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THE CELLULAR OXYGEN SENSOR PHD2 IN CANCER GROWTH

by

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To Urho and Aino

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The Cellular Oxygen Sensor PHD2 in Cancer Growth
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Abstract

Adequate supply of oxygen is essential for the survival of multicellular organisms. However, in several conditions the supply of oxygen can be disturbed and the tissue oxygenation is compromised. This condition is termed hypoxia. Oxygen homeostasis is maintained by the regulation of both the use and delivery of oxygen through complex, sensitive and cell-type specific transcriptional responses to hypoxia. This is mainly achieved by one master regulator, a transcription factor called hypoxia-inducible factor 1 (HIF-1). The amount of HIF-1 is under tight oxygen-dependent control by a family of oxygen-dependent prolyl hydroxylase domain proteins (PHDs) that function as the cellular oxygen sensors. Three family members (PHD1-3) are known to regulate HIF of which the PHD2 isoform is thought to be the main regulator of HIF-1. The supply of oxygen can be disturbed in pathophysiological conditions, such as ischemic disorders and cancer. Cancer cells in the hypoxic parts of the tumors exploit the ability of HIF-1 to turn on the mechanisms for their survival, resistance to treatment, and escape from the oxygen- and nutrient-deprived environment.

In this study, the expression and regulation of PHD2 were studied in normal and cancerous tissues, and its significance in tumor growth. The results show that the expression of PHD2 is induced in hypoxic cells. It is overexpressed in head and neck squamous cell carcinomas and colon adenocarcinomas. Although PHD2 normally resides in the cytoplasm, nuclear translocation of PHD2 was also seen in a subset of tumor cells. Together with the overexpression, the nuclear localization correlated with the aggressiveness of the tumors. The nuclear localization of PHD2 caused an increase in the anchorage-independent growth of cancer cells. This study provides information on the role of PHD2, the main regulator of HIF expression, in cancer progression. This knowledge may prove to be valuable in targeting the HIF pathway in cancer treatment.

KEYWORDS: hypoxia, hypoxia-inducible factor, prolyl hydroxylase domain protein 2, head and neck squamous cell carcinoma, cancer

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Solujen hapentunnistajaproteiini PHD2 syövän kasvussa
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Tiivistelmä

Riittävä hapensaanti on elintärkeää monisoluisille eliöille. Hapensaanti saattaa kuitenkin vaikeutua useissa olosuhteissa ja kudokset voivat kärsiä hapenpuutteesta. Tätä tilaa kutsutaan hypoksiaksi. Hapen homeostaasia ylläpidetään säätelemällä sekä hapen kulutusta että sen kuljetusta monitahoisten, herkkien ja solutyyppispesifisten hypoksiavasteiden avulla geeni-ilmentymän kautta. Enimmäkseen vaste tapahtuu yhden pääsäätelijän, hypoksia-indusoituva faktori-1:n (HIF-1) kautta. HIF:n määrä on tiukasti hapen määrästä riippuvaista. Säätelystä vastaa hapesta riippuvaisten ja happisensoreina toimivien proliinihydroksylaasien perhe, proliinihydroksylaasi-domeeni-proteiinit (PHD). Kolmen perheenjäsenen (PHD1-3) tiedetään säätelevän HIF:ia. Näistä PHD2:n ajatellaan olevan HIF-1:n tärkein säätelijä. Hapensaanti voi vaikeutua patofysiologisissa tiloissa, kuten iskeemisissä sairauksissa ja syövässä. Kasvainten hypoksisissa osissa olevat syöpäsolut hyödyntävät HIF-1:n kykyä käynnistää solujen mekanismeja eloonjäämiseen, hoidon vastustuskykyyn ja pakenemiseen hapen- ja ravinteidenpuutteesta kärsivästä ympäristöstä.

Tässä tutkimuksessa tarkasteltiin PHD2:n ilmenemistä ja säätelyä normaaleissa ja syöpäkudoksissa sekä sen merkitystä kasvainten etenemisessä. Tuloksissa havaittiin, että PHD2:n ilmeneminen lisääntyy hypoksisissa soluissa. PHD2:n havaittiin myös selvästi yli-ilmentyvän pään ja kaulan levyepiteelisyövissä ja paksunsuolen syövissä. Vaikka PHD2 on normaalisti sytoplasminen, sen havaittiin siirtyvän myös tumaan osassa syöpäsoluista. Yhdessä yli-ilmenemisen kanssa tumailmenemisen havaittiin korreloivan voimakkaasti syövän aggressiivisuuden kanssa. PHD2:n ilmeneminen tumissa lisäsi myös syöpäsolujen kykyä kasvaa kiinnittymättä alustaansa. Tämä tutkimus on lisännyt tietoa PHD2:n, tärkeimmän HIF-1:n ilmenemisen säätelijän, roolista syövän etenemisessä. Tämä tieto saattaa osoittautua arvokkaaksi kohdistettaessa syöpähoitoja HIF-reitteihin.

AVAINSANAT: hypoksia, hypoksia-indusoituva tekijä, proliinihydroksylaasi-domainiproteiini 2, pään- ja kaulan levyepiteelisyöpä, syöpä

Table of Contents

Abbreviations and PHD Nomenclature

17-AAG	17-allylaminogeldanamycin
2-OG	2-oxoglutarate
AhR	aryl hydrocarbon receptor
ARD	ankyrin repeat domain
ARNT	aryl hydrocarbon nuclear translocator
ATF	activating transcription factor
β_2AR	β_2-adrenergic receptor
bHLH	basic helix-loop-helix
CAIX	carbonic anhydrase 9
CBP	CREB (cyclic AMP response element binding protein)-binding protein
ccRCC	clear cell renal cell carcinoma
CHIP	carboxyl terminus of Hsc 70 interacting protein
CODD	C-terminal oxygen-dependent degradation domain
CTAD	C-terminal transactivation domain
EGF	epidermal growth factor
EGFP	enhanced green fluorescence protein
EGFR	epidermal growth factor receptor
EGLN	egl nine homolog
EPAS1	endothelial PAS domain protein 1
ESI-MS	electrospray ionization mass spectrometry
FIH	factor inhibiting HIF
FKBP38	FK506-binding protein 38
HAF	hypoxia-associated factor
HIF	hypoxia-inducible factor
HNSCC	head and neck squamous cell carcinoma
HPH	HIF prolyl hydroxylase
HRE	hypoxia-responsive element
Hsp90	heat shock protein 90
IGF-1/2	insulin-like growth factor 1/2
Igfbp1	insulin-like growth factor binding protein 1
IKKβ	inhibitor of NF-κB kinase beta
IL-8	interleukin-8
IL-1β	interleukin-1β
ING4	inhibitor of growth family, member 4
IPAS	inhibitory PAS domain protein
LMB	leptomycin B
LOX	lysyl oxidase
LZIP	leucine zipper
MAGE-11	melanoma antigen-11
mAKAP	muscle A kinase-anchoring protein
MAPK	mitogen-activated protein kinase
Morg1	MAPK organizer 1
MVP	major vault proteins
MYND	Myeloid translocation protein 8, Nervy, DEAF1
NES	nuclear export signal
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGF	neural growth factor

NLS	nuclear localization signal
NO	nitric oxide
NODD	N-terminal oxygen-dependent degradation domain
NTAD	N-terminal transactivation domain
ODDD	oxygen-dependent degradation domain
OS9	Osteosarcoma amplified 9
PI3K	phosphatidylinositol-3' kinase
PAS	Per-Arnt-Sim
PHD	prolyl hydroxylase domain containing protein
PET	positron emission tomography
PI3K	phosphatidyl-inositol-3' kinase
pO_2	oxygen partial pressure
PPAR-γ	peroxisome proliferative activated receptor gamma
RACK1	receptor of activated protein kinase C
Rbx1	RING-box protein 1
RCC	renal clear cell carcinoma
ROS	reactive oxygen species
SDF1	stromal derived factor 1
SENP1	SUMO1/sentrin specific protease
SLS	subcellular localization signal
SSAT2	spermidine/spermine-N(1)-acetyltransferase 2
SUMO	Small ubiquitin-like modifier
TAD	transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
TMA	tissue microarray
TNF-α	tumor necrosis factor alpha
TRiC	TCP-1 ring complex
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau protein

PHD nomenclature:

PHD1 = EGLN2 = HPH3 = HIF-PH4-1
PHD2 = EGLN1 = HPH2 = HIF-PH4-2
PHD3 = EGLN3 = HPH1 = HIF-PH4-3

List of Original Publications

This study is based on the following publications, which are referred to in the text by the Roman numerals I-IV

I Marxsen, J.H., Strengel, P., Heikkinen, P., Jokilehto, T., Wagner, T., Jelkmann, W., Jaakkola, P. & Metzen, E.: Hypoxia-Inducible Factor (HIF)-1 Promotes Its Degradation by Induction of Prolyl-4-Hydroxylases. *Biochem. J* 381: 761-767, 2004

II Jokilehto, T., Rantanen, K., Luukkaa, M., Heikkinen P., Grenman, R., Minn, H., Kronqvist, P. & Jaakkola, P.: Overexpression and nuclear translocation of HIF prolyl hydroxylase PHD2 in epithelial cancers associates with tumor aggressiveness. *Clin Cancer Res* 12: 1080-1087, 2006

III Jokilehto, T., Högel, H., Rantanen, K., Elenius K., Sundström, J. & Jaakkola, P.: Retention of prolyl hydroxylase PHD2 in the cytoplasm prevents PHD2-induced anchorage-independent carcinoma cell growth. *Exp Cell Res.* 316: 1169-1178, 2010

IV Jokilehto T. & Jaakkola, P. The role of HIF prolyl hydroxylases in tumor growth. *J Cell Mol Med* 14: 758-770, 2010

1 Introduction

Complex multi-cellular organisms appear to have evolved during a period of significant increase in the environmental oxygen levels approximately 1.6 billion years ago. At the present time oxygen is an essential element for the survival of most multi-cellular organisms. Direct diffusion of oxygen is enough to fulfill its demand in simple invertebrates, but larger multicellular organisms have developed complex structures to ensure a sufficient supply of oxygen to their cells. The levels of cellular oxygen are tightly controlled, since the consequences of too much or too little oxygen can be detrimental. While the excess of oxygen can lead to the formation of possibly disastrous oxygen radicals, an adequate supply of oxygen is essential for the survival of cells. Oxygen serves as the final electron acceptor in the mitochondrial oxidative phosphorylation chain and as a substrate in at least 100 enzymatic reactions. Mammals have developed a complex physiological system for maintaining oxygen homeostasis at the tissue level. It involves the capture, binding, transport and delivery of molecular oxygen. One essential aspect of this network is the ability to sense and respond to the circumstances of low oxygen, hypoxia.

Oxygen homeostasis is maintained by the regulation of both the use and delivery of oxygen through complex, sensitive and cell-type specific transcriptional responses to hypoxia. This is mainly achieved by one master regulator, a transcription factor called hypoxia-inducible factor (HIF). HIF helps cells to cope in hypoxic conditions by inducing the transcription of several genes involved in, for example, vascularization, erythropoiesis and glycolysis. The existence of HIF is under tight oxygen-dependent control by a family of oxygen-dependent prolyl hydroxylases (PHD) that function as the cellular oxygen sensors. Three PHD isoforms have been identified (PHD1-3). PHD2 has been suggested to be the most important regulator of HIF under normoxia.

The supply of oxygen can be disturbed in numerous pathophysiological conditions, such as ischemic disorders and cancer. Although compensatory mechanisms to hypoxia are essential for normal cells, the ability of cells to survive in hypoxic conditions can sometimes turn into a disadvantage for the host. One example are cancer cells in the hypoxic parts of the tumors, where they exploit the ability of HIF to turn on their mechanisms for survival, resistance to treatment, and escape from the oxygen- and nutrient-deprived environment.

It is important to understand the role of HIF and HIF regulators in the progression of tumors. Although the significance of hypoxic response in cancer has been studied extensively, little is known about the specific roles of the HIF regulators in tumors. The aim of this study was to characterize the expression of PHD2 in tumors and to study its significance in tumor growth. As our understanding of the molecular mechanisms underlying the pathophysiological processes increases, so will the opportunity for therapeutic intervention.

2 Review of the Literature

2.1 Hypoxia

2.1.1 General aspects of hypoxia

Molecular oxygen is required for aerobic metabolism to maintain intracellular bioenergetics, since it serves as the final electron acceptor in oxidative phosphorylation. There are also more than 100 known enzymatic reactions that use oxygen as a substrate and the majority of them are present in mammalian tissues (Vanderkooi et al. 1991). Hypoxia occurs when oxygen levels are not adequate to fulfill the demand of tissues. It is considered one of the most relevant cellular stresses.

Most mammals require ~21% atmospheric oxygen (~160 mmHg partial pressure), which is considered as a physiological environment. The majority of mammalian tissues exist at 2%-9% oxygen. Mammals have evolved a sophisticated physiological network to maintain oxygen homeostasis at the tissue level. It involves the capture, binding, transport and delivery of molecular oxygen to tissues. However, in many situations cells can become hypoxic. Physiologic hypoxia occurs during development, exposure to increased elevation, physical activity, and wound healing, among others. Hypoxic conditions are also created by increased metabolic activities in rapidly expanding tissues or by decreased blood supply in ischemic areas. Hypoxia can occur continuously, as upon ascending or staying in high altitude conditions. It can also occur intermittently, for example, in people suffering from sleep apnea, in whom airway obstruction transiently blocks oxygen uptake and causes blood oxygen levels to decrease rapidly. This wakens the person, who then breaths normally and oxygen levels rise again. Hypoxia can also be divided into an acute phase and a chronic phase. In the acute phase rapid but transient responses occur. They are mediated by post-translational modifications of existing proteins. In the chronic phase of hypoxia, delayed but durable changes occur. They are mediated through altered gene transcription and protein synthesis. Hypoxia can also be systemic as in high altitude conditions or local as in the case of myocardial ischemia. For a review see (Semenza 2010b).

Oxygen-sensing mechanisms have evolved to maintain cell and tissue homeostasis, and to allow adaptation to chronic low-oxygen conditions. Exposure to low oxygen levels initiates a response in almost all vertebrate cells that ranges from rapid changes at the cellular level to systemic changes in the whole organism. A response to low oxygen conditions has been identified in both prokaryotes and eukaryotes. Although the mechanisms differ in different organisms, they all share a common goal of preserving the critical energy levels, when extracellular oxygen concentrations decrease.

2.1.2 Oxygen tension in normal tissues

There are several methods for measuring oxygen concentrations in tissues, of which oxygen microelectrodes have been used the most. Other methods include, for example, intrinsic probes for O_2, imaging techniques such as positron emission tomography (PET) detection of radiolabeled tracers, magnetic resonance

spectroscopy, nitroimidazole binding assays and phosphorescence imaging. While different methods give somewhat varying results, the differences are not really significant and they all indicate variations in oxygen concentrations within all tissues (Vanderkooi et al. 1991).

Normoxic conditions vary greatly between the tissues and cell types and there is variation even within organs. The normal oxygen level for any tissue is therefore difficult to define and it never remains constant. Generally normal oxygen tension is given as 2-9 % (14.4-64.8 mmHg) O_2, while some tissues have even less. For example, the thymus, kidney medulla and bone marrow can exist at approximately 1% O_2 (7.2 mmHg) or even lower because of their atypical vascular system (Vanderkooi et al. 1991).

Within organs oxygen exists as a series of gradients from the vessels to the inner tissue, and its concentration varies with arterial O_2 tension, inter-capillary distance and cellular metabolic rate. The blood circulation carries oxygen to tissues and a physiological tissue distribution of oxygen occurs as a result of progressive consumption of the oxygen in the blood circulation as it passes through different organs. Tissues have an oxygen gradient across a distance of approximately 100-200 μm from a blood supply. In the blood flowing from the alveolar capillaries the amount of oxygen is around 13% (104 mmHg) and as the blood flows towards organs the concentration drops. The O_2 concentration of tissues depends on their type, rate of oxygen usage and vascularization. Some tissues can be considered even hypoxic from the molecular point of view. For example, in the rat spleen an O_2 concentration of around 2% (17 mmHg) has been measured while the thymus had a measured concentration of 1% (8 mmHg) (Braun et al. 2001). Highly metabolic tissues, such as the retina, brain and myocardium, use a lot of oxygen; thereby their measured oxygen concentrations are quite low. The rat retina has a relatively low oxygen concentration of 0.2-3% (2-25 mmHg) (Yu & Cringle 2005) and tissues in the rat brain have been reported to have even less oxygen, 0.05-1% (0.4-8 mmHg) depending on the location (Erecinska & Silver 2001). Articular cartilage is a completely avascular tissue and therefore it is a hypoxic environment. The oxygen tension in cartilage is 0.5-5% depending on the depth (Lund-Olesen 1970, Brighton & Heppenstall 1971).

2.1.3 Occurrence of hypoxia in normal tissues

Physiologic hypoxia occurs in several occasions. Systemic hypoxia occurs with exposure to increased elevation (hypobaric hypoxia) and local tissue hypoxia is connected to wound healing and other transient vascular alterations (ischemic hypoxia). During strenuous physical exercise oxygen levels drop significantly in the muscles.

Although hypoxia is mostly considered a cellular stress, it is sometimes even necessary. This is particularly true in the normal embryonic development. The differences in O_2 concentration serve as a signal to guide the development of mammalians. Hypoxic microenvironments often create specific niches that regulate cellular differentiation. They occur in both the developing embryo and the adult, and are sometimes considered essential for keeping the stem cells undifferentiated (for a review see (Simon & Keith 2008)).

2.1.3.1 Hypoxia in development

Molecular oxygen is essential for the development and growth of multicellular organisms. Normal mammalian development occurs in hypoxic conditions. During development, an embryo is exposed to varying levels of oxygen as a balance is created between vascularization and tissue growth. Development is driven by spatiotemporal differences in gene expression and cell behavior. Thereby, oxygen can be seen as a developmental morphogen, since it can modulate cell fates in a concentration-dependent manner. Also the timing of hypoxia and the release from it are key to tissue morphogenesis. Hypoxia can be seen as driving developmental morphogenesis by directing cell differentiation and cell behavior.

In the uterus, oxygen concentrations range from 1% to 5% (pO_2 0.5-30 mmHg) (Fischer & Bavister 1993). Embryonal hypoxia has been studied by means of a hypoxic marker, pimonidazole, in mice (Lee et al. 2001). In the mouse embryo, cells exposed to low oxygen ($\leq$ 2%) are distributed widely until the maternal and fetal blood interface around midgestation. After this, hypoxic cells are still detected in specific regions of the embryo, such as the developing heart and skeleton (Lee et al. 2001). Indeed, an important role for the hypoxic signaling has been shown in the development of the placenta, heart and bones (reviewed in (Dunwoodie 2009)), as well as in the development of lungs (Saini et al. 2008) and in vascularization (Ryan et al. 1998).

The development of the placenta depends on genetic responses to the hypoxic environment. Hypoxia stimulates the proliferation and differentiation of specific placental cell types and O_2 levels regulate several steps of the placentation (reviewed in (Dunwoodie 2009)). Several studies demonstrate that the physiological hypoxia encountered by developing embryos in utero is essential for generating all the components of an intact cardiovascular-pulmonary system (Simon & Keith 2008). Hypoxia is absolutely required for the embryonic vascularization (Ryan et al. 1998), since the development of the vasculature is directly dependent on the differences between tissue oxygen levels. The genetic responses to hypoxia are also essential for the development of the heart. As the mammalian heart forms, it undergoes a number of morphogenetic changes. The development of the myocardium is severely disrupted in embryos that lack components of the hypoxia-sensing machinery (Simon & Keith 2008). O_2 levels also affect the development of bones. The growth plates of developing bones are constitutively avascular and the low oxygen partial pressures experienced by the chondrocytes affect their phenotype as they evolve from a proliferative to a terminally differentiated stage (Rajpurohit et al. 1996).

2.1.3.2 Hypoxia in cellular differentiation

Hypoxia also has a role in the regulation of cellular differentiation. Stem cells and multipotent progenitor cells reside in specific niches, some of which are hypoxic. Hematopoietic stem cells seem to occupy the most hypoxic areas in the bone marrow (Parmar et al. 2007). Oxygen levels can influence stem cell phenotypes, since it can promote the differentiation of some cell types while inhibiting the others. For example, terminal differentiation of megakaryocytes into platelets is promoted by high O_2 concentrations (Mostafa et al. 2001), but the reduction of O_2 levels promotes the differentiation of cultured rat peripheral and central nervous system stem cells into neurons with specific neurotransmitter phenotypes (Morrison et al. 2000, Studer et al. 2000). Oxygen tension also regulates adipogenesis. It is thought, that hypoxia inhibits

adipocyte development from mesenchymal precursors by inhibiting the expression of peroxisome proliferative activated receptor-γ (PPAR–γ)(Tontonoz et al. 1995). There is also evidence that hypoxia is necessary in promoting the chondrocyte phenotype (for a review see (Lafont 2010)).

2.1.4 Hypoxia in pathological conditions

Hypoxia is a feature of many pathological conditions. It plays a role in, for example, acute and chronic mountain sickness, kidney and liver diseases, pulmonary hypertension, stroke, retinal degeneration, inflammation, and most forms of cardiovascular disease, including ischemic heart diseases and atherosclerosis. Wound healing and bone fractures are also usually accompanied by local tissue hypoxia due to the disruption of vasculature.

The most common causes of death in western cultures include heart disease, cancer, cerebrovascular disease and chronic lung disease, which all share the common feature of disrupted oxygen homeostasis. "Ischemia" refers to impaired blood flow below the level needed to satisfy the metabolic demands of the tissue, which leads to tissue hypoxia. For instance, cerebrovascular insult and myocardial infarction are examples of ischemic disease. Atherosclerotic stenosis of large arteries in the coronary and femoral circulations results in myocardial and limb ischemia. Cells are thereby deprived of oxygen and nutrients and the cells in the oxygen-deprived areas cannot function normally and ultimately start to die, unless the blood flow is restored. Hypoxia in ischemic cells induces adaptive homeostatic responses such as the increased production of vascular endothelial growth factor (VEGF) and other angiogenic cytokines to restore the blood flow by collateral vessel formation (Semenza 2000).

2.1.4.1 Hypoxia in cancer

Tumors exceeding the volume of $1mm^3$ outgrow their supply of oxygen, which leads to hypoxia. Hypoxia is a characteristic of locally advanced solid tumors and most of them are at least partially hypoxic. Hypoxic areas have been found in a wide range of malignancies, for example, breast cancers, uterine cancers, head and neck cancers, prostate cancer, soft tissue sarcomas, brain tumors and renal cell cancers (reviewed in (Vaupel & Mayer 2007)). Up to 50-60% of locally advanced tumors may exhibit hypoxic and/or anoxic tissue areas that are heterogeneously distributed within the tumor mass (for a review see (Vaupel et al. 2007)). In tumors the oxygen pressure is strongly reduced because of the chaotic, poorly functional architecture of tumor vessels (Folkman 2007) and the intense proliferation of cancer cells that develop distant to blood vessels. Tumor cells are therefore exposed to an oxygen gradient decreasing gradually from efficient oxygenation near the stroma to near anoxia bordering the necrotic areas.

According to Vaupel and Mayer (2007) major pathogenic mechanisms involved in the development of hypoxia in solid tumors can be classified into three groups: 1) severe structural and functional abnormalities of tumor microvessels (*perfusion-limited O_2 delivery*), 2) increased diffusion distances (*diffusion-limited O_2 delivery*) and 3) tumor-associated and/or therapy-induced anemia leading to a reduced O_2 transport capacity of the blood (*anemic hypoxia*). Tumor vasculature is frequently disorganized and leaky, which leads to inadequate perfusion of the tissue. Perfusion-limited oxygen

delivery leads to ischemic hypoxia, which is usually transient. Therefore it is also called “acute hypoxia”. Diffusion-limited oxygen delivery instead leads to so-called “chronic hypoxia”. This is often caused by an increase in oxygen diffusion distances, so that cells far away (>70 μm) from blood vessels receive less oxygen and nutrients. Anemic hypoxia is caused by tumor-associated or therapy-induced factors that reduce the red blood cell capability to transport oxygen (Vaupel & Mayer 2007).

The tumor vessels are often abnormal. As they grow, tumor cells try to attract more vessels by upregulating additional angiogenic factors that can also be upregulated by oncogenes in the malignant cells. The excessive amounts of these angiogenic factors cause changes at the vascular network as well as on the endothelial cell layer of blood vessels. Tumors often have vasculature that consists of disorganized networks of tortuous and mal-shaped vessels with a highly dysfunctional, leaky endothelial cell layer. These endothelial cells are hypermotile and irregularly-shaped. They leave gaps and are loosely connected to each other and have multiple protrusions (Mazzone et al. 2009, Jain 2005). Abnormal tumor vessels also have a defective basement membrane and pericyte coverage (Carmeliet & Jain 2000). The changes in vessels impair perfusion and the supply of oxygen. This causes hypoxia in the tumor cells that in turn upregulate factors that further cause abnormalization of vessels. This vicious cycle further aggravates tumor hypoxia. One possible outcome of this severe hypoxia is that tumor cells switch on invasive and metastatic programs, thereby accelerating tumor cell invasiveness, malignancy and metastasis (Ebos et al. 2009, Loges et al. 2009, Paez-Ribes et al. 2009).

Already in the 1920s Otto Warburg observed that cancer cells favor glycolysis even in normoxic conditions and have reduced rates of oxidative phosphorylation. Highly malignant tumor cells generate ATP primarily via glycolysis even though available oxygen levels would allow them to produce their energy through oxidative phosphorylation, which is a more efficient way to produce energy in the presence of oxygen. This is known as the Warburg effect (Warburg 1956). This effect might be connected to the oxygen-sensing mechanisms.

Tumor hypoxia can lead to different consequences. It is associated with restrained proliferation, differentiation, and cell death but it can also lead to the development of a more aggressive phenotype and promote tumor resistance to therapy. Sustained hypoxia may also lead to cellular changes resulting in a more clinically aggressive phenotype. Cells can respond to hypoxia by reducing their overall protein synthesis, which eventually leads to restrained proliferation and subsequent cell death. In anoxic conditions, most cells undergo immediate arrest and below a critical energy state, hypoxia can also lead to cell necrosis. Hypoxia can also induce programmed cell death, apoptosis. Hypoxia induces apoptotic cell death in normal and minimally transformed cells. Cancer cells, however, frequently contain mutations in their apoptotic pathways and therefore do not undergo apoptotic cell death in hypoxic conditions. The genetic alterations in apoptotic pathways, such as the deficiency of a tumor suppressor p53, have been shown in animal models to reduce sensitivity to traditional chemotherapies and radiation (Lowe et al. 1994). Hypoxia can also function as a physiologic selection pressure on the development of cancer, further enhancing the expansion of tumor cells with diminished apoptotic potential (Graeber et al. 1996). Hypoxia-induced proteome changes leading to cell cycle arrest, differentiation, apoptosis, and necrosis may explain delayed recurrences, dormant

micrometastasis, and growth retardation, which can occur in large tumors. Hypoxia also increases genomic instability, which further enhances tumor progression and aggressiveness (Koshiji et al. 2005). Hypoxia-induced changes may also promote tumor progression via mechanisms that enable cells to overcome the deprivation of nutrients, to escape from the hostile environment and to favor unrestricted growth. During the process of hypoxia-driven malignant progression, tumors may develop an increased potential for local invasive growth, perifocal tumor cell spreading and distant tumor cell metastasis. For example, lysyl oxidase (LOX) expression is connected to hypoxia. LOX expression is tightly associated to metastasis formation, and its inhibition eliminated metastasis in a breast cancer mouse model (Erler et al. 2006).

Hypoxic tumor cells appear to dedifferentiate and acquire stem cell-like properties, as seen in neuroblastoma (Jogi et al. 2002), breast carcinoma (Helczynska et al. 2003) and prostate carcinoma cells (Ghafar et al. 2003). There has been some speculation as to how this happens. It has been thought that tumor stem cells are more resistant to hypoxia parallel to many normal adult stem cells that reside in hypoxic niches, such as the hematopoietic stem cells in the bone marrow. Hypoxia might even promote the survival of these stem cells (Ceradini et al. 2004). Hypoxia might therefore have a function as a selection pressure in favor of the more stem cell-like tumor cells. Hypoxia might also promote the proliferation of the stem cells themselves and thereby increase their proportion in the tumor cell population. However, the hypoxic gene expression itself is likely to have a role in the dedifferentiation of tumor cells. Hypoxic signaling can confer many stem cell-like properties to the cancer cells and decrease the expression of cell type-specific genes (Jogi et al. 2002, Helczynska et al. 2003).

Hypoxia has been suggested to be an unfavorable prognostic factor for the outcome of cancer patients. Disease-free survival has been indicated to be worse, for example, in patients with hypoxic cervical cancer or soft tissue sarcomas (Hockel et al. 1993, Hockel et al. 1996, Nordsmark et al. 2001). In head and neck cancers, hypoxia in tumors appears to predict for poor disease-free and overall survival as well as for worse local control (Dunst et al. 2003, Nordsmark et al. 2005). The pre-treatment tumor hypoxia is highly associated with a poor prognosis for survival after radiotherapy alone or in combination with surgery or chemotherapy in patients with advanced head and neck cancer (Nordsmark et al. 2005). Hypoxic cells are more resistant to many commonly used chemotherapeutic agents because some drugs need oxygen to be maximally cytotoxic (Teicher 1994). Sustained hypoxia can also alter cell cycle distribution and the relative distribution of quiescent cells, which leads to changes in the response to radiation therapy. Already in 1909, Schwarz and colleagues observed that normal mammalian cells irradiated under hypoxic or anoxic conditions were less sensitive to radiation than those cells irradiated in oxygenated conditions (reviewed in (Bertout et al. 2008)).

2.2 Molecular mechanisms of oxygen sensing

2.2.1 Hypoxia-inducible factors

Cells respond to hypoxia through coordinated changes in gene expression. A variety of transcriptional systems, including nuclear factor κB (NF-κB), activating

transcription factors (ATFs) and p53 are responsive to ambient oxygen levels (Blais et al. 2004, Graeber et al. 1994, Zampetaki et al. 2004, Cummins et al. 2007). However, hypoxia-inducible factor-1 (HIF-1) is generally known as the master regulator of hypoxia-regulated transcription.

2.2.1.1 The family of hypoxia-inducible factors

The most extensively studied transcriptional responses to hypoxia are mediated through the transcription factor called hypoxia-inducible factor 1 (HIF-1) that regulates the expression of numerous genes in response to a reduction in the oxygen levels. HIF-1 was first identified as a factor that bound the hypoxia-responsive element in the human erythropoietin gene in hypoxic but not in normoxic conditions (Semenza & Wang 1992a). Later it was purified by ion-exchange and DNA-affinity chromatography and shown to consist of two subunits that are designated HIF-1α and HIF-1β (Wang & Semenza 1995).

In mammals three genes are known to code for the HIF-α subunits: HIF-1α, HIF-2α and HIF-3α, of which HIF-1α and HIF-2α have been the most extensively characterized. HIF-1α is expressed ubiquitously, whereas HIF-2α and HIF-3α are found in a subset of tissues. The alpha subunits are not detectable in normoxic conditions, whereas the beta subunits are constitutively expressed. HIF-1β was previously identified as mammalian aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang et al. 1995). It is widely expressed in different tissues (Carver et al. 1994). The HIF-α subunits can also dimerize with ARNT2 and ARNT3, which are highly related to HIF-1β, but are expressed in a more limited number of cell types (Hirose et al. 1996, Takahata et al. 1998).

HIF-2α (also known as endothelial PAS domain protein 1 (EPAS1), HIF-1α-like factor (HLF), HIF-1α-related factor (HRF) and member of the PAS domain family 2 (MOP2)) was the second family member identified in 1997 (Flamme et al. 1997, Tian et al. 1997, Ema et al. 1997). Whereas HIF-1α is expressed in all nucleated cells, HIF-2α expression is restricted to specific cell types that include endothelial cells, glial cells, type II pneumocytes, cardiomyocytes, kidney fibroblasts, interstitial cells of the pancreas and duodenum and hepatocytes (Wiesener et al. 2003).

HIF-1α and HIF-2α are expressed differentially both temporally and spatially. They can also activate overlapping but distinct sets of genes even within a single cell type (Elvidge et al. 2006). Target genes are often differentially activated by either HIF-1α or HIF-2α in individual cell types. Both HIF-1α and HIF-2α have non-redundant functions and are essential for normal embryogenesis. This is also true for HIF-1β. HIF-1α null embryos die at around day 10.5 of embryogenesis. They have defective cardiac morphogenesis, vascularization and neural tube closure (Ryan et al. 1998, Iyer et al. 1998). HIF-2α null mice have different phenotypes depending on their genetic background. HIF-2α null mice die during embryogenesis with defects in catecholamine production (Tian et al. 1998) or vascularization (Peng et al. 2000) or they die a few days or weeks after birth with mitochondrial dysfunction (Scortegagna et al. 2003) or due to respiratory distress (Compernolle et al. 2002). HIF-1$\beta^{-/-}$ mice die at E10.5 with placental, vascular and hematopoietic defects (Kozak et al. 1997).

HIF-3α was first identified in the mouse (Gu et al. 1998) and the human isoform was characterized in 2001 (Hara et al. 2001). Ten splice variants of HIF-3α have been

characterized, although no evidence has been found for the existence of the predicted variants HIF-3α3 and HIF-3α5 (Maynard et al. 2003, Pasanen et al. 2010). Although the role of HIF-3α is still relatively poorly known, the functions of some of the splice variants have been characterized. One of the mouse variants, that is the most homologous to human isoform HIF-3α2, is also known as inhibitory PAS domain protein (IPAS), which acts as a dominant negative regulator of HIF-1α- and HIF-2α-mediated transcription by forming a complex with HIF-1/2α, preventing its binding to hypoxia-responsive element (HRE) (Makino et al. 2001, Makino et al. 2002, Maynard & Ohh 2007). In the corneal epithelium of the eye IPAS has been shown to be required for keeping the cornea avascular (Makino et al. 2001). Also another HIF-3α isoform, human HIF-3α4, has been shown to act as a dominant-negative regulator of HIF-1α and HIF-2α by forming an abortive transcriptional complex with them and preventing their transcriptional activity in clear cell renal cell carcinomas (ccRCC) (Maynard et al. 2005, Maynard et al. 2007). HIF-3α splice variants have distinct expression patterns in tissues. Generally HIF-3α seems to be more abundant in fetal than in adult tissues (Pasanen et al. 2010). It is found at high levels in the thymus, cerebellar Purkinje cells, kidney, pancreas and the corneal epithelium of the eye (Gu et al. 1998, Hara et al. 2001, Pasanen et al. 2010, Makino et al. 2001).

2.2.1.2 Structure and function

HIFs function as heterodimers composed of α- and β-subunits. Both subunits belong to the superfamily of basic (b) helix-loop-helix (HLH) Per-Arnt-Sim (PAS) proteins (Wang et al. 1995). All members of this group contain a bHLH-domain in the amino terminus preceding the PAS domain (Figure 1). The bHLH-PAS proteins are transcriptional regulators that control a variety of developmental and physiological events (reviewed in (Crews 1998)). The basic-domain is required for DNA binding and the HLH- and PAS-domains are needed for dimerization and DNA binding (Jiang et al. 1996a). The PAS domain is subdivided into two well-conserved regions, PAS-A and PAS-B, which are separated by a spacer (Crews et al. 1988). The C-terminal half of the 826-amino-acid HIF-1α contains two transactivation domains (TAD). The amino acids 531-575 constitute the N-terminal transactivation domain (NTAD), and the amino acids 786-826 form the C-terminal transactivation domain (CTAD) and these transactivation domains are separated by an inhibitory domain (Jiang et al. 1997). HIF-1β contains only one transactivation domain. The activity of the HIF-1α transactivation domains is increased in hypoxia. This is due to the inhibitory domain that hinders the transactivation function in normoxia (Jiang et al. 1997). Amino acids 401-603 comprise an oxygen-dependent degradation domain (ODDD) that is involved in the oxygen-dependent regulation of HIF-1α stability (Huang et al. 1998).

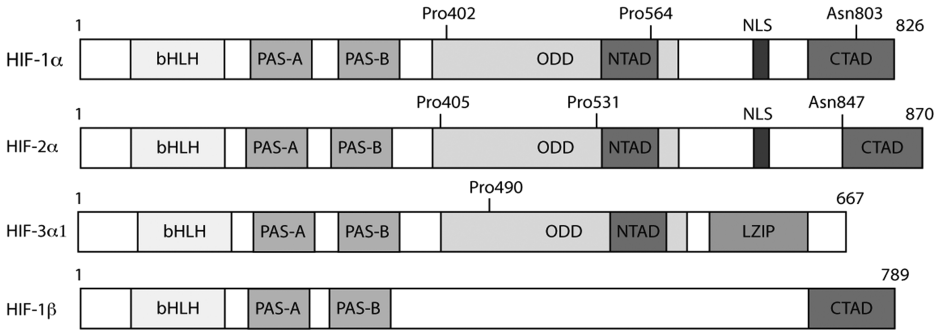

Figure 1. Structure of the hypoxia-inducible factors. HIF-1α, HIF-2α, HIF-3α and HIF-1β have a basic helix-loop-helix (bHLH) domain and a PAS (Per-Arnt-Sim) domain. HIF-αs also contain an oxygen-dependent degradation domain (ODDD) and an N-terminal transactivation domain. HIF-3α has a leucine zipper (LZIP) domain not found in other HIFs but no C-terminal transactivation domain (CTAD), which is present in other HIFs. Indicated prolines and asparagines are hydroxylated by HIF-regulating hydroxylases.

HIF-1α and HIF-2α share similar domain architecture and are regulated in a similar manner. The human HIF-2α has 870 amino acids and shares a 48% sequence identity with the human 866-amino acid HIF-1α (Tian et al. 1997). It has a high similarity to HIF-1α in the structure of key functional domains, the N-terminal bHLH and the PAS domain, but its C-terminus has more variability (Flamme et al. 1997, Tian et al. 1997, Ema et al. 1997).

The human HIF-3α1 has 677 amino acids and shares a high sequence similarity with HIF-1α and -2α over the bHLH and PAS regions but a low degree of similarity across the C-terminus (Gu et al. 1998). HIF-3α has an NTAD, but no CTAD, and therefore HIF-3α may act as a weak transcriptional activator in comparison to the other HIFs (Hara et al. 2001). Some HIF-3α splice variants have a functional ODDD and are therefore regulated in an oxygen-dependent manner (Maynard et al. 2003). HIF-3α1, HIF-3α8 and HIF-3α9 also contain a leucine zipper (LZIP) domain, not found in the other HIF-3αs or HIF-1α or -2α (Pasanen et al. 2010, Maynard et al. 2007). LZIP domains mediate DNA binding and protein-protein interactions (Landschulz et al. 1988). Another feature not shared by other HIF-αs is the signature L-X-X-L-L protein-protein interaction motif, which is found mostly in nuclear receptor co-factors (Maynard et al. 2003). These structures suggest that HIF-3α may have DNA- and protein binding functions not shared with the other HIF-alphas. The mouse inhibitory PAS domain protein (IPAS) is a splice variant of HIF-3α4. Its first 8 amino acids are coded by an IPAS-specific exon 1a instead of the exon 1b. It also lacks the ODDD, CTAD and NTAD domains (Makino et al. 2001, Makino et al. 2002).

Dimerization of HIF-1α and HIF-1β is required for the DNA-binding (Jiang et al. 1996a). Recruitment of HIF-1β is critical for enabling HIF-1α binding to HREs (Kallio et al. 1997). The HIF dimer is translocated into the nucleus where it recognizes and binds to cis-acting hypoxia-responsive elements (5'-RCGTG-3'), located usually in the promoters of hypoxia-responsive genes. A functional HRE

consists of a pair of contiguous transcription factor binding sites, at least one of which is bound by HIF-1 (Semenza et al. 1996). Active complexes then recruit cyclic AMP response element (CREB)-binding protein (CBP) / p300 transcriptional co-activators to the transcriptional complex and initiate the transcription of downstream genes (Arany et al. 1996).

2.2.1.3 Regulation of HIF activity

HIF is mostly non-functional in oxygenated cells. Its activity is mainly regulated by the stability and activity of the α-subunit. The levels of α-subunits are sensitive to cellular oxygen concentration, while the levels of the β-subunit are constant. Regulation of HIF-1α expression and activity occur at multiple levels, including protein expression and stabilization (Wang et al. 1995, Huang et al. 1998, Jiang et al. 1996b, Huang et al. 1996, Kallio et al. 1999), nuclear localization (Kallio et al. 1998) and transactivation (Jiang et al. 1996a, Jiang et al. 1997, Kallio et al. 1998, Pugh et al. 1997).

Both HIF-1α and HIF-1β are expressed constitutively under normoxia and hypoxia. There is evidence that transcription of both HIF subunits is transiently upregulated by hypoxia at least in some tissues (Wang et al. 1995, Wiener et al. 1996, Yu et al. 1998, Belaiba et al. 2007). The transcription of HIF-3α has been shown to be upregulated in hypoxic conditions and this induction is mediated specifically by HIF-1α (Pasanen et al. 2010, Tanaka et al. 2009). In another *in vivo*-study the levels of HIF mRNA remained constant under hypoxic conditions (Stroka et al. 2001). However, in many cell lines under tissue culture the hypoxic induction is absent. The importance of HIF expression in stressful conditions such as hypoxia is highlighted by the fact that HIF-α chain synthesis may be maintained during cellular stresses by the presence of an internal ribosome entry site sequence in the 5'-untranslated region, which makes the cap-directed translational initiation unnecessary (Young et al. 2008).

However, the predominant mode of HIF-α regulation happens at the level of protein stability. The levels of HIF-1α subunit increase exponentially as oxygen concentration declines (Jiang et al. 1996b). In normoxia the half-life of HIF-1α protein is only around five minutes, while in hypoxic conditions the half-life is extended to approximately thirty minutes (Huang et al. 1998). This is because under normoxic conditions the HIF α-chains are rapidly degraded by the proteasome. HIF-α protein stability is regulated by post-translational prolyl hydroxylation of the ODDD (Huang et al. 1998). In human HIF-1α there are two functionally independent hydroxylatable prolyl residues prolines 402 and 564, which reside in the aminoterminal (NODD) and carboxyterminal (CODD) degradation domains, respectively (Huang et al. 1998, Pugh et al. 1997, Masson et al. 2001). These correspond to prolines 405 and 531 in HIF-2α (Wiesener et al. 1998, Ema et al. 1999). The hydroxylation sites reside within a conserved LXXLAP sequence motif (Masson et al. 2001, Epstein et al. 2001). Mutations can be tolerated at each of these positions, except for the hydroxylacceptor proline (Huang et al. 2002). Although a substitution of flanking Leu or Ala residues has little effect on HIF-1α hydroxylation (Huang et al. 2002), an acidic amino acid at the +5 position (relative to the proline) seems to be important (Li et al. 2004). The hydroxylated α-subunit is recognized by the product of the von Hippel-Lindau tumor suppressor gene (pVHL) (Maxwell et al. 1999, Ivan et al. 2001, Jaakkola et al. 2001, Yu et al. 2001) which is a part of a multimeric E3 ubiquitin ligase that ubiquitinates the α-subunit and targets it to proteasomal degradation (Iwai et al. 1999, Lisztwan et

al. 1999). Other members of this E3 complex are elongins B and C, cullin 2 and the RING-box protein 1 (Rbx1) (Kibel et al. 1995, Pause et al. 1997, Lonergan et al. 1998, Kamura et al. 1999). Structurally, the presence of a hydroxyl group excludes a water molecule from the pVHL:HIF-α interface and allows the formation of two additional hydrogen bonds between HIF-α and pVHL, thereby stabilizing their interaction and enhancing ubiquitylation (Hon et al. 2002)

The hydroxylation of the specific proline residues in HIF-alpha subunits and thereby the control of their oxygen-dependent degradation is carried out by a family of prolyl-4-hydroxylases (Ivan et al. 2001, Jaakkola et al. 2001). In mammals, three HIF-4-prolyl hydroxylases have been characterized. They are called prolyl hydroxylase domain proteins (PHDs) 1, 2 and 3. They are also known as HIF prolyl hydroxylases (HPHs) 3, 2 and 1 or egl nine homologs (EGLNs) 2, 1 and 3, respectively (Epstein et al. 2001, Bruick & McKnight 2001, Ivan et al. 2002). They are very sensitive to cellular oxygen concentrations, since they require molecular oxygen as a cosubstrate for their reaction and therefore are effective oxygen sensors. See chapter 2.3.

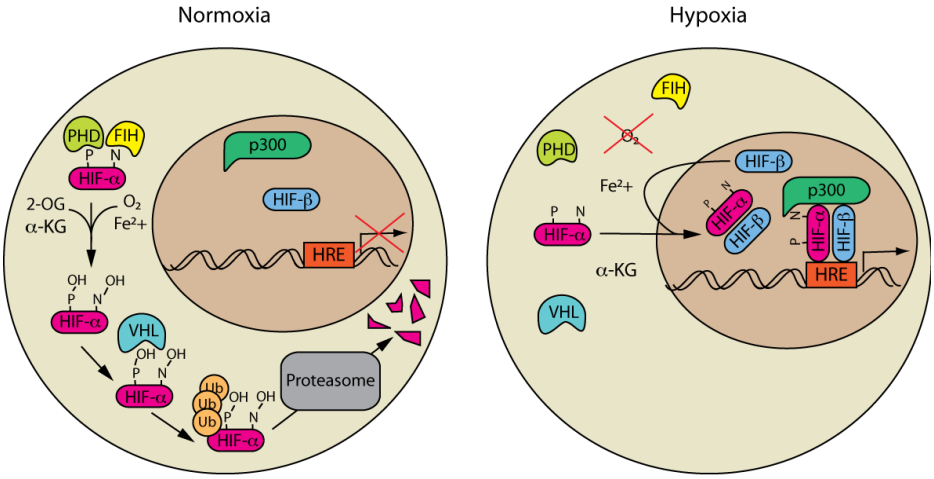

Figure 2. Oxygen-dependent regulation of HIF. In normoxic conditions HIF-α subunit is hydroxylated by prolyl-4-hydroxylases in an O_2, Fe^{2+} and 2-oxoglutarate-dependent way. The hydroxylated proline is recognized by the Von-Hippel-Lindau protein (VHL), an E3 ubiquitin ligase. The HIF-α is ubiquitinylated and degraded by the proteasome. In hypoxic conditions the hydroxylation is diminished and the α-subunit is able to escape the degradation, is translocated into the nucleus and dimerizes with the constitutively expressed β-subunit and is able to start the transcription of target genes containing the hypoxia-responsive element (HRE). Factor inhibiting HIF (FIH) hydroxylates an asparagine residue preventing the binding of the co-activator p300 and the transactivation of HIF. P and N indicate hydroxylation target proline and asparagine residues, respectively. For clarity, only one proline is shown.

The transactivation capability of HIF is also regulated in an oxygen-dependent way. Hydroxylation of an asparagine residue at the C-terminal transactivation domain of HIF-1α (N803) and HIF-2α (N851) by the factor inhibiting HIF (FIH) prevents HIF transcriptional activation by inhibiting the binding of coactivators, such as p300 and its paralogue CREB-binding protein (CBP) (Mahon et al. 2001, Lando et al. 2002).

FIH also belongs to the same protein superfamily, namely non-haem iron containing 2-oxoglutarate dependent dioxygenases, as the PHDs.

In hypoxia, the oxygen levels are not sufficient for the proline or asparagine hydroxylations. Therefore HIF-α escapes recognition by pVHL, ubiquitinylation and proteosomal degradation. This leads to increased protein levels and translocation into the nucleus where the α-subunit dimerizes with the β-subunit and interacts with the transcriptional coactivators, and the active complex binds HRE sequences of target genes and activates transcription (Figure 2).

Other proteins have been shown to take part in regulating the stability of HIF-α. They mainly have roles in facilitating HIF hydroxylation and ubiquitinylation. For example, osteosarcoma amplified 9 (OS9) binds both HIF-1α and PHD2 and stabilizes their interaction, thereby facilitating the prolyl hydroxylation (Baek et al. 2005). Major vault proteins (MVP) form complexes with HIF-1α, PHD2 and pVHL and promote the degradation of HIF, probably functioning as scaffolds (Iwashita et al. 2010). Likewise, spermidine/spermine-N(1)-acetyltransferase (SSAT) 2 binds to HIF-1α, VHL, and elongin C, stabilizing their interaction and facilitating ubiquitination (Baek et al. 2007). An anchoring protein, muscle A kinase-anchoring protein (mAKAP), was also found to function as a scaffold, clustering HIF-1α with its regulatory factors (Wong et al. 2008). HIF-1α stability can also be regulated by sumoylation. The covalent ligation of the small ubiquitin-like modifier (SUMO) has been shown to regulate HIF-1α stability either positively (Bae et al. 2004, Carbia-Nagashima et al. 2007) or negatively (Berta et al. 2007, Cheng et al. 2007). Sumoylation can even promote the binding of VHL to HIF-1α in a hydroxylation-independent manner in hypoxic conditions (Cheng et al. 2007). This is reversed in normal conditions by a SUMO1/sentrin specific protease (SENP1) preventing the degradation (Cheng et al. 2007).

Recently some oxygen-, hydroxylase-, or pVHL-independent mechanisms have been suggested in the regulation of HIF. For example, hypoxia-associated factor (HAF), which is detected in several cell lines and in proliferating tissues, has been shown to regulate HIF stability (Koh et al. 2008). HAF functions as an E3 ligase that promotes the degradation of HIF-1α independent of oxygen tension or the presence of pVHL. HAF is specific to HIF-1α, as neither its knockdown nor overexpression had any effect on HIF-2α (Koh et al. 2008). The Heat shock protein 90 (Hsp90) inhibitor 17-allylaminogeldanamycin (17-AAG) was shown to promote HIF degradation (Liu et al. 2007). The inhibition of Hsp90 allows the receptor of activated protein kinase C (RACK1) to bind HIF-1α and recruit elongin C and other components of the E3 ligase complex, thereby promoting HIF-1α ubiquitination and degradation in an oxygen-, hydroxylation-, and pVHL-independent way (Liu et al. 2007). Hsp70 and CHIP (carboxyl terminus of Hsc70-interacting protein) were also recently identified as HIF-1α interacting proteins that selectively regulate ubiquitination of HIF-1α but not HIF-2α in prolonged hypoxia (Luo et al. 2010).

In addition to hypoxia, several other conditions have been found to modulate HIF expression and to activate HIF transcriptional activity. For example nitric oxide has been shown to regulate HIF accumulation (Sandau et al. 2001). Autocrine stimulation by growth factors such as insulin and insulin-like growth factors 1 and 2 (IGF-1/2) have been shown to regulate HIF-α through the phosphatidylinositol-3' kinase (PI3K) pathway, probably through the increased rate of translation (Zelzer et al. 1998,

Feldser et al. 1999). The cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) stimulate DNA binding of HIF (Hellwig-Burgel et al. 1999). Overexpression of the v-src oncogene (Jiang et al. 1997) or the loss of tumor suppressor function of p53, pVHL or PTEN (Maxwell et al. 1999, Ravi et al. 2000, Zundel et al. 2000) can lead to HIF overexpression. Phosphorylation has also been shown to have an effect on HIF-α. For example, HIF-1α can be phosphorylated by the p44/p42 MAP kinase pathway, which increases its transcriptional activity (Richard et al. 1999).

2.2.1.4 HIF-regulated genes

Erythropoietin was the first gene found to be regulated by HIF (Semenza & Wang 1992a). Numerous other HIF target genes have been characterized since. It has been approximated that at least 1.5% of the genome is transcriptionally responsive to hypoxia (Koong et al. 2000, Denko et al. 2003, Lendahl et al. 2009). To date more than 100 HIF-regulated genes are known and the number is continuously increasing. Some genes are widely inducible, but many genes are induced only in certain cellular contexts and the battery of target genes varies greatly between different cell types (Denko et al. 2003). All HIF-1α-inducible genes contain at least one copy of the core HRE consensus sequence 5'RGTGC3' (For a review see (Guillemin & Krasnow 1997)). HIF targets include proteins needed for oxygen transport and delivery, the survival and proliferation of cells and glucose metabolism (Table 1). Overall, the genes targeted by HIF help cells to adapt to, and thereby survive in, a stressful hypoxic microenvironment.

Table 1. Target genes of HIF. Examples of HIF target genes in functional categories.

O_2 transport and delivery
Vascular endothelial growth factor A (Levy et al. 1995, Liu et al. 1995, Forsythe et al. 1996)
Endothelial-gland-derived VEGF (LeCouter et al. 2001)
VEGF receptor (Flt-1) (Gerber et al. 1997)
Plasminogen-activator inhibitor-1 (Kietzmann et al. 1999, Fink et al. 2002)
Erythropoietin (Semenza & Wang 1992b)
Leptin (Grosfeld et al. 2002)
Angiopoietin-1 & 2 (Kelly et al. 2003)
Transferrin (Rolfs et al. 1997)
Transferrin receptor (Tacchini et al. 1999, Lok & Ponka 1999)
Ceruloplasmin (Mukhopadhyay et al. 2000)
Tyrosine hydroxylase (Norris & Millhorn 1995)
α_{1B}-adrenergic receptor (Eckhart et al. 1997)
Heme oxygenase (Lee et al. 1997, Wood et al. 1998)
Inducible nitric oxide synthase (Melillo et al. 1995)
Endothelial nitric oxide synthase (Coulet et al. 2003)
Endothelin-1 (Hu et al. 1998, Minchenko & Caro 2000, Camenisch et al. 2001)
Adrenomedullin (Cormier-Regard et al. 1998, Nguyen & Claycomb 1999)
Atrial natriuretic peptide (Chun et al. 2003)
Intestinal trefoil factor (Furuta et al. 2001)
Multidrug-resistance P-glycoprotein (Comerford et al. 2002)

Cell proliferation, survival and apoptosis
Insulin-like growth factor-2 (Tazuke et al. 1998)
Insulin-like growth factor binding protein-1, 2, & 3 (Tazuke et al. 1998)
p21 (Carmeliet et al. 1998)
Nip3 (Bruick 2000)
Transforming growth factor-β3 (Caniggia et al. 2000)
Endoglin (Sanchez-Elsner et al. 2002)
Wilms' tumor suppressor (Wagner et al. 2003)
α-fetoprotein (Mazure et al. 2002)
Calcitonin-receptor-like receptor (Nikitenko et al. 2003)
Glucose metabolism
Glucose transporters 1&3 (Zelzer et al. 1998, Ebert et al. 1995, Chen et al. 2001)
Hexokinase-1 & 2 (Iyer et al. 1998, Riddle et al. 2000)
6-phosphofructo-1-kinase L (Semenza et al. 1994)
Glyceraldehyde-3-phosphatedehydrogenase (Graven et al. 1999)
Aldolase A & C (Semenza et al. 1996, Semenza et al. 1994)
Enolase-1 (Semenza et al. 1996)
Phosphoglycerate kinase-1 (Semenza et al. 1994, Firth et al. 1994)
Lactate dehydrogenase A (Semenza et al. 1996)
6-phosphofructo-2-kinase (Semenza et al. 1994)
Carbonic anhydrase 9 (Wykoff et al. 2000)
Adenylate kinase-3 (Wood et al. 1998)
Pyruvate kinase (Iyer et al. 1998)
Transcriptional regulation
DEC1 & DEC2 (Miyazaki et al. 2002)
ETS1 (Oikawa et al. 2001)
CITED2/p35srj (Bhattacharya et al. 1999)
Jumonji domain-containing histone demethylases (Beyer et al. 2008, Krieg et al. 2010)
miR-210 (Crosby et al. 2009, Kulshreshtha et al. 2007)
Cell migration
Chemocine receptor CXCR4 (Staller et al. 2003)
c-met (Pennacchietti et al. 2003)

2.2.1.5 HIF in cancer

Hypoxic regions within tumors induce HIF activity and lead to the activation of HIF-dependent gene expression. Immunohistochemical techniques have indicated that HIF is overexpressed in a wide range of human cancers (Maxwell et al. 2001, Semenza 2010a). Both HIF-1α and HIF-2α are generally overexpressed in tumors but in a differential manner (reviewed in (Semenza 2003)). HIF-1α is expressed in most of the common human cancer types. These include head and neck squamous cell carcinoma (HNSCC) and breast cancer (Zhong et al. 1999), bladder, breast, colorectal, hepatocellular, ovarian, pancreatic, prostate cancers and glioblastomas (Talks et al. 2000), non-small cell lung carcinoma and melanoma (Giatromanolaki et al. 2001), cervical and ovarian cancer (Birner et al. 2000, Birner et al. 2001), and renal clear cell (RCC) carcinoma (Raval et al. 2005). The expression of HIF-1α correlates, together with the downstream targets, such as carbonic anhydrase IX (CAIX), to poor

prognosis and resistance to treatment in multiple cancer types (Giatromanolaki et al. 2001, Hui et al. 2002, Aebersold et al. 2001, Koukourakis et al. 2002, Korkeila et al. 2009). Whereas HIF-1α is ubiquitously expressed, HIF-2α expression is more restricted to specific cancer types such as ovarian cancer, gliomas and RCC, among others (Wiesener et al. 2003, Talks et al. 2000, Raval et al. 2005).

Tumor hypoxia and the HIF-system are intensely involved in the process of tumor progression, conferring growth advantage to tumor cells and to the development of a more malignant phenotype. In general, the HIF-dependent genes have functions in most cellular responses required for the cancer cells to survive and progress in very low oxygen tension. The genes have functions in glucose homeostasis, angiogenesis, metastasis formation, growth factor activation, apoptosis regulation, protein translation and immune responses (Figure 3).

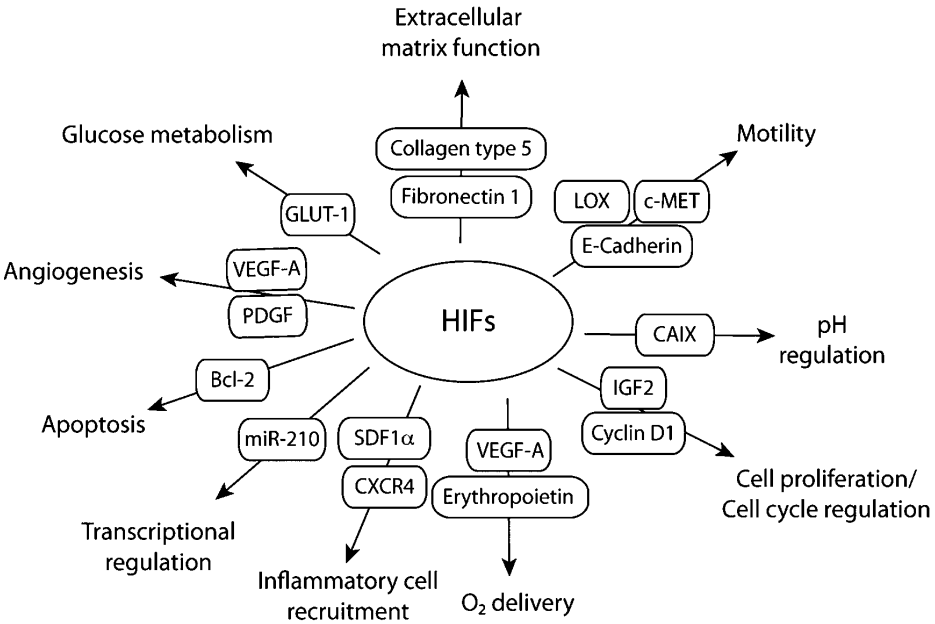

Figure 3. Examples of HIF-activated genes that are involved in tumor progression. HIF-1α and HIF-2α activate the transcription of several genes involved in the tumor initiation, growth and metastasis.

Angiogenesis has been recognized to be critical for the growth of tumors beyond a certain size. HIF can activate a number of key angiogenic factors, including VEGF and its receptor FLT-1 (Gerber et al. 1997, Levy et al. 1996). Several hypoxia-induced genes have a great influence in tumor invasion and metastasis. The levels of an extracellular matrix protein LOX are increased in hypoxic tumors (Erler et al. 2006, Denko et al. 2003). Patients with high LOX levels had lower metastasis-free and overall survival rates. LOX is important for cell invasion. It can modulate cell migration through regulating focal adhesion kinase specificity (Erler et al. 2006). It has also been shown that HIF negatively regulates E-cadherin expression. E-cadherin is essential for maintenance of epithelial cell-cell adhesion and its loss is an important

factor in the progression, aggressiveness and invasion of renal cancers (Krishnamachary et al. 2006, Esteban et al. 2006). A chemokine CXCR4 and its ligand stromal-derived factor (SDF1/CXCL12) have been shown to be hypoxia-inducible (Ceradini et al. 2004, Staller et al. 2003, Taichman et al. 2002). These factors are important for the homing of metastatic cells to different tissues, for example in prostate cancer metastasis to bone (Taichman et al. 2002). CXCR4 was also found to be expressed and active in human melanoma metastases (Scala et al. 2006).

Undisputedly the increased growth factor signaling, such as that activated by the transforming growth factor-beta (TGF-β) or epidermal growth factor (EGF), is critical for many cancers to progress. TGF-β, a strong metastasis promoting factor, is activated by HIF (Caniggia et al. 2000, Li et al. 2008). Likewise, hypoxia activates epidermal growth factor receptor (EGFR) signaling by inducing the expression of the transforming growth factor-alpha (TGF-α), an EGFR ligand, in renal cancer cells (Gunaratnam et al. 2003). Growth factors further operate in other direction by activating HIF transcriptional activity. These include autocrine stimulation of HIF-α by insulin and IGF-1 through PI3K pathway, probably through increased rate of HIF-α translation (Zelzer et al. 1998). Finally, well-known oncogenes such as c-Myc co-operate with the HIF pathway to influence, for example, cell cycle regulation. HIF-1α has been shown to repress c-Myc function in a variety of ways (Koshiji et al. 2004, Ernens et al. 2006). In contrast HIF-2α can promote c-Myc activity by stabilizing the Myc-Max heterodimer (Gordan et al. 2007). As c-Myc may induce either transformation but also senescence, HIF and the PHDs are likely to be directly involved, not only in the progression of advanced cancer, but also in the earlier steps of tumorigenesis.

HIF-1α and -2α have shared but also unique target genes. For example glycolytic gene expression is regulated mainly by HIF-1α, while HIF-2α induces the expression of cell cycle regulators and differentiation factors such as cyclin D1 and Oct4 (Covello et al. 2006). Accordingly, the HIF isoforms have different outcome in cancer. In neuroblastomas HIF-2α can be seen in well-vascularized areas and its expression is associated with more aggressive disease. The expression seems to promote growth and metastasis in later stages of cancer progression (Holmquist-Mengelbier et al. 2006).

In breast carcinoma and endothelial cells HIF-1α has been shown to be the major isoform required for the induction of most hypoxic genes, but for hypoxia-induced cell migration HIF-2α was also required (Sowter et al. 2003). In a panel of breast carcinoma cell lines HIF-1α was responsible for the regulation of hypoxia-inducible genes tested, while overexpression of HIF-2α inhibited the growth of breast cancer cells (Blancher et al. 2000). In patients with the von Hippel-Lindau syndrome the overexpression of HIF-2α is important in the development of renal carcinoma (Kondo et al. 2003).

Finally, any VHL function inactivating mutation will cause much of the above-mentioned genes to be dysregulated in a PHD-independent manner. In keeping with this, VHL deletion or mutations are seen not only in typical tumors of the VHL syndrome (hemangioblastomas, pheochromocytomas, renal clear cell carcinoma) but also approximately 70% of sporadic RCC carcinomas have mutated VHL. Taken

together, hypoxia and the PHD-HIF-VHL signaling system regulate most of the cellular responses that are essential for cancer cell survival, growth and metastasis formation. VHL disease now appears to be manifested in most circumstances as a consequence of deregulated HIF-α, which is involved in tumor progression.

2.3 HIF prolyl hydroxylases

2.3.1 Structure and function

PHDs belong to an evolutionarily conserved superfamily of non-haem iron (Fe2+)-containing 2-oxoglutarate (2-OG)-dependent dioxygenases (Aravind & Koonin 2001, Schofield & Zhang 1999). This family comprises of at least three members of HIF prolyl 4-hydroxylases, PHD 1, 2 and 3 and a HIF asparagine hydroxylase, factor inhibiting HIF (FIH). A fourth HIF 4-prolyl hydroxylase, a transmembrane domain containing prolyl 4-hydroxylase (P4H-TM), has also been reported (Oehme et al. 2002, Koivunen et al. 2007b). It is associated with the endoplasmic reticulum and it hydroxylates HIF-1α ODDD *in vitro* although it has more sequence similarity with the collagen prolyl hydroxylases than with the PHDs (Oehme et al. 2002, Koivunen et al. 2007b). Its possible *in vivo* function in HIF- hydroxylation still remains unknown.

PHD homologues have been characterized in all multicellular species, including the simplest known animal, the placozoan, *Trichoplax adhaerens*, but are not found in non-metazoan taxa (Loenarz et al. 2011). PHDs in other species include: *Caenorhabditis elegans* EGL-9 (Epstein et al. 2001) and *Drosophila melanogaster* dHPH (Bruick & McKnight 2001). The human PHD2 is the most closely related ancestrally to the HIF prolyl hydroxylases in *D. melanogaster*, *C. elegans* (Taylor 2001) and *T. adhaerens* (Loenarz et al. 2011).

Human PHD isoenzymes 1, 2 and 3 consist of 407, 426 and 239 amino acids, respectively, and they show a 42-59 % sequence similarity (Taylor 2001). PHDs have a well-conserved hydroxylase domain at their C-terminal halves, while their N-terminal halves are more divergent and more poorly characterized (Figure 4) (Epstein et al. 2001). PHD2 also contains a Myeloid translocation protein 8, Nervy, and DEAF1 (MYND)-type zinc finger domain N-terminally (amino acids 21-58) (Choi et al. 2005). The MYND- domain seems to have an inhibitory role on the catalytic activity of PHD2 (Choi et al. 2005). Recent data suggest that the MYND anchors PHD2 to mitochondrial or ER membranes through the interaction with the FK506-binding protein 38 (FKBP38) and the membrane bound PHD2 is degraded (Barth et al. 2009). The MYND-domain in PHD2 is conserved throughout the metazoans (Loenarz et al. 2011)The C-terminal region of PHD2 contains the conserved catalytic domain (amino acids 294-392).

The secondary structure of the PHD active core is shown to fold into a common β-barrel jelly-roll motif, composed of eight β-strands that are folded into a double-stranded helix, which positions a nonheme-iron co-coordinating HXD…H motif at the catalytic site (Chowdhury et al. 2009). This jelly-roll core supports three Fe(II) binding ligands, which comprise a conserved two-histidine-one carboxylate iron co-coordinating triad. In HIF hydroxylases the iron binding carboxylate is an aspartyl residue (Schofield & Ratcliffe 2005). There are two positions for binding 2-oxo-glutarate and one for molecular oxygen.

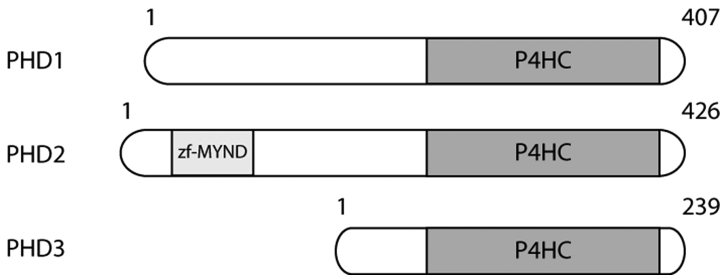

Figure 4. The structure of PHDs. All PHDs contain a prolyl hydroxylase domain (P4HC) in their C-terminal ends. PHD2 has a MYND-type zinc finger domain N-terminally. The numbers indicate the first and last amino acids.

The crystal structure of PHD2's catalytic domain has been determined in complex with Fe^{2+} and a 2-OG-competitive inhibitor, compound A {[(4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl]amino}acetic acid (McDonough et al. 2006). The catalytic domain of PHD2 crystallizes as a homotrimer, although in solution it is more likely to exist as a monomer. This crystal structure shows that in PHD2 the position of the C-terminal helix relative to the active site puts PHD2 structurally into a distinct subfamily of 2-OG oxygenases that includes enzymes involved in the biosynthesis of cephalosporin family of β-lactam antibiotics. The PHDs thus differ from the FIH subfamily, where at least for FIH, the C-terminal helices enable dimerization. The opening to the active site of PHD2 is narrower than in other known 2-OG-oxygenases, which might explain tight binding constants for Fe^{2+} and 2-OG (McDonough et al. 2006). Sequence comparisons and modeling studies indicate that the catalytic domain structure of PHD2 is highly conserved among the three human PHDs. Their specificity is therefore likely to emerge from their variable N-terminal regions as well as the sequence of the *β2-β3* finger motif that is not well conserved among the three isozymes (McDonough et al. 2006). The erythrocytosis-associated PHD2 mutant Arg371His binds HIFs significantly weaker than the wild type PHD2. This suggests the location of an active site in PHD2 that binds HIF (Percy et al. 2007).

PHDs catalyze the post-translational hydroxylation of the specific proline residues in the oxygen-dependent degradation domains of HIF-α subunits that is required for pVHL interaction and subsequent degradation of HIF-α. All PHDs can hydroxylate HIF-α in vitro (Epstein et al. 2001, Huang et al. 2002, Bruick & McKnight 2001, Hirsilä et al. 2003). However, PHD2 has been proposed as the main regulator of HIF-1α in normoxic and in mild hypoxic conditions (Berra et al. 2003, Appelhoff et al. 2004). The roles of PHD1 and PHD3 are not so well known and they might function in a more tissue-specific manner. PHD3 is proposed to regulate HIF in more severe and prolonged hypoxia. *In vitro* studies have suggested that different PHDs have different specificities towards the different HIF hydroxylation sites (Epstein et al. 2001, Hirsilä et al. 2003). PHD3 was found to be the most effective enzyme at hydroxylating CODD and the least effective or even inactive in hydroxylating NODD (Appelhoff et al. 2004). PHD2 was substantially more effective on the HIF-1α NODD than the HIF-2α NODD.

2.3.2 Reaction mechanisms

PHDs catalyze the formation of 4-hydroxyproline in HIF-alpha subunits. They require O_2, Fe^{2+}, 2-oxoglutarate (2-OG), and ascorbate as cosubstrates, and as byproducts they generate CO_2 and succinate (reviewed in (Kivirikko & Myllyharju 1998). During the reaction the molecular oxygen is split in connection with the hydroxylation of HIF and the decarboxylation of 2-oxoglutrate into succinate and CO_2 (Lando et al. 2003). The reaction is likely to proceed through the formation of a highly reactive ferryl (Fe^{IV}=O) intermediate that oxidizes the target proline. Ascorbate is needed for the full activity of the PHDs. It is likely to function as a reducer for the catalytic iron in the event of uncoupled turnover, where the failure in the HIF oxidation leaves the iron center in an oxidized and inactive form.

Somewhat different estimates of the K_m values for oxygen of these enzymes have been made using different assays. The first reported K_m values were in the range of 230-250 μM for the PHDs and 90 μM for FIH (Hirsilä et al. 2003, Koivunen et al. 2004). More recent studies using longer substrates have suggested that the K_m values are closer to 100 μM (Koivunen et al. 2006, Ehrismann et al. 2007a). In all cases, however, the K_m is thought to be significantly higher than the ambient physiological oxygen level in cells, which are in the range of 10-30 μM in most tissues. This makes the enzyme activity exquisitely dependent on oxygen concentration, allowing these enzymes to act in a fairly linear manner under a wide range of O_2 tensions and therefore to function as true oxygen sensors. The K_m of FIH for oxygen has been measured to be even lower than that of the PHDs (Koivunen et al. 2004, Koivunen et al. 2006, Ehrismann et al. 2007b). PHD2 has been shown to have a high affinity for Fe^{2+}. Analyses using soft ionization electrospray ionization mass spectrometry (ESI-MS) indicated that the binding constants for Fe^{2+} and 2-OG (in the presence of Fe^{2+}) with PHD2 are <<1 mM and, <2 mM, respectively (McNeill et al. 2005). No binding of 2-OG could be seen in the absence of Fe^{2+}, supporting the mechanism of 2-OG binding occurring after the binding of Fe^{2+}. Data from enzymatic assays suggest that the K_m values for iron for all PHDs are < 0.1 μM (Hirsilä et al. 2005) and those for 2-OG are 1-12 μM (Koivunen et al. 2007a). PHD2 forms a very stable complex with iron and 2-OG (McNeill et al. 2005, Dao et al. 2009). The reaction rate of PHD2:Fe(II):2-OG:HIF-α complex with oxygen has been shown to be very much slower than for any other 2-OG oxygenases that have been studied (Flashman et al. 2010). These observations suggest that PHD2 might reside in cells as a PHD2:Fe(II):2-OG complex that can readily bind HIF-α and its activity is regulated by oxygen availability.

PHD enzymes catalyze the forward reaction but not the reverse reaction. Thus the net reaction rate is increased with the enzyme level. Enzyme activity can be modulated by the availability of the substrate, the critical co-factor Fe^{2+} and the co-substrate 2-OG and also by the removal of the reaction product succinate. Modulation of iron levels either directly by supplementation or chelation, or indirectly through ascorbate or other redox agents clearly affects enzyme activity (Knowles et al. 2003). Succinate has also been shown to inhibit the enzyme activity and other Krebs' cycle intermediates also affect the enzyme activity (Isaacs et al. 2005). PHDs have been reported to be inhibited by citrate, isocitrate, succinate, fumarate, malate, oxaloacetate and pyruvate. (Koivunen et al. 2007a, Isaacs et al. 2005, Dalgard et al. 2004, Selak et al. 2005, Hewitson et al. 2007). The most consistent effects have been seen with

fumarate and succinate, which inhibit the PHDs competitively with respect to 2-oxoglutarate, with similar IC_{50}s (Koivunen et al. 2007a).

2.3.3 Regulation of PHDs

Several conditions have been shown to regulate the expression of PHD mRNAs, hypoxia being one of them. PHD2 and PHD3 expression levels have been shown to be upregulated by hypoxia, whereas, PHD1 levels remain stable regardless of the oxygen tension, (Epstein et al. 2001, Berra et al. 2003, Cioffi et al. 2003, D'Angelo et al. 2003) or are even reduced in hypoxia (Appelhoff et al. 2004, Erez et al. 2004). HIF-1α seems to be responsible for the hypoxic upregulation of PHD2 and HIF-2α preferentially regulates PHD3 (Aprelikova et al. 2004). The Phd2/egln1 gene contains a functional hypoxia-responsive element in its promoter area approximately 0.5 kb upstream of the translation start site and *Phd2* is a direct HIF target gene (Metzen et al. 2005). The Phd3 gene has a functional HRE as well, located within a conserved enhancer region of the first intron, 12 kb downstream of the transcription start site (Pescador et al. 2005). The *Phd1* promoter, on the other hand, contains binding sites for HIF-1β, which has been seen associated with the promoter following hypoxia, and it might be responsible for the hypoxic downregulation of PHD1 (Erez et al. 2004).

Glycolytic and citric acid cycle metabolites pyruvate and oxaloacetate have also been shown to up-regulate the mRNA levels of PHD2 and 3 (Dalgard et al. 2004). PHD2 mRNA and protein levels have also been shown to be upregulated in an aryl hydrocarbon receptor (AhR)-dependent manner in normoxia (Seifert et al. 2008). In hypoxic conditions, on the contrary, exposure to AhR-activating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) down-regulated PHD2 promoter activity. However, this downregulation did not have an influence on the stability of HIF-1α (Seifert et al. 2008). TGF-β1 has also been shown to markedly down-regulate PHD2 mRNA and protein expression levels (McMahon et al. 2006). This downregulation increased the levels of HIF-1α. Similarily, endothelin-1 was shown to inhibit the transcription of PHD2 thereby activating HIF expression in melanoma cells (Spinella et al. 2010). PHD1 mRNA levels respond to other factors as well. PHD1 has been shown to be estrogen-inducible (Appelhoff et al. 2004). It was in fact first cloned as an estrogen-induced gene in a breast cancer cell line (Seth et al. 2002). PHD1 is produced as two variants by different translational initiation sites. These variants have different protein half-lives, and differing regulation by conditions such as changes in cell confluence, but their functional significances are not known (Tian et al. 2006). PHD3, on the other hand, has been shown to be induced by p53 (Madden et al. 1996) and by stimuli that induce smooth muscle differentiation (Plisov et al. 2000, Wax et al. 1994) as well as by nerve growth factor withdrawal (Lipscomb et al. 2001).

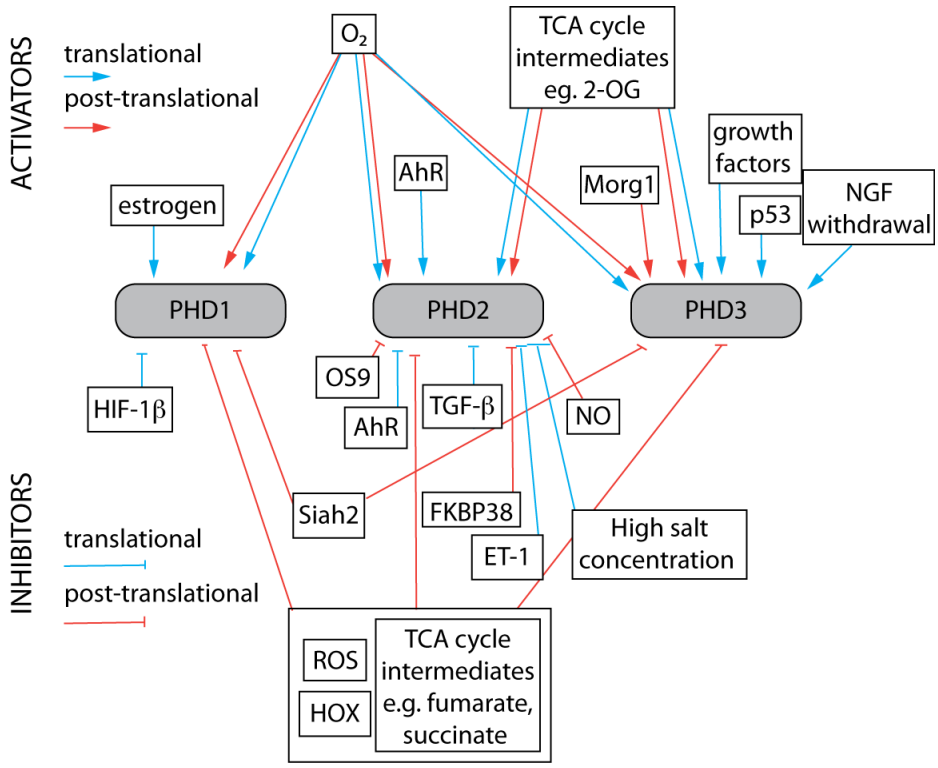

Figure 5. The PHDs are regulated by several factors. Arrows indicate activation and blunt arrows indicate inhibition. Blue represents translational regulation and red post-translational regulation. For clarity factors with similar effects on all PHDs are put in the shared box. See text for details. Abbreviations: (AhR) aryl hydrocarbon receptor, (ET-1) endothelin-1, (FKBP38) FK506-binding protein 38, (NGF) neural growth factor, (NO) nitric oxide, (OS9) osteosarcoma amplified 9, (TCA) tricarboxylic acid, (TGF-β) transforming growth factor beta,

Besides the expression, the activity of the PHDs is also regulated by several different factors. The amount of oxygen seems to be the most important in regulating their enzymatic activity, although PHD2 and PHD3 have been shown to retain significant activity in hypoxic conditions (Appelhoff et al. 2004). As mentioned, glucose metabolites pyruvate, oxaloacetate, citrate, isocitrate, succinate, fumarate and malate can also inhibit PHD activity (Koivunen et al. 2007a, Isaacs et al. 2005, Dalgard et al. 2004, Selak et al. 2005, Hewitson et al. 2007). Pyruvate and oxaloacetate were shown to bind to the 2-oxoglutarate binding sites of the enzymes (Lu et al. 2005). High-salt intake has been shown to decrease the levels of PHD2 in renal medulla (Wang et al. 2010).

Some studies have suggested that HIF induction might be regulated by reactive oxygen species (ROS). These studies have shown that intact mitochondria are required for HIF induction under hypoxia (Chandel et al. 1998, Chandel et al. 2000). Other studies, however, argue against the role of mitochondria (Srinivas et al. 2001,

Vaux et al. 2001, Enomoto et al. 2002). ROS can affect the rate of HIF hydroxylation. Possible mechanisms include direct inhibition of the PHDs or effects of ROS on levels of ascorbate, Fe(II) or Krebs' cycle intermediates. Nitric oxide (NO) has been indicated to have a role in the regulation of the hypoxic response. Whereas under normoxic conditions NO leads to HIF stabilization, likely due to direct inhibition of PHD2 (Berchner-Pfannschmidt et al. 2007, Berchner-Pfannschmidt et al. 2008), in hypoxic conditions NO blunts the HIF induction. This occurs probably by decreasing mitochondrial oxygen consumption and thereby raising intracellular oxygen availability (Hagen et al. 2003).

In addition to their expression and enzyme activity, the stability of PHDs is regulated. The E3 ubiquitin ligases Siah1 and 2 bind all of the PHDs. They control the stability of PHD1 and 3 by targeting them to ubiquitin-dependent proteosomal degradation (Nakayama et al. 2004). PHD3 was found to be most susceptible to the Siah-mediated degradation, and the ability of Siah2 to degrade PHD3 was found to be enhanced in hypoxia. The effect was seen even in moderate hypoxia (10% and 5 % O_2)(Nakayama et al. 2004). Siah2 expression is induced in hypoxia. Siah2 weakly binds PHD2, as well, but does not affect its stability. Instead, the stability of PHD2 is regulated by a ubiguitin-independent proteasomal degradation pathway, which involves FKBP38 as an adaptor protein (Barth et al. 2009). PHD3 is also a substrate for the TCP-1 ring complex (TRiC) chaperonin, which is suggested to help prevent possible aggregation-prone folding intermediates (Masson et al. 2004). A novel WD-repeat protein mitogen-activated protein kinase (MAPK) organizer 1 (Morg1) was found to interact with PHD3 and is likely to function as a molecular scaffold. Morg1 activates or stabilizes PHD3, making it more active towards HIF-1α (Hopfer et al. 2006).

2.3.4 Tissue distribution and subcellular localization

PHDs have unique but overlapping expression patterns. PHD1 mRNA is particularly abundant in the testis (Oehme et al. 2002, Lieb et al. 2002) and placenta (Cioffi et al. 2003) and slightly less expressed in the brain, liver, heart and adipose tissue. PHD2 mRNA is widely expressed in many tissues and has a more uniform expression pattern. It is particularly abundant in adipose tissue and heart (Oehme et al. 2002). PHD3 is also expressed in many tissues but is most abundant in the heart and placenta (Oehme et al. 2002, Cioffi et al. 2003, Lieb et al. 2002) as well as skeletal muscle and adipose tissue. In normoxic conditions PHD3 mRNA expression is below detection levels in many cell lines studied (Appelhoff et al. 2004). Less is known about the protein levels of PHDs in different tissues. The protein levels of PHD2 and 3 correlate with their mRNA levels, but the PHD1 protein levels are lower than would be expected from the mRNA levels (Appelhoff et al. 2004). In immunohistochemical studies PHD1 showed most prominent staining in pancreatic and salivary gland ducts, gall bladder epithelium and renal tubules (Soilleux et al. 2005). PHD1 expression was also found in myoepithelial and luminal cells of breast ducts and in testicular Leydig cells and Sertoli cells and chorionic trophoblastic cells (Soilleux et al. 2005). In this study the distribution of PHD2 was found to be mainly similar to PHD1, except that PHD2 showed strong staining in tracheal respiratory epithelial cells and the staining was weak in gallbladder epithelium (Soilleux et al. 2005). PHD3 showed strong nuclear and cytoplasmic staining in a wide range of tissues. Strong epithelial staining was found in esophagus, gastric body type mucosa, gall bladder, large intestine, pancreatic ducts, salivary gland ducts and some acini, renal tubules, endometrium,

breast myoepithelial and luminal cells, respiratory epithelium, pneumocytes and thymic epithelium. Testicular Leydig and Sertoli cells, as well as, developing spermatozoa had plenty of PHD3. Also lymphocytes and other mononuclear cells showed a lot of PHD3 staining (Soilleux et al. 2005). X-gal staining of tissues from mice hypomorphic for PHD2 and expressing the β-galactosidase reporter under *Phd2* endogenous promoter reveal strong staining in cardiomyocytes, vessel walls, throughout skeletal muscle and specific foci in the brain (Hyvärinen et al. 2010)

The subcellular localizations of different PHDs and FIH-1 were first determined with EGFP-fusion proteins. PHD1 was found to be exclusively nuclear and PHD2 and FIH-1 were found cytoplasmic. PHD3 is distributed evenly in the nucleus and the cytoplasm (Metzen et al. 2003). Overexpressed FLAG-tagged PHD1 showed predominantly nuclear localization, as well, and PHD2 and PHD3 appeared mostly cytoplasmic in COS-1 cells (Huang et al. 2002). Using monoclonal antibodies against the PHDs and FIH-1, Soilleux and others (Soilleux et al. 2005) showed similar subcellular localization. PHD1 was shown to be mostly cytoplasmic with weak nuclear staining in a proportion of cells. PHD1 has been shown to contain a functional nuclear localization signal (NLS)(Yasumoto et al. 2009), which explains its primarily nuclear localization. Hypoxia has not been shown to influence the subcellular localization of PHDs (Metzen et al. 2003).

2.3.5 Specificity of function within the PHD family

It has been speculated that the PHDs have distinct and unique functions. The specific roles of each PHD still remain inconclusive, but the creation of knock-out mouse models has shed light on their possible other functions. PHD2 is the major HIF-1α hydroxylase (Berra et al. 2003, Appelhoff et al. 2004). The other PHDs may hydroxylate HIF-1α in a context-dependent manner and they almost certainly hydroxylate HIF-2α (Hirsilä et al. 2003). Their contribution is dependent on their relative abundance in a particular cell or tissue type (Appelhoff et al. 2004). It has been shown that PHD2 has relatively more influence on HIF-1α than on HIF-2α and PHD3 has more influence on HIF-2α than on HIF-1α (Appelhoff et al. 2004). PHD2 has the highest specific activity towards HIF-1α (Huang et al. 2002). PHD2 is also closest to the ancestral forms of the hydroxylase in *C.elegans* and *D.melanogaster*. Drosophila has one gene, fatiga, but it has three different isoforms that seem to have different functions (Acevedo et al. 2010).

PHD3 was first identified as a growth factor inducible gene in smooth muscle cells (Wax et al. 1994). It has been proposed to have a function in growth arrest, differentiation, and cell death during muscle differentiation (Moschella et al. 1999, Fu et al. 2007). PHD3 has the ability to cause apoptosis in sympathetic neurons deprived of neural growth factor (NGF) (Lipscomb et al. 1999) and the ectopic overexpression of PHD3 is sufficient to cause apoptosis even in the presence of NGF in sympathetic neurons (Lipscomb et al. 2001, Lipscomb et al. 1999). In accordance, PHD3-deficient sympathetic neurons are resistant to apoptosis after NGF withdrawal (Schlisio et al. 2008). The ability of PHD3 to cause apoptosis is dependent on its hydroxylation activity (Lee et al. 2005, Rantanen et al. 2008) but not on HIF (Lee et al. 2005). Thereby, it is more likely to be caused by other yet unknown hydroxylation targets. PHD3's ability to induce apoptosis has also been connected to the formation of aggresome-like structures (Rantanen et al. 2008). PHD3-mediated neuronal apoptosis

is needed during the normal sympathetic neuronal development, which is clearly seen in the mice deficient in PHD3. They have an increased number of cells in the super cervical ganglia and in the adrenal medulla (Bishop et al. 2008). They display an adrenergic phenotype characterized by alterations in blood pressure and cardiac contractility (Bishop et al. 2008). This is likely due to the deregulation of β_2-adrenergic receptor (β_2AR), which is also a known target for PHD3 hydroxylation (Xie et al. 2009).

PHD3 also blocks the interaction between the inhibitor of NF-κB kinase β (IKKβ) and Hsp90 that is required for phosphorylation of IKKβ. PHD3 appears to be a tumor suppressor in colorectal cancer cells inhibiting the IKKβ/NF-κB signaling, independent of its hydroxylase activity (Xue et al. 2010). Decreased PHD3 expression has been observed in breast cancer (Yan et al. 2009) while PHD1 is induced by estrogen in human breast cancer cell lines (Appelhoff et al. 2004, Seth et al. 2002).

PHD1$^{-/-}$ mice are viable and grossly normal (Takeda et al. 2006, Aragones et al. 2008) but they show mammary gland hypoproliferation and do not breastfeed their pups normally (Zhang et al. 2009). It was shown that PHD1 regulates cyclin D1 in a HIF-independent manner. Cyclin D1 is not a PHD1 hydroxylation target, but the regulation of Cyclin D1 by PHD1 is indirect and involves changes in Cyclin D1 transcription or mRNA stability. Loss of PHD1 activity inhibits estrogen-dependent breast cancer tumorigenesis. PHD1 activity regulates breast cancer proliferation in response to estrogen. PHD1 depletion also affects the proliferation of other cancer cell lines. The loss of PHD1 has been linked to alterations in cellular metabolism, because the muscles and liver of PHD1$^{-/-}$ mice were found to be resistant to ischemia/reperfusion injury (Aragones et al. 2008, Schneider et al. 2009).

2.3.6 Non-HIF targets of the HIF hydroxylases

Other targets besides HIF-α for PHD-mediated hydroxylation are under extensive investigation. While such targets for PHDs have been suggested, the data on the actual hydroxylation is still mostly lacking. Only one target besides HIF-α subunits has been formally proven, the β_2 adrenergic receptor (β_2AR) for PHD3 (Xie et al. 2009). FIH, on the other hand, has been shown to hydroxylate in addition to HIF-α asparagines in many ankyrin repeat domains (ARD) (Cockman et al. 2006, Coleman et al. 2007, Ferguson et al. 2007, Zheng et al. 2008). FIH targets appear to conform to a consensus motif that is present in most of the 300 human ARD-containing proteins. The functional consequences of this ARD hydroxylation are mostly unknown, since reported effects are either subtle (Cockman et al. 2006, Coleman et al. 2007) or not contributable to hydroxylation (Ferguson et al. 2007, Zheng et al. 2008). The ARD-proteins include IKKβ, which is an inhibitor of the NF-κB signaling. It has been suggested to be a target for PHD1 hydroxylation as well (Cummins et al. 2006). No other targets besides HIF-α have been found for PHD2.

In oxygenated conditions PHD3 hydroxylates the β_2AR, which is a prototypic G protein-coupled receptor, at Pro^{382} and Pro^{395}. Hydroxylation leads to the recruitment of pVHL and ubiquitylation of the β_2AR, promoting its down-regulation by proteasomal degradation. Accordingly, hypoxia stabilizes the β_2AR (Xie et al. 2009). PHD3 has been shown to interact with and to affect the stability of the activating transcription factor-4 (ATF4), but hydroxylation of ATF4 has not been directly shown

(Koditz et al. 2007). PHD3 also interacts with myogenin stabilizing it by protecting it against VHL-mediated degradation (Fu et al. 2007). The kinesin KIF1Bβ has been shown to act downstream of PHD3 in a developmental apoptotic pathway that is activated when NGF becomes limiting for neuronal progenitor cells (Schlisio et al. 2008). However, it does not seem to be a direct hydroxylation target of PHD3. Nevertheless, PHD3 hydroxylation activity is required for the regulation of KIF1Bβ. This implies a possible hydroxylation target for PHD3 in between these two proteins in this apoptotic pathway (Schlisio et al. 2008). The degradation of RNA polymerase II has been reported to occur in a hydroxylation-dependent manner but the hydroxylase responsible for this has not yet been characterized (Kuznetsova et al. 2003).

2.3.7 PHD2

2.3.7.1 Functions of PHD2

PHD2 has been proposed to be the main regulator of HIF-1α, since the RNA interference directed against it is sufficient to induce HIF-α subunits in normoxia. In this same study, silencing of PHD1 and PHD3 had no effect on the stability of HIF-1α in normoxia or upon re-oxygenation of cells briefly exposed to hypoxia (Berra et al. 2003). This might have to do with the relative abundance of PHD2 in most cell types in comparison to the other PHDs, because PHD2 is clearly the most abundant isoform in normoxic conditions in most cell lines (Appelhoff et al. 2004). PHD2 has also been shown to specifically repress HIF-1α transcriptional activity in hypoxia. PHD2 binds HIF-1α in hypoxia as well as normoxia and inhibits HIF-1α N-terminal transcriptional activity. This was shown in a VHL-deficient cell line and thus is not due to the increased proteolysis of HIF-1α (To & Huang 2005).

PHD2 has been shown to promote growth factor-induced responses of human vascular smooth muscle cells by HIF-1α-dependent mechanisms. Endogenous PHD2 is required for normal growth factor-induced cyclin A expression in human pulmonary artery smooth muscle cells (Schultz et al. 2009). PHD2 has a critical role in normal human physiology, which is demonstrated by erythrocytosis caused by a mutation in the Phd2 gene (Percy et al. 2006). PHD2 has been shown to suppress hypoxia-induced endothelial cell proliferation in a hydroxylation-independent manner (Takeda & Fong 2007). The suppression might be due to PHD2's ability to bind and recruit binding partners to HIF-1α, such as OS-9 and inhibitor of growth family member 4 (ING4).

Mutations in the gene encoding PHD2 have been identified in patients with familial erythrocytosis. An autosomal-dominant form of erythrocytosis (erythrocytosis, familial type 3, OMIM; no. 609820) is caused by mutations in the Phd2/egln1 gene. Several different mutations leading to this disease have been found in the gene. These include two point mutations: Pro317Arg (Percy et al. 2006) and Arg371His (Percy et al. 2007). Two different heterozygous frameshift mutations in exon 1, and a nonsense mutation in exon 3 (Al-Sheikh et al. 2008) have also been characterized. These mutations render PHD2 incapable of binding and hydroxylating HIF-1α. Pro317Arg substitution is only 2 residues away from the Fe(II) binding aspartate-315 and close to the catalytic site entrance in the crystal structure. This mutation may thereby hinder both Fe(II) and substrate binding (McDonough et al. 2006). Arg371His mutation is 3 amino acids away from histidine-374, which is involved in iron binding. Proline-317

and arginine-371 lie close to each other in the PHD2 crystal structure (McDonough et al. 2006).

2.3.7.2 PHD2 in development

PHD2 has an essential role during development as is shown by the genetically modified mouse models. No viable knock-out offspring were found by two independent groups (Takeda et al. 2006, Minamishima et al. 2008). PHD2 knock-out embryos die between E12.5 and 14.5 (Takeda et al. 2006). They have severe defects in the cardiac and placental development (Takeda et al. 2006). The PHD2 knockdown was shown to upregulate insulin-like growth factor binding protein-1 (Igfbp1) (Ozolins et al. 2009), which is associated with embryonal lethality and reduced embryonal growth in several mouse models (Gay et al. 1997, Crossey et al. 2002, Ben Lagha et al. 2006). In contrast, $PHD2^{+/-}$ embryos survive to term and appear to develop relatively normally (Mazzone et al. 2009).

Broad spectrum conditional somatic inactivation of PHD2 in mice leads to premature mortality associated with venous congestion and dilated cardiomyopathy (Minamishima et al. 2008). The somatic inactivation of PHD2 promotes HIF accumulation and activation of HIF target genes including erythropoietin, leading to profound polycythemia (Minamishima et al. 2008). The PHD2 knock-out mice show that the lack of PHD2 function can not be compensated by PHD1 and PHD3 or FIH at the molecular level. PHD2 seems also to be a major negative regulator of vascular growth in adult mice as was shown by Tamoxifen-inducible PHD2 conditional knock-out mice (Takeda et al. 2007). PHD2 knock-out mice showed hyperactive angiogenesis and angietacsia in multiple organs in adult mice probably due to significantly elevated VEGF-A levels. PHD1 and PHD3 knock-out mice, on the other hand, had no significant vascular phenotypes (Takeda et al. 2007). The inactivation of PHD2 in mice is also enough to induce maximal renal EPO production, while the inactivation of all three PHDs is needed for the reactivation of hepatic EPO production in adult mice (Minamishima & Kaelin 2010). siRNA-mediated PHD2 knock-down had a protective effect in a cardiac ischemia/reperfusion model (Natarajan et al. 2006). PHD2 hypomorphic mice express clearly lower levels of PHD2 in some of their tissues and a concordant stabilization of HIF-1α and HIF-2α (Hyvärinen et al. 2010). They show no obvious adverse effects. Instead, when isolated hypomorphic PHD2 hearts were subjected to ischemia-reperfusion injury their recovery was better than in the wild type (Hyvärinen et al. 2010).

2.3.7.3 PHD2 interaction partners

Several proteins have been found to interact with PHD2 but their specific functions still remain elusive. The peptidyl prolyl *cis/trans* isomerase FKBP38 interacts with PHD2 and is suggested to be involved in the regulation of PHD2 protein stability (Barth et al. 2007). FKBP38 contains a C-terminal transmembrane domain that anchors the protein into the mitochondrial and endoplasmic reticulum membranes. It might function as an anchor to keep PHD2 cytoplasmic. OS-9 was found to interact with both HIF-1α and PHD2 (Baek et al. 2005) and it promotes the hydroxylation-dependent degradation of HIF-1α. A candidate tumor suppressor gene, inhibitor of growth family member 4 (ING4) has been shown to directly associate with PHD2. This interaction might recruit ING4 to HIF and thereby it can suppress HIF transcriptional activity (Ozer et al. 2005). Accordingly, ING4 suppresses the expression of HIF target genes, but the interaction of ING4 with PHD2 relieves this

suppressor activity (Ozer et al. 2005). The melanoma antigen 11 (MAGE-11) suppresses PHD2 activity through physical interaction (Aprelikova et al. 2009).

2.3.7.4 PHD2 in cancer

The PHD2-VHL-HIF pathway plays a crucial role in the erythropoiesis. A partial interruption of the pathway may cause erythrocytosis, while drastic alterations are associated with cancer (Percy et al. 2006, Minamishima et al. 2008, Takeda et al. 2007, Percy 2008). PHD2 has been suggested to work as a tumor suppressor in human endometrial cancer (Kato et al. 2006). Phd2/egln1 gene was found to be mutated in a significant proportion of surgically resected endometrial cancer samples studied. Significantly, introduction of wild type PHD2 into endometrial cancer cell lines bearing PHD2 mutations induced cellular senescence through the negative regulation of HIF-1 expression (Kato et al. 2006). A germline PHD2 mutation (H374R) was found in a patient with erythrocytosis and recurrent paraganglioma (Ladroue et al. 2008). In the resected tumors a clear loss of PHD2 heterozygosity was observed, which also points to a possible tumor suppressor function for PHD2. The oncogenic processes involving the loss of PHD2 are still unknown, but PHD2's role as a regulator of HIF could be involved. PHD2 has been shown to have a critical role in the regulation of tumor angiogenesis (Chan et al. 2009), which further supports its role as a tumor suppressor. It limits the expression of the angiogenic factors angiopoietin and interleukin-8 (IL-8) through the inhibition of NF-κB. PHD2 regulates NF-κB activity in a HIF- and hydroxylation-independent way (Chan et al. 2009). The mechanism of NF-κB regulation still remains unknown.

In *in vitro* studies the PHD2 heterozygous-deficient endothelial cells have been shown to be more quiescent, less proliferative and show reduced mobility upon VEGF stimulation (Mazzone et al. 2009). Haplodeficiency in endothelial cells did not affect tumor vessel density or area, tortuosity or lumen size, but induced "normalization" of the endothelial lining, barrier and stability (Mazzone et al. 2009) These changes improve tumor perfusion and oxygenation. Tumor vessels in PHD2 heterozygous deficient ($PHD2^{+/-}$) mice were normal with orderly formed endothelial cells as compared with wild type tumor vessel endothelial cells that had more fenestrations and were more leaky. This improves oxygenation in tumor cells as shown by more oxidative generation of ATP and the downregulation of many glycolytic enzymes, such as Glut-1 and phosphofructokinase (Mazzone et al. 2009). This change in the endothelial lining of $PHD2^{+/-}$ mice prevented the metastatic switch and prolonged overall survival. Their tumors were overall more benign than in wild type mice (Mazzone et al. 2009). Also angiopoietin-1 –induced normalization of immature vessels is associated with reduced PHD2 expression (Chen & Stinnett 2008).

PHD2 seems to have dose-dependent effects. Haplodeficiency or reduction of PHD2 levels in half sufficed to induce endothelial normalization in cancerous vessels, while complete loss of PHD2 leads to other vascular changes. PHD2 levels are inversely correlated with tumor-forming potential, HIF-1α activity, glycolytic rates, VEGF expression, and ability to grow under hypoxic stress (Lee et al. 2008). However, when PHD2 is completely inhibited, tumorigenesis is reduced, presumably because of a HIF-dependent pro-apoptotic response (Lee et al. 2008). These findings on a dual role of PHD2 in tumor biology highlight that PHD2 may regulate, in a dose-dependent manner, different oncogenic versus tumor-suppressing mechanisms

3 Specific Aims

PHDs have an important role in the regulation of the hypoxia-inducible factors, which in turn are needed for tumor cells to survive and flourish in a poorly oxygenated microenvironment. This study aims to advance understanding of the role of HIF prolyl hydroxylase PHD2 in cancer and its influence on the regulation of HIF.

The specific aim of this study was to further characterize the expression and significance of PHD2, focusing on its role in cancer cells.

Specifically,

1. To study the hypoxic regulation of PHD2 expression

2. To study the mRNA and protein expression levels of PHD2 in cancers, primarily in head and neck squamous cell carcinoma and colon carcinoma.

3. To study the significance of the subcellular localization of PHD2 on cancer cell properties.

4 Materials and Methods

4.1 Patient samples (II,III)

HNSCC samples from both genders were collected from surgically removed oral and oropharyngeal cancer (n=44), between the years 1990-2002. For comparison to HNSCC, normal tissue samples (n=21) were collected from patients undergoing uvulo-palato-pharyngoplasty. Samples were from both genders and the patient age ranged from 29 to 87 years. Paraffin-embedded tissue array material from both genders was obtained from 114 patients with colon adenocarcinoma treated during 1993-2000 in Turku University Central Hospital. All samples were removed before any treatment, either radiation therapy or chemotherapy. For colon adenocarcinomas the Dukes' class was A for 14 patients, B for 62 patients, C for 21 patients and D for 17 patients. The age of the patients ranged from 31 to 82 years. Corresponding normal tissue samples were obtained from areas surrounding the tumors.

The patients gave their informed consent and the use of tumor samples was approved by the National Authority for Medicolegal Affairs. Participants gave their informed consent, and the studies were conducted according to the declaration of Helsinki.

4.2 Cell culture (I,II,III)

RCC4, HeLa, HaCaT and HepG2 cell lines were obtained from the American Type Tissue Collection (Rockville, MD). Human SCC cell lines were established from primary tumors (UT-SCC2, UT-SCC8), recurrent tumors, or metastasis (UT-SCC7, UT-SCC9) of head and neck squamous cell carcinomas (Lansford et al. 1999). Normal human skin fibroblasts were established from a healthy male volunteer donor. HUVEC cells were a gift from Dr. E. Iivanainen. Cells were kept in Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg streptomycin and 6 nmol/l L-glutamine. SCC cells were also supplemented with nonessential amino acids (Sigma, St Louis, MO). Cells were cultured in humidified air containing 5% CO_2 at 37°C. For hypoxia treatments air was replaced by nitrogen to reach 1% oxygen in a hypoxia workstation (Ruskinn Technology Ltd., UK).

4.3 Transient transfections and siRNA treatments

Transfections were performed on subconfluent cells with FuGene HD transfection reagent (Roche, Germany) or Effectene transfection reagent (Qiagen, Chatsworth, CA) according to the manufacturers' instructions. For siRNA treatments cells were grown to 70-80% confluence and the medium was changed to DMEM without supplements. Double-stranded RNA oligonucleotides were transfected with Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA sequences have been published in (Berra et al. 2003). After 4 hours of incubation the supplements were returned to the medium. The efficiencies of the transfections and RNAi treatments were controlled by western blotting.

4.4 Plasmid constructs (II,III)

For studying PHD2's putative endogenous localization signals two deletion constructs were generated based on full length Flag-tagged PHD2. Subcellular localization signals were searched with NetNES server 1.1 (http://www.cbs.dtu.dk/services/NetNES/) and PSORT II (Nakai & Horton 1999). Based on the searches nucleotides 299-349 were deleted from delSLS and nucleotides 564-584 from delNES. For targeting PHD2 to specific cellular compartments, well-known target sequences were fused to the N-terminus of Flag-PHD2. The constructs were made with PCR primers containing the added sequences. For nuclear targeting of PHD2 a double SV40 small T nuclear localization signal (NLS), PKKKRKVAPKKKRKV, (CCC AAG AAG AAG CGC AAG GGT) GGC (Kalderon et al. 1984) and for cytoplasmic targeting the HIV-1 rev nuclear export signal (NES), LQLPPLERLTL, (CAG CTG CCC CCC CTG GAG CGC CTG ACC CTG)(Fischer et al. 1995) were used. Hydroxylase-deficient PHD2 mutants (FlagPHD2H313K, FlagPHD2H374K) were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The active site iron-coordinating histidines (H313, and H374) were point mutated into lysines (CAT → AAA). HIF constructs were a kind gift from Dr. Chris Pugh (Oxford University, UK) and PHD2-EGFP construct was a kind gift form Dr. Eric Metzen (University of Luebeck, Germany).

4.5 RNA isolation and cDNA synthesis (I,II)

RNA was extracted with the acid guanidium thiocyanate-phenol-chloroform method. After removal of culture medium, the cells were first washed with ice cold PBS. Then 1ml TRIzol® (Invitrogen, Karlsruhe, Germany) was added and the cells were homogenized into the reagent, moved into an eppendorf tube, and incubated for 5 minutes at room temperature. Chloroform was added 100 µl/1 ml and the suspension was mixed thoroughly and centrifuged for 15 minutes, 12 000 x g at +4°. The RNA containing phase was moved into a clean eppendorf tube and 500 µl isopropanol was added. After 10 minutes of incubation at room temperature the centrifugation (12 000 x g at +4°, 10 minutes) was repeated. The supernatant was removed and the pellet was washed with 75% ethanol and centrifuged 7 500 x g at +4°C for five minutes. Then the pellet was dried and solved into diethylpyrocarbonate (DEPC)-treated water and incubated for 10 minutes at 55°C.

Before cDNA synthesis the RNA was treated with DNAseI to eliminate possible DNA contaminations. For this 1 µg RNA was pipetted into an eppendorf tube with 1 µl of 10X DnaseI reaction buffer, 1 µl DnaseI (1U/µl) and DEPC-treated water was added until a total volume of 10 µl. Reaction was incubated at room temperature for 15 minutes and then stopped by adding 1 µl of 25 mM EDTA and incubating at 65°C for 10 minutes.

First strand cDNA synthesis was performed with 200U Moloney murine leukemia virus (MMLV) RNase H^- reverse transcriptase (Promega) using 0.5 µg poly$(dT)_{15}$ primers (Promega) or random hexameric primers (Promega) and 1 µg of DNAseI treated RNA. RNA and oligos were first incubated in 70°C for 5 minutes for annealing and moved to ice for 5 minutes. Then 10 ml 5X reaction buffer was added with 2.5 ml of PCR nucleotide mix (10mM each) and 200U of reverse transcriptase

and water for total volume of 50 ml. Reaction was incubated at 40°C for 60 minutes and inactivated by incubating at 70°C for 15 minutes.

4.6 Quantitative reverse transcription-PCR analysis (TaqMan) (I,II)

Quantitative real time reverse transcription PCR was used to analyze the RNA levels of PHD2. Primers and fluorogenic probes for RT-PCR were designed with the Primer Express computer software (PE Biosystems) and obtained from MedProbe (Oslo, Norway). EF-1α (Eukaryotic elongation factor-1α, Unigene number Hs.439552) was used as a reference gene. It was chosen because of the stability of its levels also in hypoxic conditions. The sequences of the primers and probes are:

EF-1α: fwd 5'-CTGAACCATCCAGGCCAAAT-3',
rev 5'-GCCGTGTGGCAATCCAAT-3',
probe 5'(FAM)-AGCGCCGGCTATGCCCCTG-(TAMRA)-3'.
PHD1: fwd 5'-CGAGGTGAAGCCAGCCTATG-3',
rev 5'-CCGCTCCTTGGCATCAAA-3'.
probe 5'(FAM)-CACCAGGTACGCCATCACTGTCTGGTA-(TAMRA)-3'.
PHD2: fwd 5'-AGCAGCATGGACGACCTGAT-3',
rev 5'-TCGTCCGGCCATTGATTT-3',
probe 5'-(FAM)-CCCAGCTTCCCGTTACAGTGGCG-(TAMRA)-3'.
PHD3: fwd 5'-CGAAGTGCAGCCCTCTTACG-3',
rev 5'-TTTTGGCTTCTGCCCTTTCTT-3',
probe 5'-(FAM)-AACCAGATATGCTATGACTGTCTGGTACTTTGATGCT-(TAMRA)-3'.
GLUT1:fwd 5'-GTGGGCATGTGCTTCCAGTA-3',
rev 5'-AAGAACAGAACCAGGAGCACAGT-3',
probe 5'-(FAM)-CAACTGTGTGGTCCCTACGTCTTCATCATCT-(TAMRA)-3'.

RT-PCR reactions were performed using 2 μl of diluted cDNA, 5 μl of the TaqMan universal PCR master mix (Applied Biosystems), 300 nM oligonucleotide primers and 200 nM FAM-labeled probes. The amplification was detected with ABI Prism 7700 sequence detector (PE Biosystems) as an increase in the FAM fluorescence. Cycling was initiated with 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The accumulation of target sequence was detected as an accumulation of fluorescence in real-time. The accumulation of fluorescence was plotted against cycle value to determine C_T values, which is the number of cycles at which the fluorescence signal exceeded a C_T value of 0.05 relative fluorescence units. Each determination was done in duplicate and normalized to simultaneous EF-1α values of the same samples. The relative expressions of the analyzed genes were calculated using the formula: relative expression = $2^{-\Delta CT}$, where $\Delta C_T = C_T$(target gene) - C_T(EF-1α) for each sample.

4.7 Soft-agar anchorage-independent growth assay (III)

To study the anchorage-independency the cells were transfected with appropriate PHD2 expression constructs or an empty control vector. The next day they were transferred to grow in soft agar. Bottom layers (1.5 ml) composed of DMEM containing 0.5% agar (Bacto-agar, Biokar Diagnostics, Beauvais, France), 10 % FCS,

penicillin-streptomycin and L-glutamine were poured on 6-well-plate wells. The top layers (1.5 ml) with 0.35% agar and 5000 cells in medium were poured on top of the solidified bottom layers. Cells were grown at 37°C for 8 days and supplemented with 200 µl fresh growth medium every two days. The nuclei were stained with Hoechst 33342 (Sigma) and the colonies were photographed on a fluorescence microscope (Zeiss SteREO Lumar V12). Colony sizes were determined using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007)

4.8 Subcellular fractionation (III)

Cells were grown on 10 cm plates and transfected with appropriate constructs 24 hours before harvesting. Cells were washed twice with ice-cold PBS and harvested by scraping into 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM DTT and protease inhibitor mix. Cells were incubated on ice for 15 minutes, vortexed, and then centrifuged for 20 seconds. This reveals the cytosolic fraction, which was collected. The remaining pellets were washed with PBS and resuspended into 20 mM Hepes (pH 7.9), 400 mM NaCl, 0.25 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 0.5 mM DTT and incubated on a shaker at 4°C for 20 minutes. The samples were centrifuged at 16 000 X g for 2 min at 4°C and the nuclear fractions were collected. PHD2 localization was assessed by western blot analysis.

4.9 Western blot analysis (II,III)

For western blotting proteins were extracted in Laemmli buffer and separated by SDS-PAGE gel electrophoresis. Then the proteins were transferred to Immobilon-P membrane (Millipore). The proteins were detected with specific primary antibodies followed by HRP (horse radish peroxidase) -conjugated secondary antibodies. The antibodies used are summarized in table 2. The immunoblotted proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Table 2. Antibodies used in immunological applications. Abbreviation used are: WB western blotting, IHC immunohistochemistry, ICC immunocytochemistry.

	Application				
Antibody	**WB**	**IHC**	**ICC**	**Source**	**Used in**
Primary antibodies					
PHD1	X			BL525, Bethyl Laboratories	II
PHD2	X	X	X	NB100-137 Novus Biologicals	II,III
PHD3	X			NB100-139 Novus Biologicals	II
β-actin	X			AC-40, Sigma	II,III
HIF-1α	X	X		BD transduction laboratories	II,III
HIF-2α	X			Abnova	III
PARP	X			Sigma	III
c-jun	X			60A8, Cell Signalling	III
CD34		X		BD Biosciences	II
Secondary antibodies					
Anti- mouse -HRP	X			DAKO	II,III
Anti-Rabbit-HRP	X			DAKO	II,III
Anti-rabbit-Cy3			X	Jackson Immunochemicals	II,III

4.10 Immunohistochemistry (II,III)

Paraffin-embedded tumor sections were sliced into 5-micrometer thick sections. Tissue microarrays (TMAs) were prepared from formalin-fixed and paraffin-embedded archive material by selecting representative areas of colorectal cancer for 3-mm thick tissue cylinders. The sections were stained with antibodies against PHD2 (1:500) and Hif-1α (1:100). Visualization of primary antibodies was done with Vectastain ABC reagent and diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CO, USA), which is based on an indirect streptavidin-biotin method. Slides were counterstained with hematoxylin. Antigen retrieval was carried out using a microwave oven. The staining for CD34 (dilution 1:100) was performed using the TechMate 500 immunostainer and a peroxidase/diaminobenzidine (DAB) multilink detection kit (DAKO, Denmark).

In each HNSCC sample, the PHD2 staining intensity was assessed with an x40 objective in three separate evaluation areas with a total of 300 squamous cell carcinoma cells by two independent observers. Staining intensity was assessed on a scale of 0–3 where 3 indicated an intense staining corresponding to the staining intensity of endothelial cells. HIF-1α staining intensity was assessed on a scale of 0 to 2. Nuclear PHD2 staining was evaluated in the whole tumor area with an x20 objective. This gave an estimation of the percentage of cells showing nuclear staining

in the whole sample. Thereafter, the most representative tumor area was identified and the previous estimation was verified by quantitative assessment of the percentage of cells showing nuclear staining with an x40 objective in three separate optical fields in a total of 300 carcinoma cells. Each tumor was scored for the amount of cells with nuclear staining as a percentage of all cells. Finally, nuclear staining and staining intensity were combined into a histoscore by multiplying the percentage of nuclear positivity with the staining intensity.

In each colorectal TMA-cylinder sample, the proportion of carcinoma cells showing nuclear staining for PHD2 was assessed with an x40 objective in the most intensive area by making use of a 10x10 griddle, with a total of 300 adenocarcinoma cells. Staining intensity was assessed on a scale of 0–3 where 3 indicated an intense staining corresponding to the staining intensity of endothelial cells that show intense PHD2 expression.

4.11 Cell imaging (II,III)

For immunocytochemistry, cells were plated on cover slips and for live cell imaging on MatTek glass bottom culture dishes (MatTek Corporation, Ashland USA). For visualizing endogenous or transfected PHD2, cells were fixed with PTEMF (100 mM PIPES [pH 6.8], 10 mM EGTA, 1 mM $MgCl_2$, 0.2% Triton X-100, 4% formaldehyde) and stained with PHD2 antibody followed by Cy3-conjugated secondary antibody. The nuclei were stained with Hoechst 33342 (Sigma). Fluorescent images were obtained with Zeiss LSM 510 META confocal microscope (Carl Zeiss Corporation, Jena, Germany).

For live cell microscopy, cells were grown to 60% confluence and transfected with PHD2-EGFP. The experimental conditions for fluorescence recovery after photobleaching (FRAP) have been described elsewhere (Phair & Misteli 2000). Shortly; images were acquired with the confocal microscope and Physiology software. GFP fluorescence was excited using the 488nm laser and detected with LP505 filter. All experiments were performed at 37^{o}C and 5% CO_2 in a humidified cell culture chamber. Each FRAP-experiment started with an image scan followed by a bleach pulse of 100 iterations and 100% laser power. A series of 60 images were collected at 5s intervals. The mobile fraction was calculated by using the equation y=A(1-exp(kt)) and custom designed software (Dr. Rolf Sara, Turku Centre for Biotechnology). In this analysis, bleaching compensation is calculated from the declining tail of the bleached region of interest (ROI) instead of using the reference cell ROI.

4.12 Cell proliferation assay (III)

For proliferation assays cells were plated in equal amounts on 96-well plates and allowed to attach. The following day cells were transfected with appropriate PHD2 expression constructs or an empty vector control. The next day cell proliferation was determined with a colorimetric BrdU incorporation assay (Roche, Germany) according to the manufacturer's instructions. Briefly, 10 µl bromodeoxyuridine (BrdU) was added to each well for labeling and the cells were allowed to grow for another 6 hours. Then the labeling medium was removed and the cells were fixed and denatured. The incorporated BrdU was determined with a peroxidase conjugated

BrdU antibody and a substrate solution. The absorbance of the samples was measured with an ELISA reader at approximately 492 nm.

4.13 Flow cytometry (III)

The amount of apoptotic cells was assessed by flow cytometry. HeLa cells were transfected with appropriate constructs and fixed with ice cold 80% ethanol. The next day cells were stained with propidium iodide solution. Flow cytometric analysis was done using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Propidium iodide was measured using 670 longpass filter. For FACS analysis CellQuest software (Becton Dickinson) was used.

4.14 Statistical analyses (II,III)

Correlations between PHD2 expression and tumor characteristics were calculated with Spearman's correlation coefficients. P-values less than 0.05 were considered statistically significant. Welch two-sample t-test was used for statistical analyses of soft agar colonies. All analyses were two-sided at the 5% level of significance. Statistical computations were performed using SAS System for Windows, release 8.2 and R 2.9.0 statistical software (http://www.R-project.org).

5 Results

5.1 Hypoxia induces PHD2 and PHD3 in cell lines (I, unpublished)

PHD2 and 3 mRNAs have been reported to be hypoxia-inducible in several cell lines, such as HeLa (Epstein et al. 2001, Berra et al. 2003), U2OS osteosarcoma cells (Metzen et al. 2003), and C6 rat glioma cells (D'Angelo et al. 2003). The hypoxic induction of PHD expression was further studied in primary SCC cell lines derived from cancer of the head and neck region. Several cell lines were studied. These included UT-SCC2 (primary tumor of mouth), UT-SCC7 (cutaneous metastasis of the neck), UT-SCC8 (primary tumor of the larynx) and UT-SCC9 (primary tumor of the gingiva). Cells were subjected to hypoxia and results were obtained by real time PCR analysis and western blotting (Figure 6). The results show that PHD1 mRNA expression is not induced in hypoxia, but longer hypoxia rather lowers its mRNA levels. PHD2 expression is moderately induced in all the cell lines, whereas the hypoxic induction of PHD3 is substantial in all the cell lines. Protein levels were seen to be in accordance with the mRNA levels.

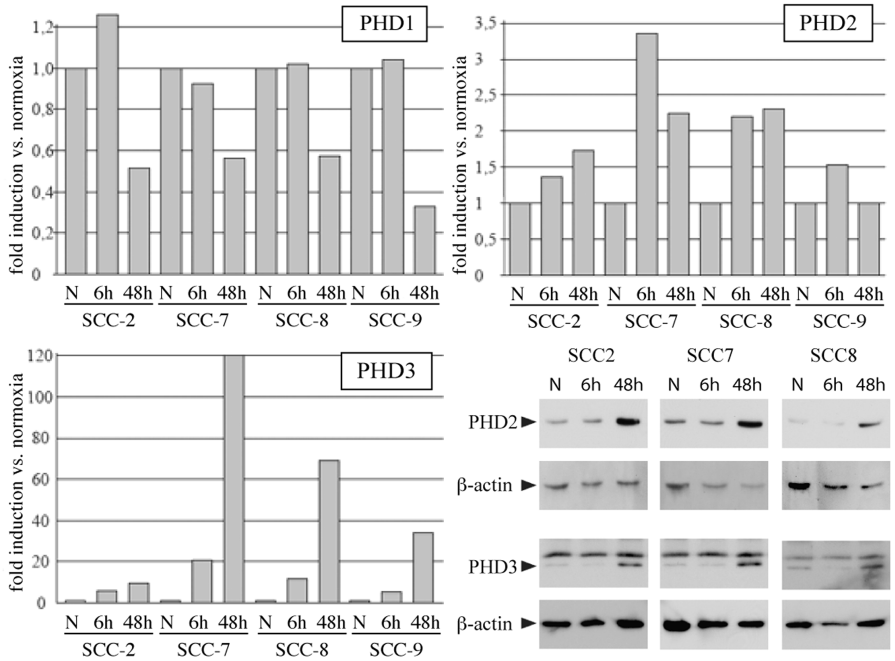

Figure 6. Hypoxic induction of PHDs in SCC cell lines. SCC cell lines were kept in hypoxia for 6 and 48 hours and the levels of PHDs were assessed by quantitative RT-PCR and western blotting. The levels of hypoxic mRNA were compared to the normoxic levels as fold induction. Clear hypoxic induction is seen for PHD2 and 3, whereas the levels of PHD1 mRNA are lower in the longer hypoxia. The protein levels of PHD2 and 3 were studied in some of the cell lines and the hypoxic induction was also seen on the protein level. β-actin serves as a loading control in western blotting.

VHL is a key component in the HIF degradation pathway, since it provides the target recognition in the E3 ubiquitin ligase complex. In order to study the VHL-dependency of hypoxic PHD induction, RCC4 cells were used. They do not express VHL and therefore show high normoxic HIF levels. Stable transfection of a VHL expression vector restores the normal HIF regulation in these cells. These cells were used for studying, whether the dysregulation of HIF is connected to the loss of hypoxic upregulation of PHD2 and PHD3 in RCC cells. The PHD mRNA induction was measured with real time PCR. We saw that the hypoxic induction of PHD2 and 3 was clearly reduced in wild type RCC4 when compared to VHL recompleted cells, although some induction could still be seen. (I, Fig. 3). This is indicated that the hypoxic regulation of PHD2 and 3 occurs through HIF.

5.2 Expression of PHD2 in normal tissue (II, III)

PHD2 is expressed abundantly in many different tissues but the available data on the PHD2 expression in most tissues has been on the level of mRNA. Immunohistochemistry was used for studying the distribution of the PHD2 protein in several normal cell types from the oral and pharyngeal region. First the specificity of the commercial antibody used was validated using western blotting. The analysis detected no cross-reactivity with the other PHD isoforms in transiently transfected cell samples and showed hypoxic induction of the endogenous PHD2 (II, Fig. 1A). Localization of PHD2 in the normal pharyngeal tissue, which included blood vessels, muscle cells, salivary glands, neural tissue, fibroblasts, and epithelial cells, as well as placental tissue was studied. The strongest PHD2 expression was found in the endothelial cells regardless of the anatomic site (II Fig. 1 B-F). The particular staining of endothelial cells was further verified by CD34 endothelial cell marker staining from parallel sections (II, 1B). The strong expression was very clear in all sections studied and it was seen in the endothelial lining of large blood vessels as well as in capillaries. Equally robust staining was seen in the specialized endothelial cells, such as neuroendothelial cells (II, Fig. 1E), endothelial cells of the muscle tissue (II, Fig. 1C) and salivary glands (II, Fig.1D) as well as endothelial cells of the placenta (II, fig.1F). Muscle cells (II, Fig. 1C) and the mucous acini of the salivary glands (II, Fig. 1D) showed clearly less staining and fibroblasts did not show any detectable staining. In the normal epithelium a dual expression pattern was seen. The basal proliferating layer of stratified squamous epithelium showed strong PHD2 expression, which decreased towards the surface of the epithelia being completely lost in the flattened superficial cells (II, Fig. 2B). In addition to endothelial and proliferating epithelial cells, placental trophoblasts showed robust PHD2 expression (II, Fig. 1F). Normal colon tissue showed clear PHD2 staining in the mucosal glands (III, Fig. 1A).

5.3 Expression of PHD2 in cancerous tissue (II,III)

In order to understand the role of PHD2 in HIF regulation in tumors, as well as the correlation of PHD2 with the clinicopathologic features of human cancers, PHD2 expression was studied in the human HNSCC both at the mRNA and protein levels and in colon adenocarcinomas at protein level. The mRNA levels were studied by the quantitative real time reverse transcription PCR (TaqMan). The PHD2 expression of twenty-nine HNSCC tumor samples from oral and pharyngeal cancers was studied and compared to 13 normal samples derived from surgically removed oropharyngeal tissue. The levels were normalized to the EF-1α housekeeping gene, instead of actin. EF-1α was chosen because of its constant levels also in hypoxic conditions. The

expression of PHD2 was clearly higher in the cancer samples as compared to the normal tissue samples (II, Fig. 2A). The fold change in the mean expression levels of the cancer group compared with the normal group was 4.5. The highest values for PHD2 exceeded the mean expression levels of the control group by 14-fold. These results indicated that PHD2 mRNA is overexpressed in HNSCC tumors.

PHD2 protein levels were studied by immunohistochemical staining of histological sections derived from HNSCC patients (n=44) and colorectal adenocarcinoma patients (n=114) and compared with normal pharyngeal epithelia (n=21) or normal colon tissue. PHD2 expression was clearly elevated in HNSCC samples. Moderate elevation was seen in 43% of tumors and strong up-regulation in 57% of the tumors (II, Fig.2B, Table 1). In colon adenocarcinomas PHD2 overexpression could also be seen (III, Fig. 1A), although to a lesser extent compared to HNSCC.

The PHD2 protein levels were studied in primary human cells and cells derived from HNSCC by western blotting. In accordance with the histological data, primary human fibroblasts showed very little PHD2 whereas primary human umbilical vein endothelial cells (HUVEC) showed high basal PHD2 levels. The HNSCC cell lines showed varying levels of PHD2 ranging from low expression (UT-SCC8) to high expression (UT-SCC9) (II, Fig. 2C).

So far, hypoxia was the only known stimulus of PHD2 expression. However, the increased PHD2 expression in HNSCC did not superimpose with hypoxic areas and equal PHD2 expression was detected far from any vessels as well as close to the vessels. The expression of PHD2 in hypoxia was therefore studied in the HNSCC cell lines. The cell lines with low or moderate PHD2 expression (e.g. UT-SCC8) showed clear hypoxic up-regulation of PHD2, while in the cell lines with high normoxic expression of PHD2 (e.g. UT-SCC9) the levels were not further up-regulated in hypoxia (II, Fig. 2C), although these cells did retain normal hypoxic HIF-1α induction. This indicates possible other means of PHD2 up-regulation or changes in the regulation of PHD2 expression in cancer cells.

When the staining intensity was correlated with the histological grade of the tumors, a clear trend between the two was seen, as the PHD2 expression levels were clearly elevated in higher-grade tumors (II, Fig. 3A; Table 2). Grade 1 tumors showed low PHD2 expression (17 out of 19 tumors), whereas grade 2 tumors (18/20) and grade 3 tumors (5/5) showed clearly increased PHD2 expression. The aggressivity of tumors was further confirmed by Ki67 proliferation marker from adjacent sections.

5.4 PHD2 is seen in the nuclei in tumor cells (II,III)

Originally PHD2 has been shown to reside mostly in the cell cytoplasm (Metzen et al. 2003). In all the normal tissue cells in this study as well, the expression of PHD2 was only seen in the cytoplasm of the cells (II, Fig. 1, 3A; III, Fig 1A). However, in a subset of HNSCC and colon adenocarcinoma tumor cells PHD2 expression was also detected in the cell nuclei and the localization was studied further. The percentage of PHD2 positive nuclei in HNSCC tumor samples was counted and correlated with the grade of the tumor. A strong association between nuclear staining and higher-grade tumors was seen. In grade 1 tumors 0%-5% positive nuclei were seen and the rest of the cells showed normal cytoplasmic expression. However, in grade 3 tumors 50%-90% of the cells showed both cytoplasmic and nuclear staining (II, Fig. 3A; Table 2).

For further analysis the histoscore values for each specimen were calculated. For histoscore the nuclear staining and the overall staining intensity were combined by multiplying the percentage of nuclear positivity with the staining intensity. This showed an even stronger association between the PHD2 expression and tumor grade compared with PHD2 intensity alone. The Spearman correlation coefficient for nuclear staining and tumor grade was 0.80 ($P < 0.0001$) and the histoscore value was 0.83 ($P < 0.0001$). No correlation between PHD2 expression and tumor size, lymph node status, or metastasis were seen in HNSCC.

Colon adenocarcinoma samples showed substantial variation in their PHD2 localization patterns. PHD2 was cytoplasmic in a large part of the cells, but in a subset of cells nuclear expression was also seen. Specifically, 23% of tumors (26/114) had increased nuclear PHD2 expression. In these samples 11% - 80% of the cells had nuclear PHD2 expression.

In all cultured cells some nuclear PHD2 staining was seen in addition to cytoplasmic expression. Little nuclear expression was found in primary fibroblasts and immortalized epithelial cells (HaCaT). Primary endothelial cells on the contrary showed a lot of nuclear PHD2 staining. This pattern is in agreement with the staining pattern seen in the tissues with immunohistochemistry. The HNSCC-derived cell lines, particularly UT-SCC8, showed increased nuclear expression of PHD2 (II, Fig. 3B). Hypoxia had no effect on the localization of PHD2 (III, Fig. 5F).

5.5 PHD2 has different dynamics in the nuclear and cytoplasmic compartments (II)

To study the differences in the dynamics of cytoplasmic and nuclear PHD2, cells were transfected with enhanced green fluorescent protein tagged-PHD2 (EGFP-PHD2) and fluorescence resonance after photobleach (FRAP) was used. The localization of the EGFP-PHD2 was comparable to the localization of endogenous PHD2 in the cell lines used (II, suppl. Fig.) Both the nuclear and cytoplasmic compartments showed active movement of PHD2. The kinetics of the nuclear fraction were fast with the half-time of approximately 5 seconds. The cytoplasmic fraction had 2- to 3-fold longer half-time (II, Fig. 3C). This suggests differential PHD2 complex formation within the different cellular compartments.

5.6 PHD2 expression correlates partially with HIF-1α downregulation (II)

PHD2 regulates the levels of HIF-1α protein, and on the other hand PHD2 expression is enhanced by HIF-1α. Normally HIF-1α is not detectable in most tissues, but most carcinoma tissues show up-regulated HIF-1α protein. HIF-1α expression is usually seen at severely hypoxic perinecrotic areas. Parallel HNSCC sections (n=14) were stained with HIF-1α and PHD2 and first the tumors were scored for overall expression of these proteins. Most sections that had negative or low HIF-1α expression were moderately or highly PHD2 positive. In a subset of tumors, however, both high HIF-1α and PHD2 expression was seen. Since the overall staining in the sections does not reveal the causal relation of PHD2 and HIF-1α, their levels were studied in consecutive optical fields (n=21) in the HNSCC sections and scored for HIF-1α and PHD2 staining intensity. Most regions that showed strong PHD2 staining had low or no HIF (73%), as expected. However, areas with both high expression of

PHD2 and HIF-1α could be seen (27%) (II, fig. 4, suppl. Table). This is a strong indication that in some cases PHD2 expression is not sufficient to down-regulate HIF-1α.

5.7 PHD2 is shuttled between the nuclear and cytoplasmic compartments (III)

The differences in PHD2 subcellular localization suggested an active transport mechanism for PHD2. In order to study the transport of PHD2, the nuclear export of proteins was inhibited by leptomycin B (LMB, 10 ng/ml). The cells were stained for endogenous PHD2 and its localization was studied with confocal microscopy. In LMB treated cells the accumulation of PHD2 in the nuclei was evident up to 6 hours of treatment, while the cytoplasmic PHD2 diminished in HeLa and HaCaT cells (III, Fig.1B and C, suppl. Fig.1) The vehicle treated cells showed a more invariable PHD2 distribution between the cell compartments. This implied that the distribution of PHD2 protein is regulated and that PHD2 might contain regions that control its intracellular localization.

5.8 Nuclear localization of PHD2 promotes anchorage-independent growth of HeLa cells (III)

Because the association of PHD2 nuclear localization with the aggressiveness of the tumors was so evident, it was thought that the nuclear localization might provide a growth advantage for the tumor cells. To study this, we decided to use PHD2 plasmids targeted to be expressed either in the nucleus or in the cytoplasm. For this, compartment-specific PHD2 construct plasmids were made. For nuclear localization an SV-40 small T nuclear localization signal (PKKKRKVAPKKKRKV) was fused to the N-terminus of the Flag-tagged PHD2. This construct was called NuclPHD2. For cytoplasmic localization, the HIV-1 rev-protein's nuclear export signal sequence (LQLPPLERLTL) was added to the aminoterminus of Flag-PHD2. This construct was called CytoPHD2. The localization of these proteins was confirmed by fluorescence microscopy (III, Fig. 2A) and quantitation of compartmental PHD2 expression with Image J image analysis software (III, Fig. 2B) as well as with subcellular fractionation followed by western blot analysis (III, Fig. 2C). In fluorescence microscopy the wild type PHD2 showed mostly cytoplasmic expression but had some nuclear expression as well. This was clear when compared to CytoPHD2, which was almost completely cytoplasmic. NuclPHD2 showed clear nuclear localization.

To study the possible cancer specific role of PHD2 localization, several assays were used to assess the cancerous properties of the PHD2-transfected cells. No differences were found in the cell cycle regulation or the amount of apoptosis in cells transiently transfected with wild type PHD2, NuclPHD2 or CytoPHD2, as compared with empty control vector transfected cells. This was determined by FACS analysis (III, Suppl. Fig. 2) and western blot analysis of the polyADP ribose polymerase (PARP) cleavage (III, Fig. 2E). An approximately 20% increase in the proliferation rate of the wild type PHD2 and NuclPHD2 transfected cells was observed as well as a small decrease in the proliferation of the CytoPHD2 transfected cells compared to the empty vector transfected cells (III, Fig. 2D). However, the most prominent effect could be seen in the anchorage-independent cell growth as determined by soft-agar growth assay. Cells expressing either the wild type or NuclPHD2 grew statistically significantly larger colonies in soft agar, as compared with empty control vector transfected cells. Cells

expressing CytoPHD2 had no growth advantage and grew somewhat smaller colonies as compared with control vector transfected cells (III, Fig. 3). An approximately 20% increase in the colony size was quantitated for the WT- and NuclPHD2. The data clearly demonstrated that PHD2 has cellular compartment specific effects on anchorage-independent cell growth.

5.9 A putative nuclear localization signal in PHD2 does not function as a localization signal

Because PHD2 seems to be actively transported into the nucleus in the cells, it is likely to contain a nuclear localization signal. Database searches (NetNES) revealed a putative bipartite nuclear localization signal (RRDNASGDAAKGKVKAK) at the N-terminus (aa 98-114) of PHD2. This has also been predicted in (Dupuy et al. 2000). To study the functionality of this NLS, a PHD2 construct with targeted deletion (aa 100-115) of the signal sequence was made and named delSLS for subcellular localization signal. The construct was transfected into cells and its localization was examined using confocal microscopy and cell fractionation. The deletion of the SLS sequence did not prevent the nuclear localization of PHD2, as was expected, but enhanced it compared to wild type PHD2 (III, Fig. 4B,C). This implies that SLS does not function as a nuclear localization signal that was predicted by its sequence. When compared to the control vector transfected cells, the delSLS transfected cells showed no difference in cell proliferation, as determined by BrdU incorporation or cell cycle regulation and apoptosis activity as determined by FACS analysis (III, Supl. Fig. 3). An increase in the anchorage-independent growth, however, was observed with delSLS transfected cells compared with control vector transfected cells. The size of the colonies was considerably increased after one week (III, Fig. 4D). Compared with control vector-transfected cells, a 72% increase in size was observed in the delSLS transfected cell colonies (III, Fig. 4E).

In database searches a putative nuclear export signal (NES) was also seen in PHD2. To study its functionality it was deleted from the delNES-PHD2 construct. The deletion of this putative NES made PHD2 somewhat more nuclear, but the effect was not as clear as with the deletion of the putative NLS (Figure 7).

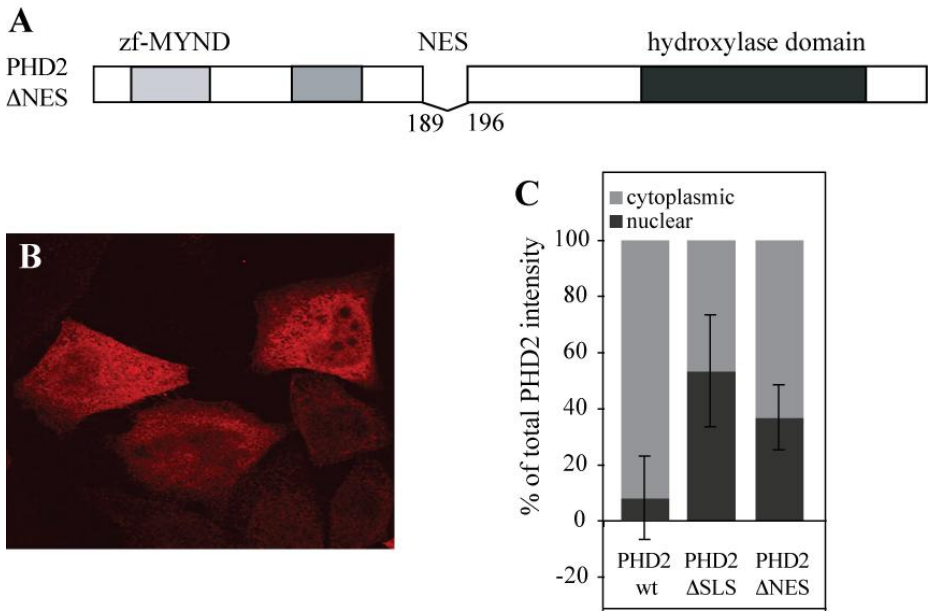

Figure 7. The effects of a putative nuclear export signal in PHD2. A) The sequence between amino acids 189-196, containing the putative NES, was deleted from PHD2 delNES. **B**) Confocal microscopy image of PHD2 delNES in HeLa cells. **C**) Quantitation of nuclear and cytoplasmic staining in cells transfected with indicated constructs as means of three optical fields. For comparison the levels of WT and delSLS PHD2 are shown.

5.10 Promotion of anchorage-independent growth is independent of hydroxylase activity

Since PHD2 is known to be the main regulator of HIFs in normoxic conditions, the effect of PHD2 localization on HIF expression was studied, as well as the effect of HIF-1α and -2α on the anchorage-independent cell growth. Cells transfected with compartment specific PHD2 constructs were kept in hypoxia for 6 hours and the amount of HIF-1α was studied by western blotting. HIF-1α expression was most efficiently inhibited by the nuclear PHD2 (III, Fig.5A). The forced normoxic expression of HIF-1α and HIF-2α in HeLa cells decreased the size of the colonies in soft agar in comparison to the empty vector transfected cells (III, Fig.5A). In keeping with this, the silencing of either HIF-1α or HIF-2α by siRNA increased the colony size (III, Fig.5D).

To study the dependence of anchorage-independent growth on PHD2 hydroxylase activity I mutated the iron coordinating histidines (H313 and H374) within the PHD2 hydroxylase domain into lysines both in the NuclPHD2 and CytoPHD2. The mutated PHDs lost their ability to hydroxylate HIF. The effects of the mutated PHD2s did not differ significantly from the non-mutated nuclear or cytoplasmic PHD2. A small reduction in growth was seen in H313K CytoPHD2 transfected cells and H374K NuclPHD2 transfected cells. In conclusion, the silencing of the PHD2 hydroxylase-activity has no or only a modest effect on the anchorage-independent growth of HeLa cells, while the growth seems to be dependent on HIF activity.

6 Discussion

6.1 Hypoxia is an inducer of PHD2 expression

HIF-1 is the master regulator of hypoxic gene expression and PHD2 has been implicated as the main regulator of HIF-1α in cell culture conditions (Epstein et al. 2001, Bruick & McKnight 2001, Berra et al. 2003). Hydroxylation of prolines 564 and 402 leads to the binding of pVHL and recognition by the E3 ubiquitin ligase complex and subsequent degradation of HIF. Transcription of PHD2 and 3 is hypoxia-inducible (Epstein et al. 2001, Berra et al. 2003, Cioffi et al. 2003, D'Angelo et al. 2003, Metzen et al. 2003). These results were confirmed in SCC cell lines.

Regulation of PHD expression was assessed in pVHL-deficient RCC4 cells, which show normoxic upregulation of HIF. The hypoxic induction of PHD2 and PHD3 was shown to be diminished in a renal cell carcinoma cell line RCC4. In contrast, the induction was clearly seen in RCC4 cells that had the VHL expression restored by stable transfection of a VHL expression vector. This implied that the dysregulation of HIF is connected to the loss of hypoxic upregulation of PHD2 and PHD3. Similar results were obtained by another group in pVHL-deficient 786-O cells (del Peso et al. 2003). They show that the hypoxic induction was lost in these cells.

These results suggest that the hypoxic induction of PHD2 is dependent on normal regulation of HIF. This was further suggested by the inhibition of HIF activation by RNA interference, which completely blocked the hypoxic induction of PHD2 and 3. Later it has been shown that PHD2 is a direct HIF-1 target gene and has a functional HRE in its promoter region (Metzen et al. 2005).

As PHD2 is a direct target gene of HIF-1 and regulates the degradation of HIF-1, they form a direct negative feedback mechanism, where HIF regulates its own expression. This allows fast restriction of HIF activity upon reoxygenation. Indeed, it has been shown that the downregulation of HIF-1α happens faster after long periods of hypoxia (Berra et al. 2001a). The increase in the amount of PHD enzyme might also alter the kinetics of the reaction, as it is known that PHDs retain activity in hypoxic conditions (Appelhoff et al. 2004). Therefore, the increased PHD expression is likely to restrict excessive HIF expression. It has been shown that *in vivo* PHD2 and 3 remain upregulated over more than ten days after hypoxia (Stiehl et al. 2006). PHD2 also suppresses HIF-1α transcriptional activity in hypoxia (To & Huang 2005). This might happen through the recruitment of ING4 (Ozer et al. 2005). The negative feedback function of PHD2 and 3 has been shown in gliomas, as well (Henze et al. 2010), where their induction limits the hypoxic HIF-response. The increased levels of PHDs compensate for their reduced activity. The negative feedback loop protected the cells against hypoxia-induced cell death. The inhibition of PHDs facilitated the induction of cell death by apoptosis-inducing factors. Therefore the increased PHD expression is thought to be a more general way for cells to adjust to their prevailing oxygen concentrations and besides restricting HIF expression, to be able to sense decreases in the oxygen tension even in tissues with generally low oxygen tensions.

6.2 Expression of PHD2 in normal tissues

The previous data on PHD2 expression across tissues was mostly on the mRNA level and little data was available on the protein expression of PHD2. In this study the protein levels of PHD2 in several normal tissues were investigated by immunohistochemistry. Variable levels of PHD2 were found across tissues. Strong PHD2 expression was seen in endothelial cells and in the basal layer of cutaneous epithelium. Low expression was found in the muscle cells and PHD2 was undetectable in fibroblasts, neural cells and non-proliferating, more differentiated epithelial cells.

While we found the endothelial expression of PHD2 very strong across all the tissues studied, another study using different antibody saw very variable PHD2 expression in endothelial cells. The expression ranged from non-existing to very strong (Soilleux et al. 2005). Strong PHD2 expression was also found in the basal, proliferating layer of the normal epithelium. Epithelial expression of PHD2 seemed to vary strongly. We found no expression in the flattened superficial cells and in between these cell layers the expression was gradually diminished. In the other study, PHD2 showed strong staining in the tracheal respiratory epithelial cells and low expression in the gallbladder epithelium (Soilleux et al. 2005).

The expression patterns of PHD2 are not completely similar in different studies. Differences in the protein and mRNA expression levels have been found on other PHDs as well, on cellular and tissue levels. Generally, the mRNA levels seem to be higher than the protein levels in tissues. For example, the protein levels of PHD1 in several cell lines were found to be lower than expected from the mRNA levels (Appelhoff et al. 2004). In the same study the PHD2 mRNA levels correlated well with the protein levels in cultured cells (Appelhoff et al. 2004). Undetectable PHD2 protein levels were seen in fibroblasts, neural cells and non-proliferating, more differentiated epithelial cells of the skin. In muscle cells the PHD2 protein levels were very low, although the mRNA levels of PHD2 have previously been shown to be quite high in the skeletal muscle tissue (Hirsilä et al. 2003). Similarly, the PHD2 protein levels were very low in adipose tissue, although it has previously been reported that the PHD2 mRNA is abundant in adipose tissue (Oehme et al. 2002). However, in adipose tissue, low PHD2 mRNA levels have also been reported (Cioffi et al. 2003). The variation in PHD2 expression levels in different tissues might reflect the distinctions in the relative roles of different PHDs in keeping HIF levels low in respective tissues. For example, PHD3 has been shown to be very abundant in muscle tissue and might be responsible for the regulation of HIF in muscles.

The discrepancies between the mRNA and protein levels might reflect post-translational regulation of PHD2. Indeed, a ubiquitin ligase, Siah2, has been shown to weakly bind PHD2 (Nakayama et al. 2004). It does not seem to affect PHD2 stability, even though it targets PHD3 to proteasomal degradation (Nakayama et al. 2004). Another protein, FKBP38 is also known to interact with PHD2 and is suggested to be involved in the regulation of PHD2 protein stability (Barth et al. 2007). There might also exist other, still unknown, mechanisms for the regulation of PHD2 stability. This clearly demonstrates the need for knowledge on *in vivo* PHD protein levels, as the known mRNA levels do not constantly reflect the distribution of the PHD proteins.

6.3 PHD2 expression in cancers

The expression of PHDs in the tumor tissues has been studied lately in different cancers. Variable levels have been detected, but mainly increased staining has been noticed. For example, all PHDs are expressed in pancreatic endocrine tumors (Couvelard et al. 2008) and breast carcinomas (Soilleux et al. 2005, Tan et al. 2009). Overexpression of PHDs was also seen in pancreaticobiliary tumors (Gossage et al. 2010). In contrast, decreased expression of PHDs has been detected in broncogenic carcinomas, renal cell carcinoma, and follicular lymphomas (Soilleux et al. 2005). Our results show that PHD2 is overexpressed in HNSCC, as well as in colon adenocarcinomas. The expression levels of PHDs in colorectal cancers have also been studied on mRNA levels. In this study PHD1 seemed to be upregulated and PHD2 levels were unchanged, whereas PHD3 levels were decreased (Xue et al. 2010). Decreased PHD3 expression has been observed in breast cancer, as well (Yan et al. 2009). In contrast, PHD1 expression is increased in breast cancers (Zhang et al. 2009). In a set of 60 cell lines derived from nine different tissues, PHD2 expression was found very variable. Even cells originally from the same tissue showed varying levels of PHD2. Reduced levels of PHD2 were seen in a number of cancer cell lines (Chan et al. 2009). These studies also show that the levels of PHD2 mRNA are significantly lower in tumors compared with normal tissue. This was particularly apparent in colon carcinoma. Similar results were obtained with immunohistochemistry of colon carcinomas (Chan et al. 2009). These studies highlight the complexity of PHD2 expression, which varies strongly between cancer types but also within one type of cancer.

In a subset of tumor cells we also saw clear nuclear localization of PHD2. The PHD2 overexpression with its nuclear localization correlated with the aggressiveness of the tumors. That is, the tumors were more proliferating and less differentiated. In line with this, it was shown that the nuclear expression of PHD2 in HNSCC also correlated with lowered sensitivity to radiation (Luukkaa et al. 2009). Specific subcellular localization of other hydroxylases has also been shown to associate with tumor characteristics. Cytoplasmic FIH has been shown to associate with tumor grade in invasive breast cancer (Tan et al. 2009) and in pancreatic endocrine tumors (Couvelard et al. 2008). In pancreatic endocrine tumors, overexpression of PHDs and FIH was also shown to associate with tumor aggressiveness and tumors with high nuclear PHD1 or PHD3 expression were associated with poorer survival (Couvelard et al. 2008). Predominantly cytoplasmic localization of PHD2 was seen in breast carcinomas and low PHD2 levels in tumors correlated significantly with shorter survival times (Bordoli et al. 2010). In clear cell renal carcinomas, there was no correlation between PHD1, 2, 3 and RCC patient survival. All PHDs were also located in the nuclei in these tumors, but in contrast to other cancer types, increased nuclear PHD levels rather correlated to less aggressive (grade 1) carcinoma (Kroeze et al. 2010). The low nuclear expression of FIH was instead found to be a strong independent factor for poor overall survival in ccRCC (Kroeze et al. 2010). In pancreaticobiliary cancers or normal tissues from the same areas, no PHD2 nuclear reactivity was seen (Gossage et al. 2010). Taken together, it appears that in some carcinomas the nuclear expression of PHD2 may be an important feature of more aggressive phenotype.

Since hypoxia is a known inducer of PHD2 expression, the possibility of PHD2 overexpression being caused by hypoxia was examined. However, we saw PHD2

expression throughout the tumor area, both in the regions that seem hypoxic and regions that were close to vessels and seemingly well oxygenated. Similarily, PHD3 has been shown to have strong expression in apparently non-hypoxic tumors (Boddy et al. 2005). The expression of PHD2 did not fully correlate with the expression of HIF either. Although we saw areas with high PHD2 expression having low or no expression of HIF-1α, we also saw areas with high expression of both PHD2 and HIF-1α. In another study, a similar lack of correlation in PHD2 and HIF expression was seen in a panel of 60 tumors from nine different tissues (Chan et al. 2009). In prostate cancer cells, PHD2 has been shown to inversely correlate with HIF-2α, but no correlation was seen for HIF-1α (Boddy et al. 2005). Although, hypoxia is a known inducer of PHD2 expression and PHD2 is a direct HIF target, it is not the only known trigger for PHD2 expression. Other mechanisms have been shown to regulate the PHD2 mRNA and protein levels. For example, TCDD upregulates both of them in an AhR-dependent manner in normoxia and downregulates in hypoxia (Seifert et al. 2008). TGF-β1 and endothelin-1 have also a downregulating effect on PHD2 levels (McMahon et al. 2006, Spinella et al. 2010). Moreover, the longer half-life of PHD2 as compared to HIF may also partially explain the discrepancy. Other mechanisms for the regulation of PHD2 levels might still be revealed as well.

PHD2 activity may be impaired in tumors, which might explain the simultaneous expression of PHD2 and HIF. Insufficient levels of ascorbate (Knowles et al. 2003), elevated levels of succinate (Selak et al. 2005) or 2-oxoacids (Dalgard et al. 2004) could suppress its hydroxylase activity. For full activity PHD2 also requires interaction partners, such as OS-9 (Baek et al. 2005). The melanoma antigen 11 (MAGE-11) suppresses PHD2 activity through physical interaction (Aprelikova et al. 2009) and a candidate tumor suppressor gene, ING4 has been shown to directly associate with PHD2. This interaction might recruit ING4 to HIF and thereby suppress HIF transcriptional activity (Ozer et al. 2005). Accordingly, ING4 suppresses the expression of HIF target genes, but the interaction of ING4 with PHD2 relieves this suppressor activity (Ozer et al. 2005).

6.4 Subcellular localization of PHD2

There are contradictory studies on the subcellular localization of PHD2. It was first shown to be primarily cytoplasmic as an EGFP-tagged protein (Metzen et al. 2003). Similarly, all PHDs were shown to be mainly cytoplasmic in lung cancer cells (Giatromanolaki et al. 2008). Then it was shown that the endogenous PHD2 is mostly nuclear in several cell lines and that PHD2 seems to be responsible for all the nuclear hydroxylation activity (Berchner-Pfannschmidt et al. 2008). Similarily, by fractionation endogenous PHD2 was shown to reside in both nuclear and cytoplasmic compartments in equal amounts (Ozer et al. 2005).

By immunohistochemistry we could see a difference in the subcellular localization patterns of PHD2 between normal and cancerous tissues. This difference seems not to be as clear in cultured cells. Exclusively cytoplasmic expression was seen in, for example, epithelial, nerve, endothelial and mucosal tissue cells. However, in cultured carcinoma cells at least some degree of nuclear expression of PHD2 is constantly seen. Even primary human umbilical vein endothelial cells showed some degree of nuclear PHD2. In tumors we saw strong nuclear expression of PHD2 and this nuclear expression correlated with the aggressiveness of the tumors. To study further the subcellular localization of PHD2, we used leptomycin B (LMB), which blocks the

CRM-1/exportin dependent nuclear export of proteins that contain a nuclear export sequence (NES)(Fornerod et al. 1997), and studied the movement of PHD2 between the cellular compartments. We saw that LMB-treated cells accumulated PHD2 in their nuclei. This implicates an active transport mechanism for PHD2 and a presence of a NES sequence. This has been shown in other studies, as well (Yasumoto et al. 2009, Steinhoff et al. 2009). In addition, a direct interaction between PHD2 and CRM-1 has been seen (Steinhoff et al. 2009). In computer predictions, PHD2 does contain a NLS sequence as well as a NES. To study the functionality of these subcellular localization signals (SLS), we deleted a part containing the putative localization signal from PHD2. The deletion of the putative NES resulted in the PHD2 being more nuclear. Surprisingly, the deletion of the putative NLS also caused PHD2 to be more nuclear. This implicated that this putative NLS was not a functional localization signal. This has since been shown by another group, as well (Yasumoto et al. 2009). Deletion of this SLS deletes a part of the FKBP38 interaction site. FKBP38 was proposed a role in keeping PHD2 cytoplasmic, since it is anchored in the endoplasmic and mitochondrial membranes. FKBP38 also regulates the stability of PHD2 (Barth et al. 2009, Barth et al. 2007). Therefore, it may be possible that FKBP38 could also regulate the subcellular localization of PHD2. It was hypothesized that the FKBP38-bound PHD2 is constantly degraded, while the free cytoplasmic PHD2 is stable. The hindering of the PHD2 degradation might also elevate its amount to levels forcing it to the nuclear compartment.

The localization of PHD2 has been studied much lately. PHD2 was shown to translocate into the nuclei of the cells in several cell lines, but it was shown not to interact with the importin αs or importin β (Steinhoff et al. 2009). In contrast, the nuclear accumulation of PHD1 clearly occurs dependent on importin α/β and relies on a NLS. It has been predicted that the region between amino acids 181 and 220 is required for the nuclear localization of PHD2 (Steinhoff et al. 2009). The region essential for nuclear export of PHD2 is likely to be located in the N-terminal region (aa 6-20) of PHD2, since the deletion of this region caused a shift of PHD2 localization from the cytoplasm into the nucleus (Yasumoto et al. 2009). A mutant lacking this region showed a clearly reduced effect towards HIF compared with wild-type PHD2.

It has been shown that the trapping of HIF-1α either in the nucleus or in the cytoplasm does not prevent its degradation (Berra et al. 2001b). This clearly demonstrates that all of the components needed for the degradation of HIF are present in both compartments of the cell. In hypoxia, HIF-α localizes almost exclusively into the nucleus. Therefore it is reasonable that its degradation machinery also relocalizes into the nucleus. Indeed, it has been shown that the nuclear-cytoplasmic trafficking of pVHL is required for the ubiquitinylation of HIF (Groulx & Lee 2002) and same sort of mechanism might be expected for PHD2. It has also been shown that the different localization of PHD2 affects HIF-1α stability (Yasumoto et al. 2009).

We also showed that PHD2 has different dynamics in the cytosolic and nuclear compartments. It shows clearly faster movement in the nuclei. This implicates a smaller complex formation. PHD2 might also be held in the cytosolic compartment by some protein. In accordance, FKBP38 binds PHD2 and is involved in the regulation of PHD2 stability (Barth et al. 2009, Barth et al. 2007)

6.5 The effects of PHD2 localization

Since we saw such a difference in the subcellular localization of PHD2 in tumor cells as opposed to the cells in normal tissues, we studied further the effects of PHD2 localization. We made PHD2 constructs, which localized either in the cytoplasm or in the nucleus. We did not see any effects on cell proliferation or apoptosis, but we saw an increase in anchorage-independent growth with nuclear PHD2 overexpression using either wild type or nuclearly targeted PHD2. Similar effect was seen with the SLS deletion mutant, which also localized more nuclearly. In contrast, the cytoplasmically targeted PHD2 reduced the anchorage independent growth of cells. Anchorage-independent growth is a characteristic of cancer cells.

PHD2 has been proposed to function as a tumor suppressor via downregulation of HIF. Nevertheless, we saw that the forced overexpression of HIF-1α and 2α decreased the anchorage-independent growth. Accordingly, their depletion by siRNA increased cell growth. This correlates to the function of PHD2 being the regulator of HIF. PHD2 has also been suggested to be enzymatically more active in the nucleus than in the cytoplasm (Berchner-Pfannschmidt et al. 2008). We also saw this in our experiments. The effect of PHD2 on the anchorage-independent growth, however, was not dependent on its hydroxylase activity, since the inactivating mutation of the iron coordinating histidines into lysines did not alter the effect. This suggests that the effect might not be dependent on the degradation of HIF. Indeed, PHD2 has been shown to have hydroxylation-independent effects on HIF. For example, it inhibits the proliferation of endothelial cells in a hydroxylation-independent manner (Takeda & Fong 2007) and represses HIF transcriptional activity directly (To & Huang 2005). Nuclear PHD2 could also exclude HIF from specific growth promoting genes. In line with this, PHD2 interacts with ING4, a nuclear protein, which represses HIF activity (Ozer et al. 2005). PHD2 has also been reported to interact with OS9 independently of its hydroxylase activity (Baek et al. 2005). Other still unknown mechanisms might apply as well.

While nuclear PHD2 caused an increase in the anchorage-independent growth of cells, cytoplasmic PHD2 decreased the growth of cells. This suggests that the regulation of intracellular PHD2 distribution is an effective pathway for the control of the hypoxic response and the inhibition of PHD2 nuclear localization might slow down the aggressive growth of cancer. This highlights the importance of proper regulation of PHD2 subcellular localization.

In HNSCC, PHD2 expression is increased in less differentiated phenotypes and it is further translocated into the nuclei following dedifferentiation. This pattern suggests a novel biomarker for the differentiation status of carcinomas. In breast cancer, tissue marker detection for gene expression profiles indicating prognosis and treatment response has become common practice (Wolff et al. 2007).

6.6 The role of PHD2 in cancer

The roles of PHDs in cancer are not yet clear. They are often functionally inactivated in cancer, and it appears that they can also be genetically or epigenetically inactivated in tumors via downregulation or mutation (Ladroue et al. 2008, Chan et al. 2009, Hatzimichael et al. 2010). The tumor suppressor roles have mostly been suggested for PHD1 and PHD3.

There is conflicting data on the function and significance of PHD2 in cancers. For PHD2 there are reports supporting a role as a tumor suppressor as well as a role as a tumor promoter. PHD2 has been proposed to function as a tumor suppressor, since it regulates the expression of HIF in cancers. There is much evidence for this role for PHD2. For example, *in vivo* silencing of PHD2 induces neoangiogenesis by the regulation of many angiogenic factors through the stabilization of HIF-1α (Wu et al. 2008, Knowles et al. 2004). In melanoma, PHD2 seems to function as a tumor suppressor, since its loss accelerates tumor growth. In normoxic melanoma cells, endothelin-1 can increase both HIF-1α and 2α by the downregulation of PHD2 and thereby promote vascularization and tumor cell invasion (Spinella et al. 2010). Similarly, the downregulation of PHD2 leads to increased tumor growth in a hormone-dependent mammary carcinoma mouse model (Bordoli et al. 2010). PHD2 has also been shown to induce cellular senescence in endometrial cancer cells through the regulation of the HIF pathway (Kato et al. 2006). In a xenotransplantation assay, it was shown that the loss of PHD2 in cancer cells confers a significant growth advantage over control tumors. The improved tumor growth was not dependent on HIF, but the decrease in the levels of PHD2 activates NF-κB, which regulates vasculogenesis through the induction of interleukin-8 and angiopoietin (Chan et al. 2009).

Our results point more towards the role of a tumor promoter for PHD2, at least in HNSCC, since its overexpression is seen in more aggressive and less differentiated tumors. PHD2 may also function as a tumor promoter by affecting the tumor vasculature, and this may occur HIF-independently (Mazzone et al. 2009, Chan et al. 2009). In addition, the ectopic expression of Drosophila Hph has been shown to be enough to increase cellular growth (Frei & Edgar 2004). It has also been proposed that PHD2 has a biphasic role in cellular transformation (Lee et al. 2008). In this study, moderate decreases in PHD2 activity lead to malignant transformation, whereas further loss of PHD2 activity produced cells that do not form tumors. This effect of PHD2 did not require hydroxylase activity. This implies that the effect of PHD2 is not, at least completely, due to its effect on the degradation of HIF. We also detected that the nuclear overexpression of PHD2 increases the anchorage-independent growth of cells. Our results implicate that the localization of PHD2 might have a key role in the progression of cancer.

PHD2 knockdown xenograft tumors have been shown to grow more rapidly and have increased angiogenesis, independently of HIF-1α (Chan et al. 2009). The wild-type xenograft tumors were less aggressive when grown in a $PHD2^{+/-}$ background (Mazzone et al. 2009). These data point to the roles for PHD2 in tumorigenesis and the interaction between a tumor and its environment. Although a number of these effects are likely to be dependent upon HIF, there appear to be HIF-independent activities of PHD2 that are important in tumorigenesis.

PHD3 appears to function as a tumor suppressor in colorectal cancer cells by inhibiting IKKβ/NF-κB signaling (Xue et al. 2010). PHD3 expression is decreased in colorectal cancer. The decreased expression is associated with higher tumor grade and metastasis. PHD3 inhibits phosphorylation of IKKβ and activation of NF-kB, independent of its hydroxylase activity (Xue et al. 2010). Loss of PHD3 has been associated with the development of pheochromocytomas.

PHD1 seems also to have a tumor suppressive role in colon carcinoma, since its ectopic expression in a mouse xenograft model inhibited tumor growth. It correlated with increased necrosis and decreased microvessel density (Erez et al. 2003). In one study, PHD1 appears to have a positive role in tumorigenesis. Its overexpression promotes breast cancer cell proliferation (Seth et al. 2002) and the knockdown of PHD1 causes hypoproliferation *in vitro* and *in vivo* (Zhang et al. 2009).

Taken together, the effects of PHD2 in tumor growth seem to be more complicated as compared to, for example, PHD1 and 3. In my studies on HNSCC, PHD2 functions as a tumor promoter and this was attributed to its nuclear localization.

7 Summary and Conclusions

In this study the expression of the HIF-regulator PHD2 was studied in normal and cancer cells as well as in resected tumors. First it was shown that PHD2 is a hypoxia-inducible gene in SCC cell lines. The regulation of PHD2 was shown to be dependent on the functional VHL system, which pointed towards the regulatory role for HIF in the hypoxic regulation of the PHD2 expression. Indeed a role for HIF has been later proven.

Little data is still available on the expression of PHDs in normal tissues. In normal tissues, the expression of PHD2 was found to be variable ranging from non-existing to very strong. Markedly strong expression was seen in endothelia across the tissues studied. This might reflect the relative roles of PHD isoforms in different tissues. The expression of PHD2 was further studied in HNSCC and colon adenocarcinomas. The results show that PHD2 is clearly overexpressed in both cancers and that it translocates into the nucleus in a subset of tumor cells. This nuclear localization correlates with the aggressiveness of the tumors. In line with this, in all normal tissues the expression of PHD2 was completely cytoplasmic.

The subcellular localization was studied further in cultured cells. It was noticed that PHD2 is, at least partially, shuttled between the nuclear and cytoplasmic compartments. Although the translocation can be restricted by the inhibition of the nuclear exportin activity, the supposed subcellular localization signals in PHD2 were shown not to be functional. This points towards more uncommon localization signals in PHD2.

It was shown that the nuclear translocation of PHD2 is a common phenomenon in carcinomas, at least in HNSCC and colon adenocarcinomas. It was also shown that while cytoplasmic PHD2 expression prevents anchorage-independent growth of cancer cells, the nuclear expression of PHD2 promotes the anchorage-independent growth of carcinoma cells. PHD2 and HIF expression do not coincide in all the tumor regions. This indicates that the regulatory connection between the two proteins may be broken in cancer. The question remains as to how PHD2 promotes the growth and what is the possible role of HIF.

In summary, this study provides new information on the expression and function of PHD2 in normal tissues and in tumors. The significance of the subcellular localization of PHD2 in tumor progression is also highlighted.

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