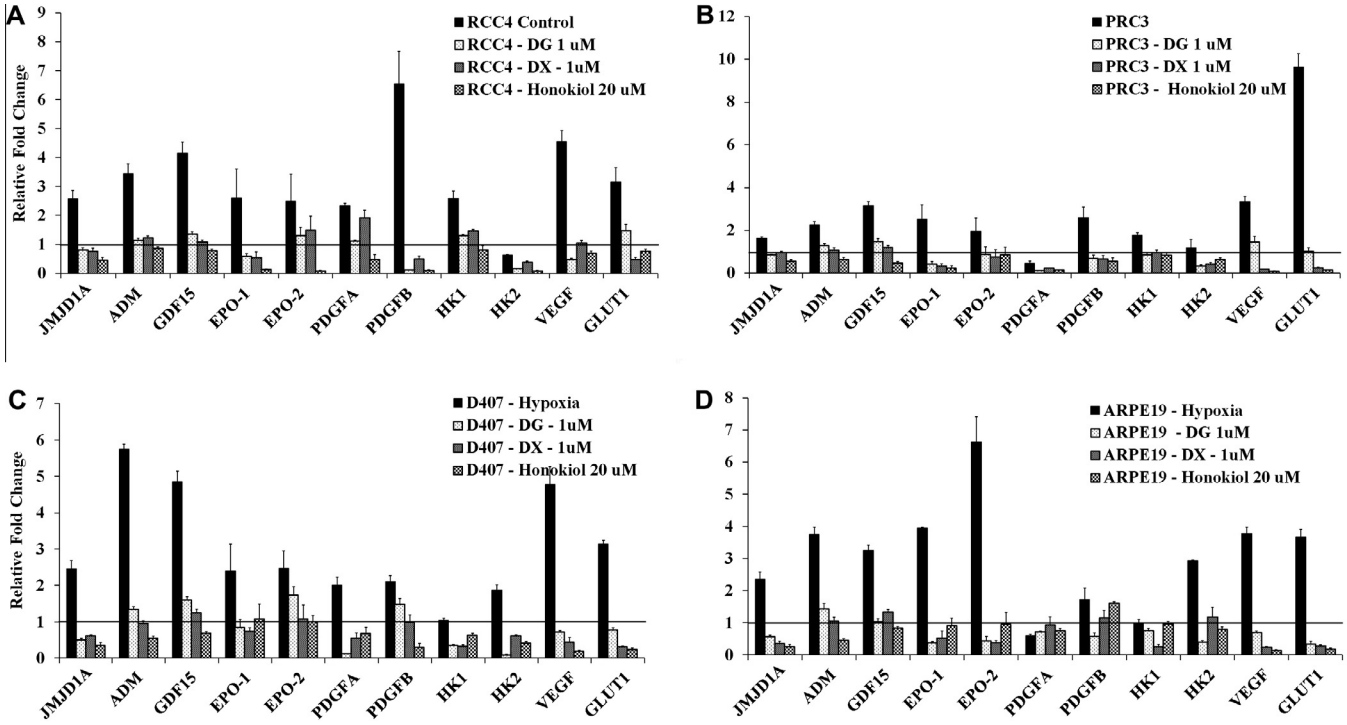

Fig. 1. Relative fold change in the mRNA levels of indicated pro-angiogenic genes in: RCC4 (Panel A), PRC3 (Panel B), D407 under hypoxia (Panel C), and ARPE19 under hypoxia (Panel D) in the presence or absence of 1 μM digoxin (DG), 1 μM doxorubicin (DX), and 20 μM honokiol. Solid, beveled, grey, and checked bars represent relative mRNA fold change ± standard error for control, 1 μM DG, 1 μM DX, and 20 μM honokiol treatments, respectively. Normoxic levels are represented by the horizontal line.

Cluster of differentiation 24 (*CD24*), Cluster of differentiation 34 (*CD34*), Cluster of differentiation 44 (*CD44*), Cluster of differentiation 133 (*CD133*), Cluster of differentiation 144 (*CD144*, also known as VE-cadherin), *NANOG*, and *NOTCH1*].

Upon activation of the HIF pathway, either due to the loss of functional pVHL or due to hypoxia, we detected overexpression of 2 out of 3 Wnt pathway markers (*i.e. LEF1*, *LGR5*), 1 out of 3 drug resistance proteins (*i.e. MDR1*/*P-GP*), and 4 out of 8 other stem cell/cancer stem cell markers (*i.e. CD133*, *CD144*, *NANOG*, and *NOTCH1*) in most of the cell lines (Figs. 2 and 3). In general, over expression of target genes was more pronounced when the HIF pathway was activated by the exposure of cells to hypoxic condition. Taken together, these results show that mutations in the *VHL* gene leads to the activation of the HIF pathway. This activated pathway induces the expression of stem cell/cancer stem cell markers, in addition to overexpression of pro-angiogenic factors. Thus our results suggest a possible mechanism for the origin of neoplastic stromal cells in hemangioblastomas.

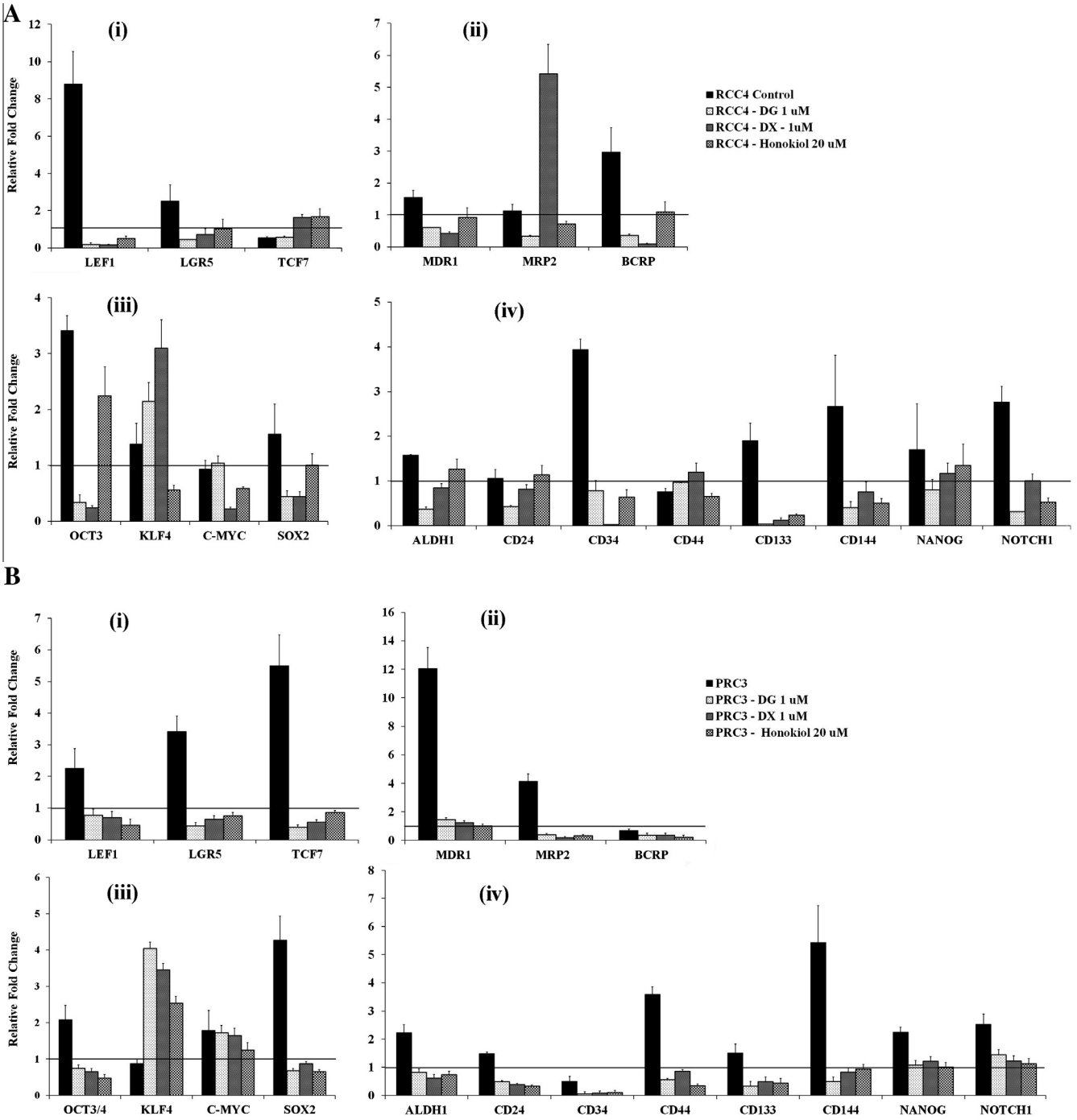

Fig. 2. Relative fold change in the mRNA levels of indicated stem cell/cancer stem cell markers in: RCC4 (Panel A) and PRC3 (Panel B) in the presence or absence of 1 μM digoxin (DG), 1 μM doxorubicin (DX), and 20 μM honokiol. In both panels (i) represents Wnt pathway genes, (ii) represents drug resistance genes, (iii) represents Yamanaka factors, and (iv) represents cancer stem cell-associated markers. Solid, beveled, grey, and checked bars represent relative mRNA fold change ± standard error for control, 1 μM DG, 1 μM DX, and 20 μM honokiol treatments respectively. Normoxic levels are represented by the horizontal line.

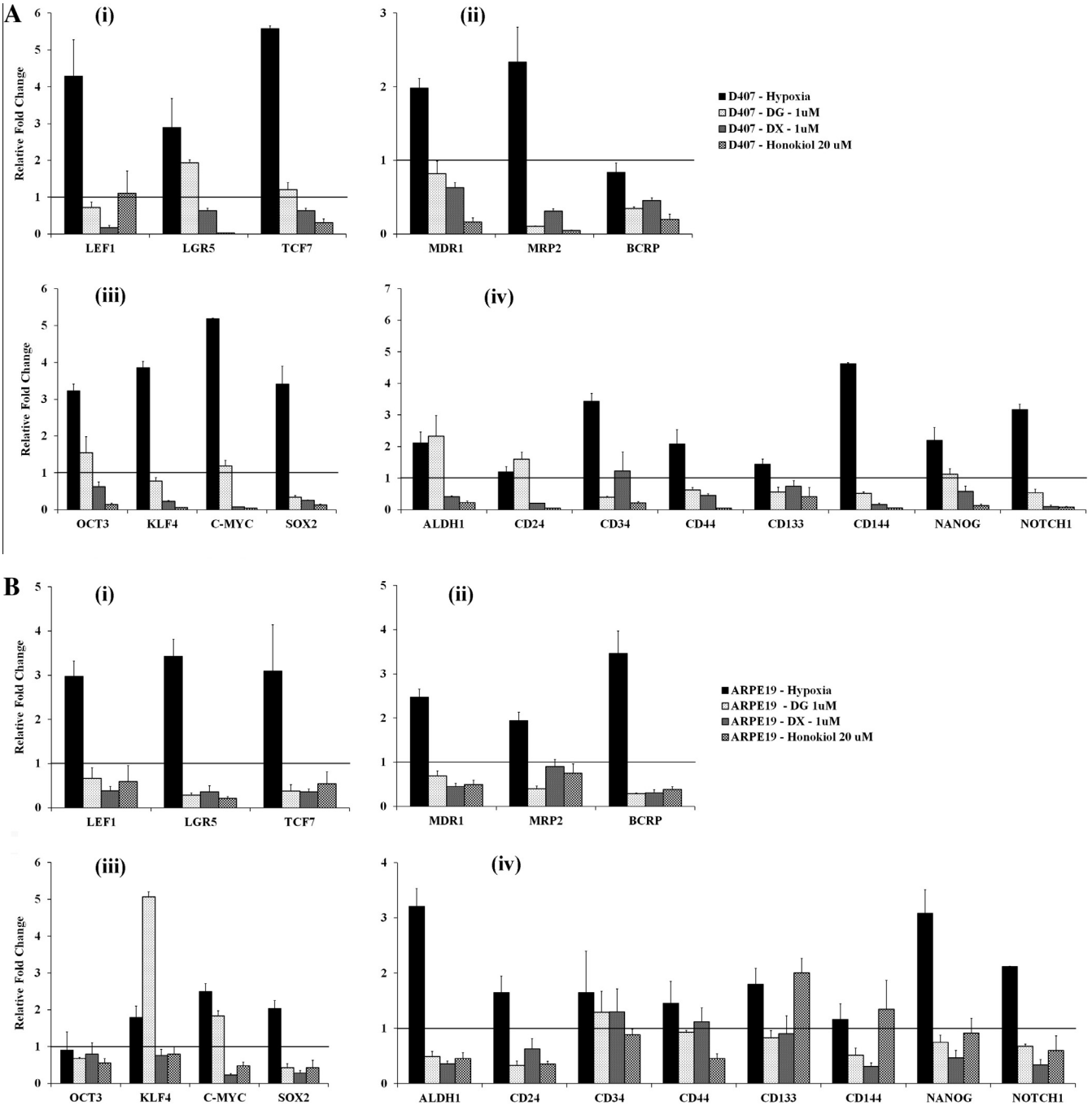

Fig. 3. Relative fold change in the mRNA levels of indicated stem cell/cancer stem cell markers in: D407 (Panel A) and ARPE-19 (Panel B) in the presence or absence of 1 μM digoxin (DG), 1 μM doxorubicin (DX), and 20 μM honokiol under hypoxia. In both panels (i) represents Wnt pathway genes, (ii) represents drug resistance genes, (iii) represents Yamanaka factors, and (iv) represents cancer stem cell-associated markers. Solid, beveled, grey, and checked bars represent relative mRNA fold change ± standard error for control, 1 μM DG, 1 μM DX, and 20 μM honokiol treatments respectively. Normoxic levels are represented by the horizontal line.

3.3. Inhibitors of the HIF pathway down-regulates the expression of stem cell markers

In order to conclusively prove that the transcription of pro-angiogenic and stem cell markers is due to the HIF pathway, we evaluated the effectiveness of digoxin, doxorubicin, and honokiol, three recently identified HIF inhibitors from natural sources, to suppress the transcription of selected markers. We found that all the three HIF inhibitors strongly (≈50–90% for most genes) inhibited the expression of HIF-dependent transcription of selected markers in the retinal pigment epithelial cell lines (D407 and ARPE-19) under hypoxia, and *VHL*−/− renal cell carcinoma cells under normoxic conditions (Figs. 1–3). These results confirm that the inhibition of the HIF pathway by digoxin, doxorubicin, and honokiol, is conserved across multiple cell lines without any noticeable cytotoxicity both under hypoxic and normoxic conditions. Importantly, no significant change in the transcription of HIF independent housekeeping genes (ribosomal protein L32, actin and GAPDH) was noted (data not shown). These results suggest that digoxin, doxorubicin, and honokiol are not general transcription inhibitors, but rather are specific inhibitors for the HIF pathway.

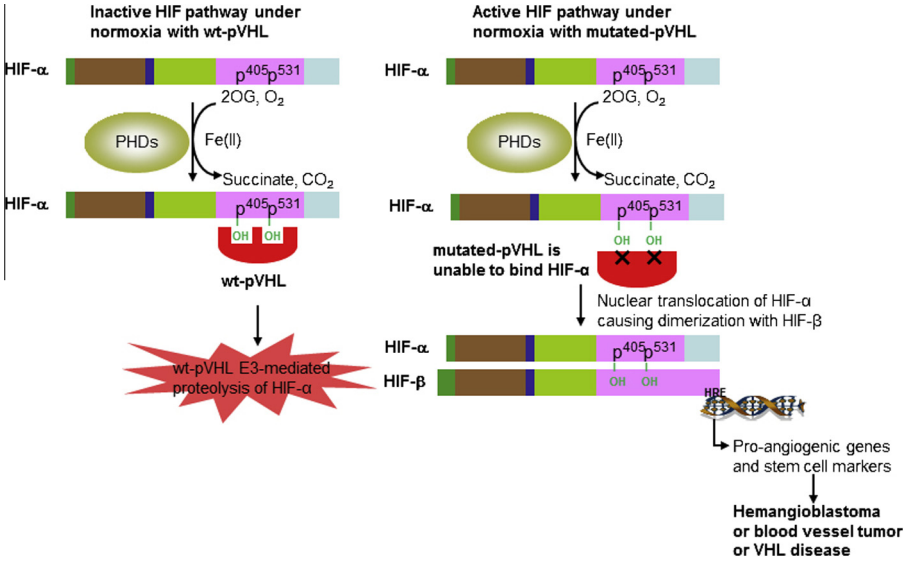

Fig. 4. A model showing modulation of the HIF pathway by pVHL under normal conditions or in hemangioblastomas. Under normal normoxic conditions (left panel), PHDs hydroxylate HIF-α on proline (P405/531) residues, leading to ubiquitin ligase pVHL E3-mediated destruction of HIF-α. However, in patients with mutations in pVHL, although HIF-α gets hydroxylated at the proline residues, it escapes proteosomal degradation, allowing its translocation and dimerization with HIF-β in the nucleus leading to transcription of hypoxia response genes even under normoxic conditions, which includes a number of pro-angiogenic and stem cell markers in neoplastic stromal cells in hemangioblastomas (right panel). The fragment colors of HIF subunits represent different protein domains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The treatment of RCC4, RCC4–T314, APRE-19 and D407 cells with honokiol under normoxia or hypoxia did not alter their proliferation in cell culture (data not shown). These results are consistent with a number of cell-based studies showing that the inhibition of the HIF pathway in *VHL*−/− RCC cells, by reintroduction of wt-*VHL* in *VHL*−/− cells, does not inhibit their proliferation in cell culture [18,21,22]. However, such inhibition of the HIF pathway abolishes the tumor forming ability of *VHL*−/− 786-O cells in mouse models [18,21,22]. Therefore, the inhibitors of the HIF pathway evaluated in this study, all of which are used by humans for other conditions, can be used for the treatment of pVHL-associated hemangioblastomas.

VHL disease is an autosomal dominant tumor syndrome with a predilection for the retina, CNS, and kidney. The incidence of VHL disease is ≈1 in 36,000, and it is estimated that there are ≈7000 patients with VHL disease in the US alone. The VHL disease is associated with significant mortality with an average life-expectancy of patients is ≈50 years. The mean age at diagnosis of hemangioblastomas in VHL disease is ≈25 years. Ocular hemangioblastoma is observed in upto 85% patients with VHL disease and is the earliest manifestation of the disease. The VHL-associated ocular hemangioblastomas can cause massive exudation, subretinal edema, inflammation, retinal detachment, or glaucoma, resulting in loss of vision. Current treatment with laser photocoagulation or cryotherapy can cause subretinal and vitreous hemorrhage. Further, using such methods, it is difficult to specifically target and treat optic disc lesions. Anti-VEGF therapies have been largely ineffective [8,9], possibly due to contribution of other pro-angiogenic factors, such as EPO, PDGF, etc. in hemangioblastomas. Thus, there remains an unmet medical need to develop more effective treatments for hemangioblastomas. Based on our studies, we propose a model where mutations in the *VHL* gene lead to activation of the HIF pathway in patients with hemangioblastomas. This results in the expression of stem cell/cancer stem cell markers, in addition to overexpression of pro-angiogenic factors, in neoplastic stromal cells (Fig. 4). Finally, the current study evaluates the use of HIF inhibitors as an effective therapeutic agent for the treatment of hemangioblastomas, for which limited treatment options are currently available.

Acknowledgments

This research has been funded by UMKC internal support in the form of start-up to M.M. The RCC4, RCC4–T314, PRC3, and WT8 cell lines were generously provided by Dr. M. Celeste Simon (University of Pennsylvania Cancer Center), while D407 cell line was generously gifted by Dr. Richard Hunt (University of South Carolina). The authors would like to thank Dr. Kun Cheng for allowing us to use the real-time qPCR instrument.

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