

Award Number: **W81XWH-08-1-0112**

TITLE: **Epigenetic control of prolyl and asparaginyl hydroxylases in prostate cancer**

PRINCIPAL INVESTIGATOR: **Adam J. Case**

CONTRACTING ORGANIZATION: **University of Iowa**
Iowa City, IA 52242

REPORT DATE: **July 2009**

TYPE OF REPORT: **Annual Summary**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 31/07/2009		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 July 2008- 30 June 2009	
4. TITLE AND SUBTITLE Epigenetic control of prolyl and asparaginyl hydroxylases in prostate cancer				5a. CONTRACT NUMBER W81XWH-08-1-0112	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Case, Adam J. Email: adam-case@uiowa.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Iowa Iowa City, IA 52242				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In many solid tumors, including prostate cancer, hypoxia-inducible factors (HIF) are up-regulated compared to their normal tissue counterparts. These HIF molecules are transcription factors, and supply the cancer with the proper proteins necessary for metabolism as well as angiogenesis. Recently, a set of proteins known as prolyl and asparaginyl hydroxylases (PHD and AHD respectively) have been shown to be essential in the regulation of HIF, and in some cancers have been transcriptionally and translationally silenced. We therefore proposed a study that focuses on the epigenetic control of these crucial enzymes. In addition, recent evidence has demonstrated a potential role for androgen insensitivity on the effect of suppression of these enzymes. In this report, we present data demonstrating our first findings of PHD expression in prostate cancer cell lines. Furthermore, we begin to identify specific epigenetic mechanisms that may play a major role in the transcriptional and translational control of these enzymes. Last, we will explain our future direction of the project for the remainder of the award period.					
15. SUBJECT TERMS Hypoxia Inducible Factor, HIF, Prolyl Hydroxylase, PHD, Prostate Cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-7
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusion.....	8-9
References.....	9
Appendices.....	10

Introduction

In many solid tumors including prostate cancer, hypoxia-inducible factors (HIF) are up-regulated compared to their normal tissue counterparts. These HIF molecules are transcription factors, and supply the cancer with the proper proteins necessary for metabolism as well as angiogenesis. Recently, a set of proteins known as prolyl and asparaginyl hydroxylases (PHD and AHD respectively) have been shown to be essential in the regulation of HIF, and in some cancers have been transcriptionally and translationally silenced. We therefore proposed a study that focuses on the epigenetic control of these crucial enzymes. In addition, recent evidence has demonstrated a potential role for androgen insensitivity on the effect of suppression of these enzymes. In this report, we present data demonstrating our first findings of PHD expression in prostate cancer cell lines. Furthermore, we begin to identify specific epigenetic mechanisms that may play a major role in the transcriptional and translational control of these enzymes. Last, we will explain our future direction of the project for the remainder of the award period.

Body

We first set out to characterize the expression level of PHD and AHD in an array of cell lines by quantitative real-time PCR. To obtain a larger-scale picture of PHD expression, some breast cell lines were also examined in addition to the prostate cancer cell lines discussed in the proposal. It was observed that the expression of PHD3 (the main focus of our research) varied greatly amongst the cells lines queried (Figure 1). In addition, the level of AHD was not significantly different at the mRNA level (data not shown) and as such the remainder of our attention was focused on PHD3. In one prostate cancer cell line, PC3, the mRNA level was essentially undetectable which made this cell line promising to study any epigenetic modifications that may be present. Western blots were performed to ensure that PHD3 was also absent at the protein level (Figure 2).

With this novel observation, we attempted to understand what, if any, epigenetic modification was responsible for the decreased PHD3 expression in PC3 prostate cancer cells. A hallmark of epigenetic control is DNA methylation, so we attempted to identify if the promoter of PHD3 in PC3 cells was aberrantly methylated. To identify this, we used a method previously discussed in the proposal known as sodium bisulfite

conversion sequencing. Briefly, DNA is extracted from the tissue and exposed to sodium bisulfite. This process converts all non-methylated cytosine's to uracil, whereas methylated cytosines are unaffected. The bisulfited DNA is then PCR amplified at a region of interest and sequenced. Because methyl-cytosine and uracil are not incorporated during PCR, any cytosines that are remaining indicate methylation in the original sequence prior to bisulfite treatment. It was observed that a large section of the PHD3 promoter (which contains the putative HIF binding site) in PC3 cells was hypermethylated (Figure 3). In addition, the breast cancer line MB-435, which also demonstrated decreased PHD3 expression, was also hypermethylated. Last, the remaining cell lines were shown to have unmethylated promoter regions, which is consistent with their detectable PHD3 expression levels.

DNA methylation is a powerful method of gene suppression, and many genes when methylated are unable to be activated by their regular cellular signals. For example, it has long been established that PHD expression is controlled by hypoxia through the HIF's. In other words, HIF's are able to control their own degradation by up-regulating PHD's in times of hypoxia. To understand if this control has been lost due to DNA methylation, quantitative real-time PCR for PHD3 was performed on PC3 cells in times of normoxia (21% O₂) and hypoxia (1% O₂). It was observed that even during hypoxia, PHD3 expression was unchanged (Figure 4). Furthermore, in another cell line (DU145), the expression of PHD3 increased almost 10-fold in response to hypoxia, demonstrating the transactivation of the gene by HIF. This lack of an up-regulation of PHD3 in PC3 cells could also be explained by a lack of proper HIF up-regulation during times of hypoxia. To test this hypothesis, western blot analysis for HIF1 α and HIF2 α was performed (Figure 5). From this analysis, it was determined that PC3 cells demonstrate a proper HIF response to hypoxia, which suggests that the DNA methylation at the HIF responsive element in the PHD3 promoter is responsible for aberrant expression during times of normoxia and hypoxia.

To complete Aim 1 as outlined in the statement of work, we attempted to reactivate the expression of PHD3 by use of 5-aza-deoxycytidine (5-aza-dC). 5-aza-dC is a DNA methyltransferase inhibitor, which can elicit global DNA demethylation within a treated cell population. Treatment with this drug incited an almost 5 fold increase PHD3 expression in PC3 cells (Figure 6). Treatment with 5-aza-dC on two low-expressing breast

cancer cell lines also increased PHD3 expression, suggesting that DNA methylation may be a common mechanism shared amongst different cancer types. In addition to treatment with 5-aza-dC, a histone deacetylase inhibitor (i.e. Trichostatin A (TSA)) was also applied to the cells. Histone deacetylation is another epigenetic modification in which cells may silence gene expression by altering histone modifications. Histone deacetylase inhibitors (HDACI's) are drugs that inhibit the removal of acetyl groups, allowing for more open chromatin and active gene expression. Interestingly, the use of TSA alone and in combination with 5-aza-dC only slightly increased, had no significant effect, or even lowered PHD3 expression in any of the cell lines examined (data not shown). This finding suggests that histone modifications may not play a major epigenetic silencing role with PHD3, and as such our attention should focus elsewhere.

To further examine our hypothesis that epigenetic modifications are at the root of silencing PHD3 in PC3 cells, we have begun preliminary studies on chromatin accessibility as outlined in Aim 2 of the original statement of work. This assay will tests if the chromatin is in a relatively more open (e.g. transcriptionally active) or closed (e.g. transcriptionally silent) state. It was discovered that the PC3 cells demonstrated a much more closed state of chromatin when compared to MCF7 cells, which are highly PHD3 expressing (Figure 7). These studies have only been performed once, and will have to be repeated to identify statistical significance, but preliminary results are quite promising.

With the completion of Aim 1 and part of Aim 2 of our original statement of work, we are shifting our focus to experiments outlined in Aim 3 as well as new assays that we hope will serve as an adjuvant to our original hypothesis. Unfortunately, in our original hypothesis we proposed that there may have been some type of hormone responsiveness that may have played a role in the control of epigenetic mechanisms regulating PHD3. From a preliminary experiment using the hormone responsive MCF7 and the hormone insensitive line MCF7-5C, we determined there to be a minimal difference in the expression level of PHD3 (data not shown). As such, we have focused our attention to the primary means behind PHD3 methylation in certain cancer cell lines. We have acquired two new tools that we hope aid in elucidating more about the aberrant expression of these genes. Specifically, we have obtained HIF-1 α promoter¹ and oxygen-dependent degradation domain (ODD) luciferase plasmids² from Dr. Konstantin Salnikow at the National Cancer Institute.

The HIF-1 α promoter construct will allow for identification of proper HIF transactivation within a cell population to aid in ruling out HIF functionality as a cause for loss of PHD3. Along with that, the ODD construct produces a reporter protein that contains the same degradation domain as HIF. By this methodology, we will be able to understand the capacity in which the cells are able to regulate hypoxia responsive genes. These powerful tools serve to improve the impact of our data by truly elucidating the mechanism of PHD3 loss.

Last, a recent publication from our group demonstrated that certain reactive oxygen species (ROS) such as superoxide (O₂^{•-}) are able to regulate the stabilization of HIF proteins³. Since ROS are known to be elevated in cancers, this allows for another aspect of study to fully understand PHD3 control. We have recently obtained mice that are floxed (flanked by loxP sites) for the *SOD2* gene locus⁴. *SOD2* encodes for manganese superoxide dismutase, which is a mitochondrial antioxidant enzyme whose function is to neutralize superoxide. By breeding with a mouse that expresses Cre-recombinase in a tissue specific manner (i.e. prostate by way of PSA or probasin promoter), we are able to make conditionally specific *SOD2* knock-outs. This *in vivo* model will allow us to understand if there may be any direct link between ROS and epigenetic silencing of the PHD enzymes. We are in the very early stages of this directive and are obtaining the proper mice and to initiate breeds, but this aspect provides great promise and novelty to the study of epigenetics and free radical biology.

Key Research Accomplishments

- Identification of PHD3 mRNA expression and protein level in prostate cancer cell lines
- Observation of no change of ASD expression in prostate cancer cell lines
- Detection of methylated promoter region in low PHD3 expressing cell lines
- Identification of lack of hypoxia response for PHD3 in PC3 cells
- Reactivation of PHD3 by way of DNA methylation epigenetic modifying drug
- Observation of no significant response with histone specific epigenetic modifying drug
- Identification of closed chromatin around PHD3 locus in PC3 cells

Reportable Outcomes

Peer-reviewed publications:

- Nam, C., **Case, A.J.**, Hostager, B.S., and O'Dorisio, M.S. (2009) The role of vasoactive intestinal peptide (VIP) in megakaryocyte proliferation. *Journal of Molecular Neuroscience* **37**(2): 160-167.
- Venkataramen, S., Fitzgerald, M., Jettawatana, S., **Case, A.J.**, Oberley, LW., and Domann, FE. (2008) *Epigenetics, a key factor in hormone-independent prostate and breast cancers: New concepts for developing tailored therapies. Free Radical Biology and Medicine* **45**: S57-S58.

Book Chapters:

- **Case, A.J.** & Domann, F.E. *Hypoxia-Induced Gene Regulation through Hypoxia Inducible Factor-1 Alpha*, In Redox Signaling and Regulation in Biology and Medicine (eds. Jacob, C. & Winyard, P.) (Wiley-VCH) 2009. – See appendix for full text.

Abstracts:

- **Case, A.J.**, Johns, A., Takahashi, T., Waldschmidt, T., and Domann, FE. (2008) *Aberrant thymic development in a T-lymphocyte specific SOD2 knock-out mouse. Free Radical Biology and Medicine* **45**: S133-S134.

Awards:

- **Case, A.J.**, Young Investigator Award, Annual Meeting of the Society for Free Radical Biology and Medicine, Indianapolis, IN, November 21, 2008.

Conclusion

In summary, this project progressed as outlined in the statement of work. Aim 1 was completed, Aim 2 has begun before schedule, and we have already improved Aim 3 and are obtaining the proper reagents and tools to expand our knowledge of PHD3 control in prostate cancer. Our studies have required us to make adaptive changes in our original plan so as to truly understand the mechanism underlying this epigenetic silencing, and much has been learned from our approaches. Finally, we hope that the addition of our *in vivo*

model allows for a more clinically relevant study, and as such may lead to novel epigenetic or antioxidant therapies that could effectively treat the growing population suffering with prostate cancer.

References

1. Salnikow, K., An, W.G., Melillo, G., Blagosklonny, M.V. & Costa, M. Nickel-induced transformation shifts the balance between HIF-1 and p53 transcription factors. *Carcinogenesis* **20**, 1819-23 (1999).
2. Salnikow, K. et al. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J Biol Chem* **279**, 40337-44 (2004).
3. Kaewpila, S., Venkataraman, S., Buettner, G.R. & Oberley, L.W. Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide. *Cancer Res* **68**, 2781-8 (2008).
4. Ikegami, T. et al. Model mice for tissue-specific deletion of the manganese superoxide dismutase (MnSOD) gene. *Biochem Biophys Res Commun* **296**, 729-36 (2002).

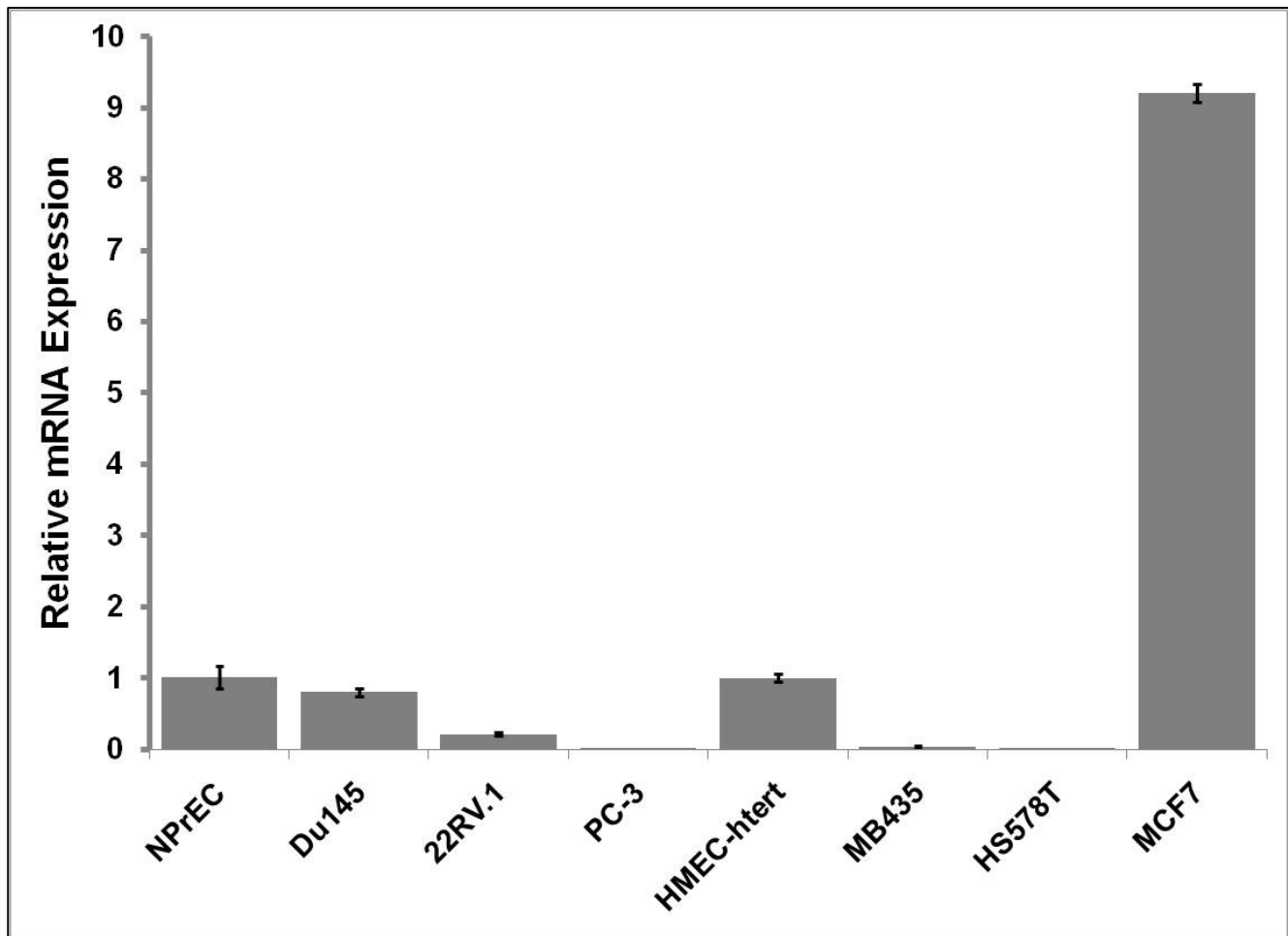


Figure 1. PHD3 mRNA levels vary amongst different prostate and breast cancer cell lines.

RNA was extracted using Trizol reagent and reverse transcribed by using the ABI cDNA Archive Kit. Quantitative real-time PCR analysis was performed using primers specific to the PHD3 mRNA transcript, and data was normalized using the $\Delta\Delta CT$ method to the normal prostate and normal breast cell lines NPrEC and HMEC-htert respectively. PC3 prostate cancer cells and MB435 breast cancer cells demonstrate the most abundant knock-down of PHD3 transcript.

Appendices – Figure 2

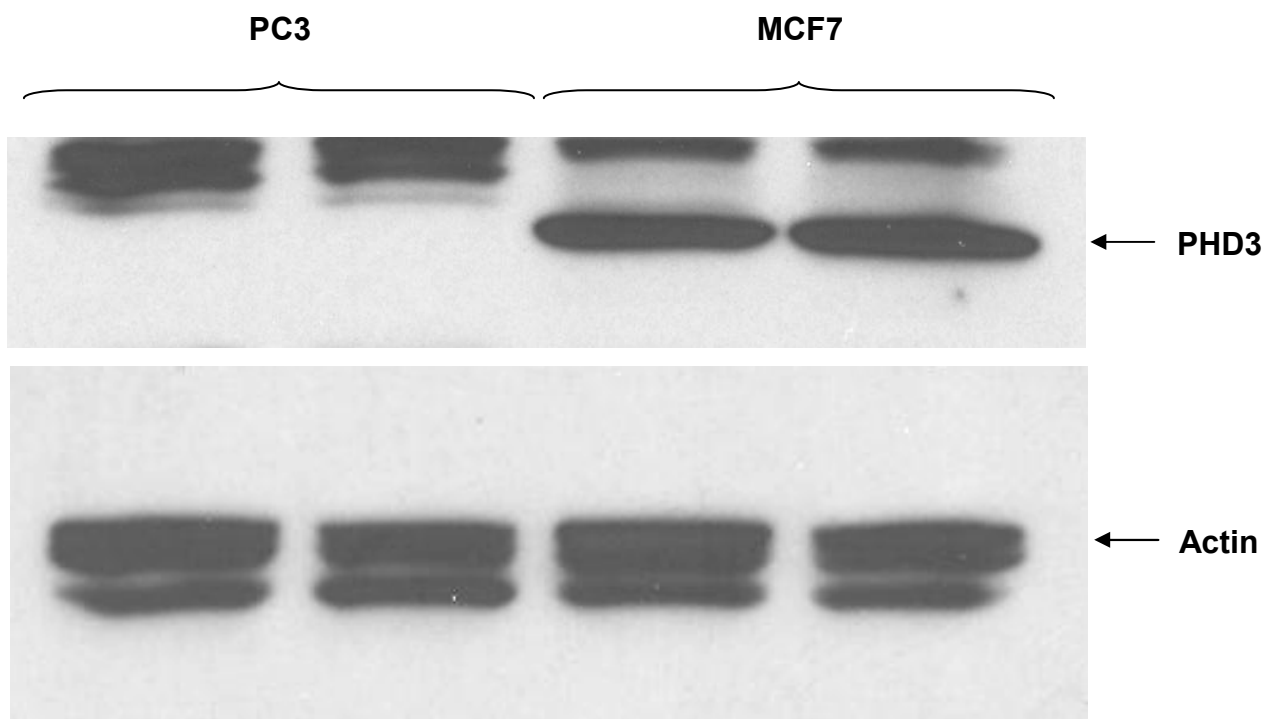


Figure 2. PHD3 protein is non-detectable in PC3 prostate cancer cells. Protein was extracted from both PC3 and MCF7 cells using a modified RIPA buffer. Western blot analysis shows highly abundant protein in MCF7 cells, and virtually absent protein in PC3 cells (run in duplicate). This data supports the mRNA expression data as seen in Figure 1.

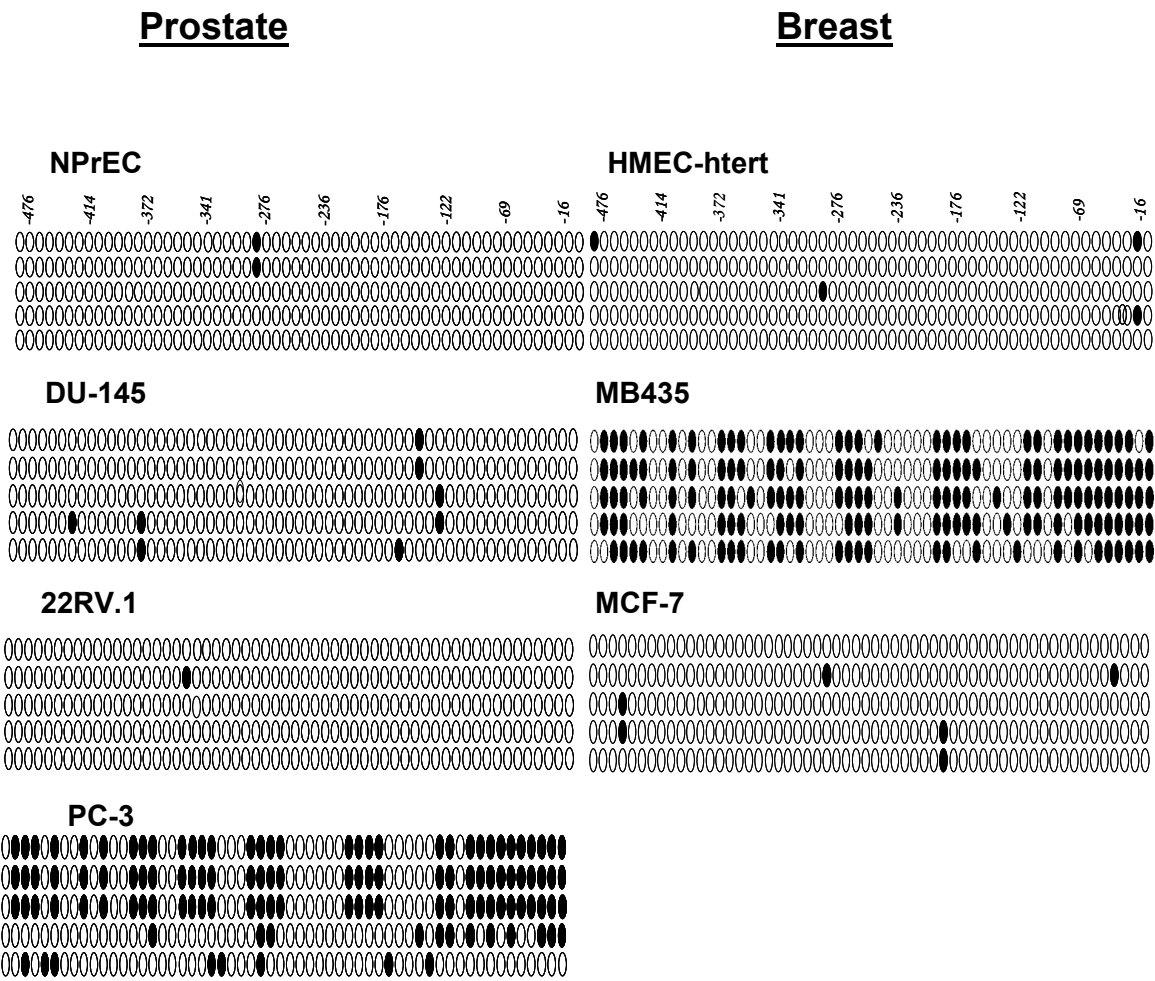


Figure 3. DNA methylation analysis reveals the promoters of PC3 and MB435 cancer cells are hypermethylated. Genomic DNA was bisulfite converted using the Zymo Research EZ-DNA Methylation kit, and then PCR amplified using primers specific to the promoter region of PHD3. PCR products were cloned into bacterial colonies for amplification and then followed by sequencing. Closed/Black circles represent a specific methylated CpG within the promoter region while Open/White circles represent a non-methylated CpG. Data demonstrate that only the cell lines with minimal expression are highly methylated.

Appendices – Figure 4

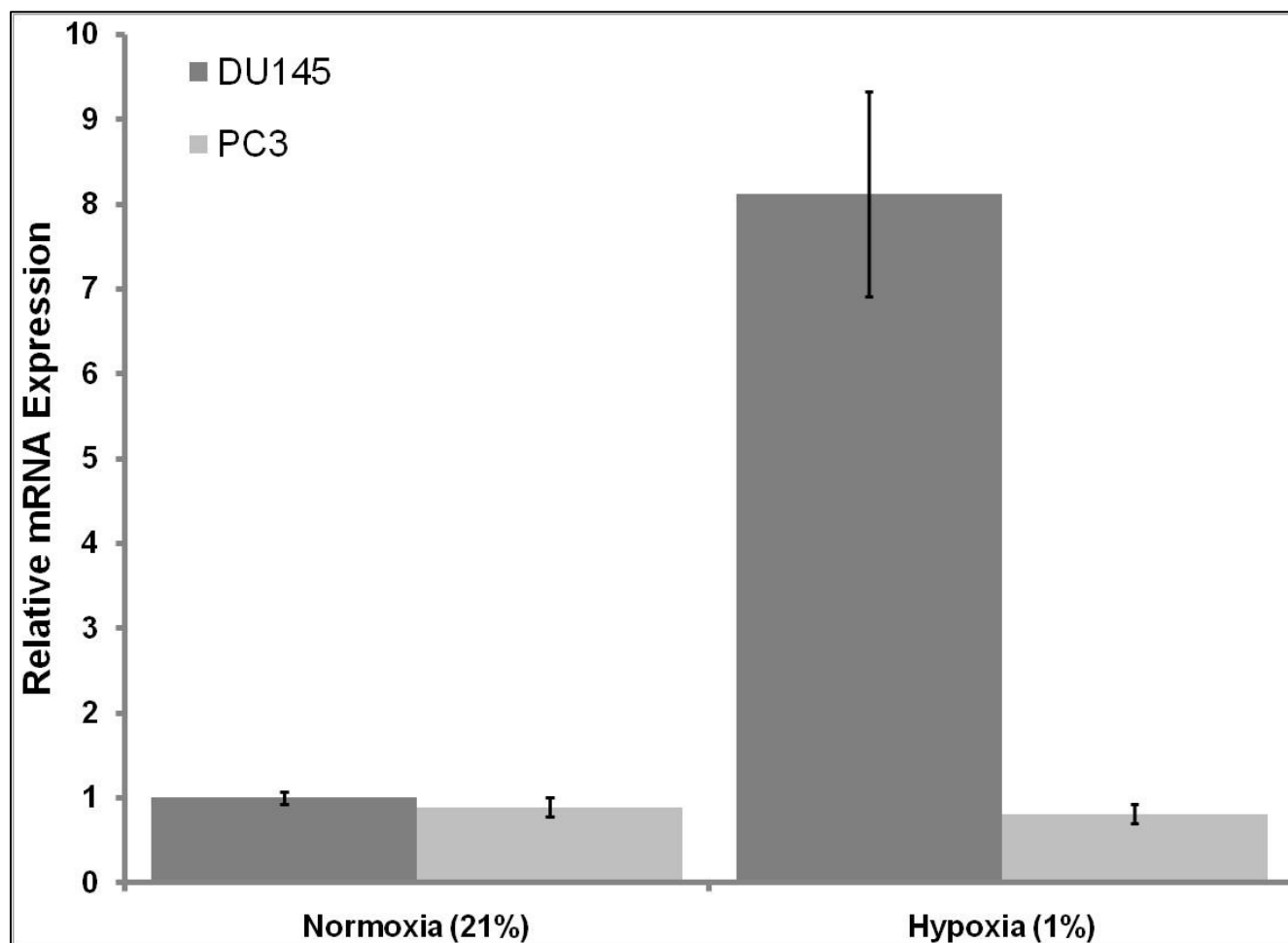


Figure 4. PC3 cells do not exhibit proper PHD3 hypoxia inducible response. Cells were exposed to normoxia or hypoxia for 16 hours. RNA was extracted using Trizol reagent and reverse transcribed by using the ABI cDNA Archive Kit. Quantitative real-time PCR analysis was performed using primers specific to the PHD3 mRNA transcript, and data was normalized using the $\Delta\Delta CT$ method to the hypoxia responsive cell line DU145. PC3 prostate cancer cells demonstrate no induction of PHD3 in response to hypoxia.

Appendices – Figure 5

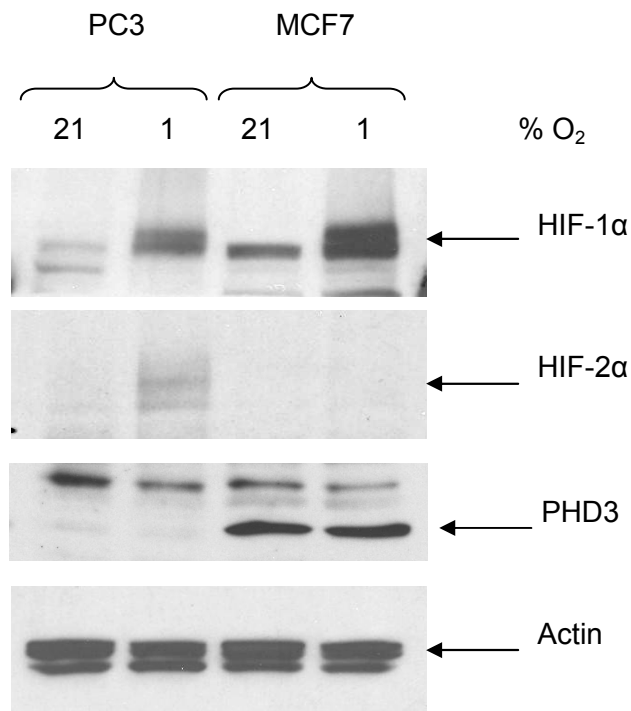


Figure 5. PC3 cells demonstrate proper HIF induction even in absence of PHD3. Cells were exposed to normoxia or hypoxia for 16 hours. Protein was extracted using a modified RIPA buffer. Western blot analysis demonstrates a strong induction of both HIF-1α and HIF-2α in response to hypoxia in PC3 cells, while PHD3 remains unchanged. MCF7 cells also show proper induction of HIF proteins, but due to over-abundance of protein in normoxia the hypoxia response is not dramatic.

Appendices – Figure 6

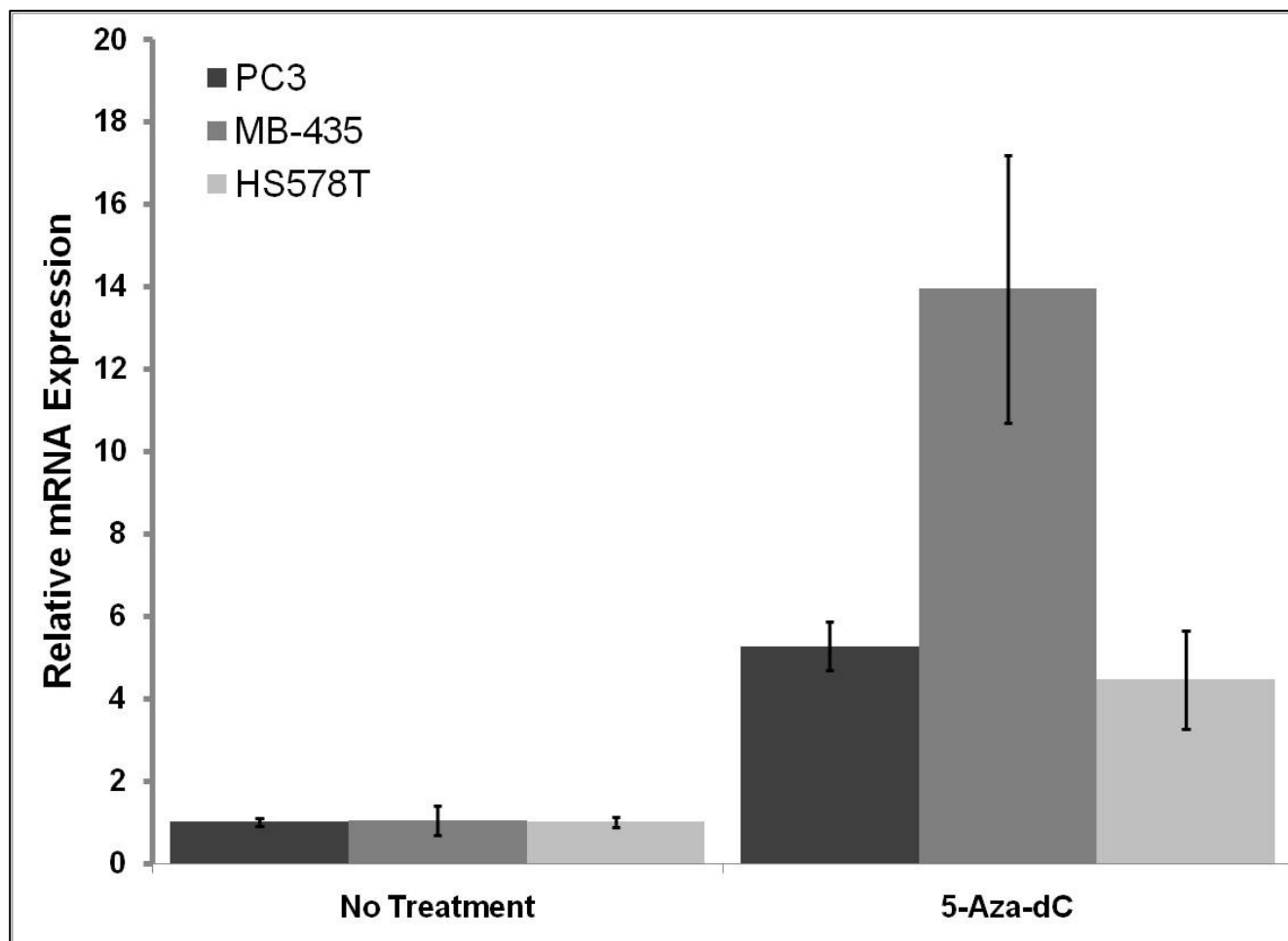


Figure 6. All low PHD3 expressing cell lines show reactivation with 5-aza-dC. Cells were treated with 5 μ M 5-aza-dC every other day for 7 days. RNA was extracted using Trizol reagent and reverse transcribed by using the ABI cDNA Archive Kit. Quantitative real-time PCR analysis was performed using primers specific to the PHD3 mRNA transcript, and data was normalized using the $\Delta\Delta$ CT method to PC3 prostate cancer cells. Data show induction of PHD3 in all cell lines treated with 5-aza-dC.

Appendices – Figure 7

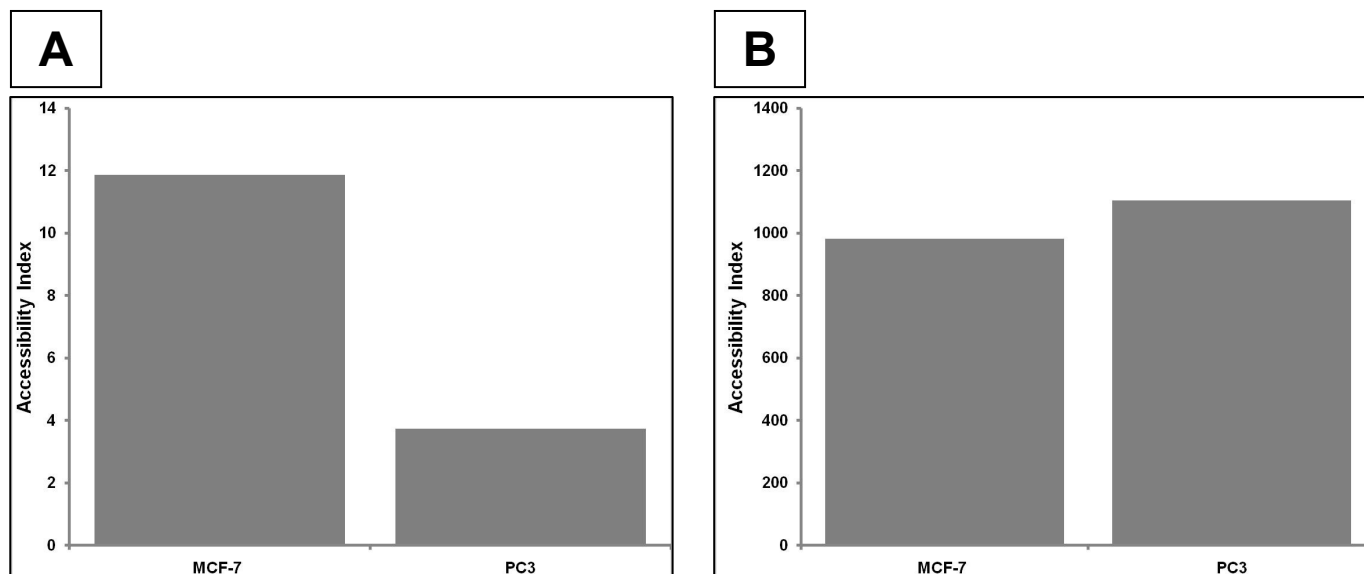
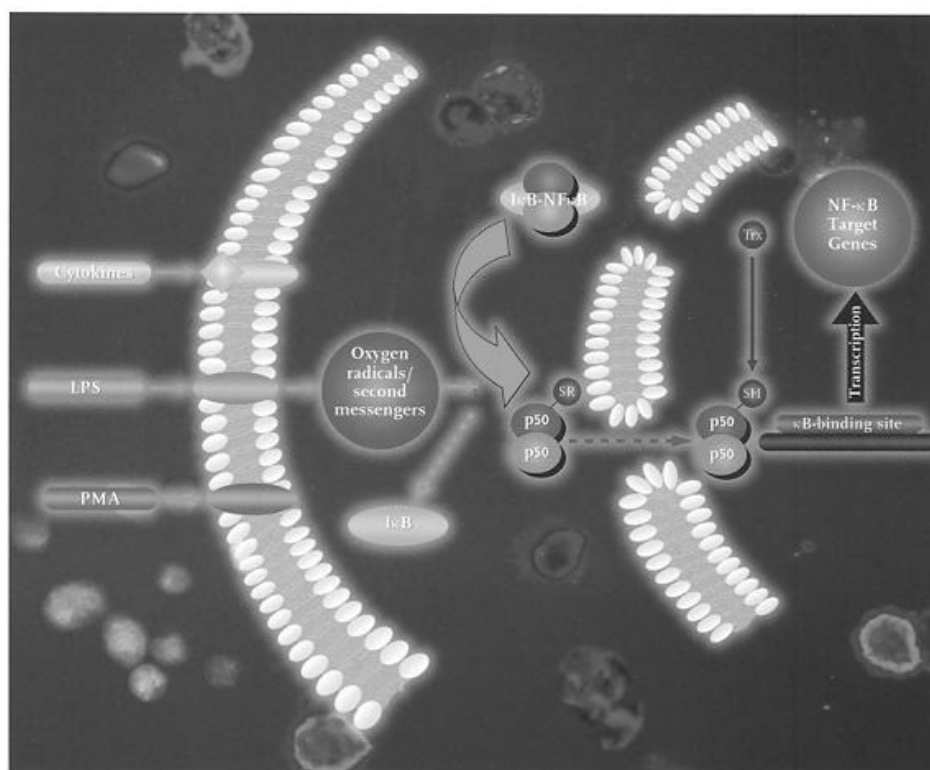


Figure 7. PC3 cancer cells demonstrate decreased chromatin accessibility at the PHD3 locus compared to MCF7 breast cancer cell lines. Isolated nuclei from the cell lines were treated with DNase I to understand susceptibility to digestion by the endonuclease. DNA was extracted post-digestion and analyzed by quantitative real-time PCR for both the PD3 locus (panel A) as well as GAPDH (panel B) as a relative control. PC3 cells appear to have a more closed chromatin structure at the PHD3 locus than the high PHD3 expressing cell line MCF7.

Edited by Claus Jacob,
and Paul G. Winyard

 WILEY-VCH

Redox Signaling and Regulation in Biology and Medicine





Adam J. Case is a fifth-year combined MD/PhD student at the University of Iowa. He is currently pursuing a PhD in free radical and radiation biology, and his research interests include elucidation of the role of antioxidant enzymes in cancer, epigenetics of regulatory enzymes, and the use of antidiabetes medication in the prevention of oncogenesis. Following his thesis dissertation, Adam plans to pursue a medical residency in pediatrics where he hopes to practice both clinical and research medicine in the field of pediatric oncology.

Adam J. Case, The University of Iowa, Holden Comprehensive Cancer Center, Carver College of Medicine, Department of Radiation Oncology, Iowa City, IA 52242-1181, USA



Edith Charlier is a PhD student at the GIGA-Research Center, Liège, Belgium, in the division of Jacques Piette in the subgroup of Geoffrey Gloire. She studied biochemistry at the University of Liège and she joined the division of Jacques Piette for her thesis. She is working on an inositol phosphatase which influences the sensitivity of T lymphocytes to CD95-induced cell death.

Edith Charlier, University of Liège, Virology-Immunology Unit, GIGA-Research, 1, avenue de l'Hôpital, 4000 Liège, Belgium



Silvia Cristofanon received her PhD from the University of Rome Tor Vergata in spring 2008 after three years of research at the LBMCC Lab in Luxembourg. Her major interests are related to apoptotic cell signaling pathways in human leukemia cells induced by glutathione depletion.

Silvia Cristofanon, Hôpital Kirchberg, Fondation Recherche sur le Cancer et les Maladies du Sang, Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (LBMCC), 9, rue Edward Steichen, 2540 Luxembourg and Università di Roma "Tor Vergata", Dipartimento di Biologia, via Ricerca Scientifica, 00133 Roma, Italy



Alison Curnow As medical research director of Cornwall Dermatology Research (Peninsula Medical School, UK), Alison Curnow has established one of the top dermatological photodynamic therapy groups in the UK. The clinical and experimental research program aims to improve the effectiveness and diversify the application of this noninvasive light-mediated treatment modality. In addition, the laboratory also investigates the causation and potential prevention of skin carcinogenesis.

9	Hypoxia-Induced Gene Regulation through Hypoxia Inducible Factor-1α	207
	<i>Adam J. Case and Frederick E. Domann</i>	
9.1	Introduction	207
9.2	The Proteins and Mechanism	208
9.3	Hypoxia Inducible Factor Target Genes	213
9.4	Non-Hypoxia-Induced Activation of Hypoxia Inducible Factor	218
9.5	Diseases Involving Hypoxia Inducible Factor	220
9.6	The Promise of Hypoxia Inducible Factor-Targeted Therapies	223
9.7	Conclusions	226
	References	226
10	Eicosanoid-Based Signaling	229
	<i>Valerie B. O'Donnell</i>	
10.1	Introduction	229
10.2	Biosynthesis and Structures of Eicosanoids	231
10.2.1	Lipoxygenases	234
10.2.2	Cyclooxygenases	235
10.2.3	Cytochrome P450	237
10.3	Signaling by Eicosanoids	239
10.4	Metabolism of Eicosanoids	240
10.5	Summary	242
	References	243
11	Redox-Controlled Transcription Factors and Gene Expression	245
	<i>Gregory I. Giles</i>	
11.1	Introduction	245
11.2	Redox Signaling and Gene Microarray Data: The Global Picture	248
11.3	The Antioxidant Response Element	251
11.4	The Transcription Factor Nrf2: The Master Regulator of Antioxidant Transcription	253
11.5	The Keap1-Nrf2 Complex: A Sensor for Cellular Stress	255
11.5.1	Reactive Oxygen Species, Reactive Nitrogen Species and Electrophile Sensing by Keap1	256
11.5.2	Structural Basis for the Sensing Function of the Keap1-Nrf2 Complex	257
11.6	Redox Reactions of Transcription Factors	261
11.6.1	Nuclear Factor κ B: Redox Control of the Immune Response	262
11.6.2	Activator Protein 1 (AP-1): Redox Control of Proliferation and Apoptosis	264
11.6.3	The Role of the Nuclear Redox State in Gene Expression	265
11.7	Summary	266
	References	267

9

Hypoxia-Induced Gene Regulation through Hypoxia Inducible Factor-1 α *Adam J. Case and Frederick E. Domann*

9.1

Introduction

It has long been appreciated that most eukaryotic organisms depend upon oxygen for survival. Oxygen is involved in many cellular processes: first, oxygen acts as the final four-electron acceptor in the electron transport chain during aerobic respiration; second, oxygen acts as a one-electron acceptor for production of superoxide during innate immunity such as by phagocytic white blood cells in attempts to control invading pathogens; and third, oxygen acts as a cofactor for dioxygenases such as the cytochrome P450 enzymes in the liver which use molecular oxygen to oxidize potentially toxic compounds rendering them hydrophilic and easier to excrete.

In spite of the requirement of molecular oxygen for these diverse functions, eukaryotic cells are still able to survive and function when oxygen availability is limited. Thus, cells have adapted a mechanism for sensing oxygen, and through this mechanism are able to adapt their metabolism and energy production from primarily aerobic to anaerobic processes to enhance cellular viability under hypoxic conditions.

Hypoxia is defined broadly as a deficiency in oxygen. In complex organisms, hypoxia usually arises due to two major causes. The first is an environmental lack of oxygen, such as occurs in areas of high altitude where the concentration of oxygen is insufficient for proper delivery to tissues. The other is during times of ischemia, or lack of blood flow. Ischemia may come about due to either a constriction of an artery, or by blockage of an artery. These occlusions commonly lead to infarctions, and infarctions of cardiovascular and cerebral tissues are the cause of over 800 000 deaths each year in the United States [1]. Hypoxia does not lead to instant cellular death or damage, and most organisms that depend on oxygen for survival have devised strategies to counteract the negative effects of hypoxia.

In 1992, Semenza and Wang extensively studied promoter and enhancer sequences of the erythropoietin (EPO) gene [2]. These investigators focused on an enhancer region 3' of the coding region, denoted the hypoxia response element

interacted with the DNA, but only one protein that was bound during hypoxic conditions. They named this protein the hypoxia inducible factor (HIF), and it was thought at that time to be an EPO-specific transcriptional regulator. Subsequent studies showed HIF DNA binding activity in EPO-deficient models, leading to the hypothesis that HIF may play an extensive role in hypoxia-induced gene regulation involved in numerous cellular processes [3]. Since that time, investigations in the field of hypoxia-induced gene regulation have grown exponentially.

Hypoxia inducible factor controls the expression of a variety of genes including those that control angiogenesis, anaerobic and aerobic metabolism, and a multitude of other cellular processes [4]. In addition, aberrant HIF expression has been noted in certain disease states including cancer [5]. This chapter will address the role of HIF in transcriptional regulation of such genes and the current thinking of how HIF itself is regulated to function as an effector of cellular oxygen concentrations. A discussion will follow that takes an in-depth look at HIF in different disease states as well as current concepts focused on hypoxia-induced gene regulation and the predicted future of this area of inquiry.

The topic of HIF-mediated gene expression and the factors that govern HIF activity are on the cutting edge of genetics and molecular biology investigations, and it appears that exciting discoveries await.

9.2

The Proteins and Mechanism

HIF is a heterodimeric transcription factor composed of two subunits denoted HIF- α and HIF- β . Both subunits are members of the basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain family [6]. It is believed that the PAS domains on each of the subunits mediate dimerization of the transcription factor, while the bHLH domains constitute the HIF DNA-binding domain. The β -subunit was originally known as the aryl hydrocarbon receptor nuclear translocator (ARNT), and contains the nuclear localization sequence necessary to translocate the heterodimer from the cytosol into the nucleus [7]. The β -subunit appears to be found in almost all tissue types and is expressed at constitutively low levels with no response to oxygen levels. Unlike the one isoform of the β -subunit, the α -subunit has three different homologs [8]. HIF-1 α is uniformly expressed in a similar manner to the β -subunit, while HIF-2 α and HIF-3 α display a more heterogeneous expression pattern both spatially and temporally. Much of the expression and cellular role of the latter two forms has still yet to be fully elucidated, and as such the focus of HIF in this chapter will center on the HIF-1 α subunit.

As would be expected by the name hypoxia inducible factor, the protein expression of all the α -subunits may be upregulated by hypoxia in certain tissue types [6]. However, the expression of these effector proteins is not regulated at the level of transcription, but rather by posttranslational modifications to the protein through

Box 9.1: Hypoxia, Cancer and Disease-causing Viruses

Most of the themes covered in this book deal with oxidative stress (OS) and abnormally increased levels of oxidizing species. In contrast, there are various tumors that exhibit an unusually reducing intracellular redox environment, at least in cells located toward the center of the tumor. Blood flow is restricted in these hypoxic regions, and cells therefore have to deal with significantly reduced levels of dioxygen. One way of adapting to this situation is a change in the levels of specific intracellular proteins. Such cells often exhibit increased levels in enzymes such as DT-diaphorase and NADPH:cytochrome *c* reductase, which in essence serve to reduce various small molecules.

Interestingly, a range of natural and synthetic molecules are comparably nontoxic in their oxidized forms but develop considerable cytotoxicity upon reduction. Such substances, which are collectively known as "bioreductive" agents, are therefore preferentially "activated" in cancer cells which contain significant amounts of reducing enzymes. Among these bioreductive drugs, the natural compound mitomycin C (MMC) has been tested as an anticancer agent since the 1950s and is currently approved for clinical use. Since then, a wide range of synthetic compounds have been developed, including quinones, *N*-oxides, nitroaromatic and heterocyclic compounds, and even metal complexes, such as a cobalt (Co^{3+}) complex which releases a toxic nitrogen mustard ligand upon reduction to Co^{2+} .

Importantly, OS and hypoxia are not mutually exclusive. In fact, there have been various reports recently indicating that the above-mentioned changes in hypoxic cells also result in increased levels of OS. In some ways, this apparent "paradox" is not even surprising: ROS formation does not depend on dioxygen levels *per se*, but is the result of unusual – and often incomplete – reduction processes. It is therefore feasible that unusual reductive processes also result in reductive activation of ROS, for instance by reduction of O_2 to $\text{O}_2^{\bullet -}$ or by reduction of H_2O_2 to $\text{HO}^{\bullet}$.

another group of enzymes that appear to act as the primary oxygen sensors. Intuitively, it would be disadvantageous to cells if transcription and translation of new proteins were required in response to hypoxia. Hypoxia will damage cells quickly, and thus cells need to respond to this lack of oxygen in a timely manner. Therefore, eukaryotic cells have evolved a clever posttranslational methodology of control that acts to sense oxygen changes rapidly, and can flip the switch to hypoxia-induced gene regulation just as quickly.

The mechanism and regulation of HIF-1 α levels in response to oxygen involves several other proteins and is quite unique in nature. In 1999, Maxwell *et al.* discovered that an E3 ubiquitin ligase could interact with HIF-1 α to cause its polyubiquitination, and ultimately target HIF-1 α to the 26S proteasome for degradation [9]. The protein was named VHL after von Hippel–Lindau disease, a condition in which the ligase is

absent due to a genetic deletion. Patients with this disease are subject to numerous types of cancers due to the loss of this important tumor suppressor gene, as described in greater detail later in this chapter.

Many more studies followed this initial work, and it was shown that only in normoxic conditions could VHL polyubiquitinate HIF-1 α . The mantra of HIF regulation was simple: HIF-1 α is constitutively transcribed and translated under both normoxic and hypoxic conditions, but the protein is degraded in the presence of oxygen and not in hypoxia. This hypothesis accounted for the observed nature of the HIF transcription factor, but still neglected to answer the quintessential question: how does HIF-1 α stability change in response to oxygen, and what is the sensor of changes in oxygen concentration that leads to changes in HIF-1 α protein levels? There was no evidence that VHL had any structural or functional differences in the presence or absence of oxygen, suggesting that it was not the oxygen sensor. So the molecule(s) that served as the oxygen sensor to regulate the stability of the HIF effector molecule remained a mystery.

In 2001 a discovery was made that helped answer this question. It was shown that VHL could only interact with HIF-1 α if two crucial proline residues were hydroxylated [10]. Prolines 402 and 564 of the HIF-1 α protein reside in an area of the protein termed the oxygen-dependent degradation domain (ODD). X-ray crystal structures of the HIF-1 α -VHL complex showed that the hydroxylated proline residues of HIF-1 α displaced a water hydrogen-bonding interaction between a histidine and serine molecule in the hydrophobic core of VHL (figure 9.1). These hydroxyl groups make the interaction with VHL very specific, and without the presence of these hydrogen-bonding groups HIF-1 α and VHL could not interact.

With the understanding that specific proline residues were essential to the stability of the HIF-1 α by affecting its interaction with the VHL ubiquitin ligase, the search began to identify the enzymes necessary to perform the hydroxylation of these residues. A proline hydroxylase with activity toward collagen had long been known to be central to ascorbate-deficient scurvy [11, 12]. Thus, candidate gene searches were carried out looking for enzymes that may act as HIF-1 α -targeted proline hydroxylases [13]. Three very similar enzymes with hydroxylase activity toward proline residues were discovered, and were named prolyl hydroxylase domain-containing proteins (PHD1, 2, and 3). Each of these proteins possesses an iron molecule at its active site, and in addition requires ascorbate (as a reducing agent) to keep the iron in the ferrous state), α -ketoglutarate and molecular oxygen as cofactors for the reaction to occur.

Current thinking on the mechanism of action of this enzyme is that when molecular oxygen and α -ketoglutarate are present, they compete with water for conjugation with the iron moiety. When the target proline residue from HIF-1 α enters the active site of this "primed" PHD enzyme, electrons shift to a more energetically favorable state, producing succinate, carbon dioxide and a hydroxylated proline residue (figure 9.2). The PHD enzymes identify a specific amino acid sequence on HIF-1 α shown to be LXXIAP, which allows for the proper proline

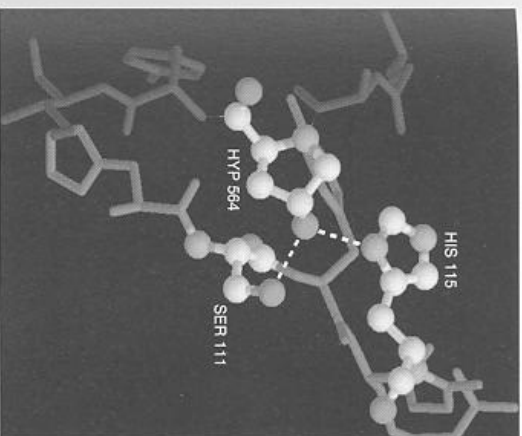


Figure 9.1 Interaction of the hydroxylated proline residue (Hyp564) of the HIF-1 α transcription factor with the VHL protein. The hydroxylated proline is able to form two new hydrogen bonds (dashed lines) with histidine (His115) and serine (Ser111) residues found on the VHL protein. This area of VHL is highly hydrophobic, and the region is normally stabilized by the interaction of these two residues with a water molecule. This hydroxylated residue acts to displace the water, and thus increases the specificity of the binding of HIF to VHL.

residues to be hydroxylated [14]. The aforementioned VHL ubiquitin ligase is able to recognize these hydroxylated prolines and target the HIF-1 α for degradation by the 26S proteasome.

Furthermore, the exact mechanisms of each of the three different isoforms of PHD are still not fully understood, but new findings suggest a complex pattern of regulation of these proteins. The subcellular location of the three enzymes is as follows: PHD1, nucleus; PHD2, cytoplasm; and PHD3, equal between both nucleus and cytoplasm [8]. By the use of siRNA in normoxia, one study found that knocking down of PHD2 demonstrated the greatest stabilization of HIF-1 α [15]. This finding led to the hypothesis that the PHD2 isoform may play the major role in oxygen-sensing for hypoxia-induced gene regulation.

The mechanism described above appears to be the primary form of HIF-1 α regulation, but HIF-1 α is an important transcriptional regulator for adaptive responses to hypoxic conditions and as such has developed a second mode of control as well. The C-terminal end of HIF-1 α contains a transactivation domain (CTAD) that

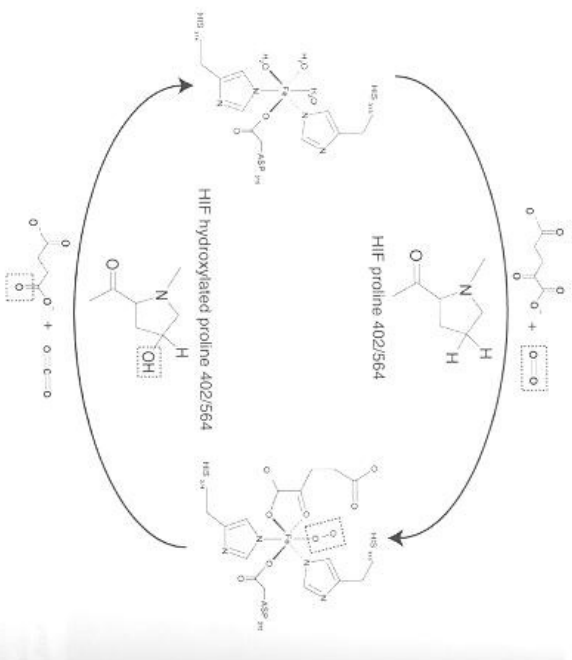


Figure 9.2 Proline hydroxylase enzymes use molecular oxygen to hydroxylate proline residues 402/564. The mechanism of the enzyme is as follows: Molecular oxygen and α -ketoglutarate displace three water molecules conjugated to iron at the active site of the PHD. The HIF-1 α proline residue easily becomes oxidized within its ring structure, while the remaining molecules rearrange to succinate and carbon dioxide. Water once again reclaims its conjugation to the iron, and the enzyme is prepared to catalyze another reaction. The dotted box indicates the fate of molecular oxygen throughout the process.

possesses the ability to interact with essential coactivators of transcription such as CBP/p300 [16]. For HIF-1 α to be transcriptionally active, it is essential that these other proteins are able to aggregate with the CTAD. Interestingly, at position 803 of the HIF-1 α protein is a crucial asparagine residue. In a reaction comparable to that of the proline hydroxylation, this asparagine is hydroxylated in the presence of oxygen by a specific asparaginyl hydroxylase, named factor inhibiting HIF-1 α (FIH-1). This hydroxylated asparagine residue prevents binding of the CBP/p300 coactivators, rendering HIF-1 α inactive to transactivate its target genes. It is proposed that in certain disease states where the PHD/VHL mechanism of degradation is defective the FIH-1 hydroxylation of the CTAD acts as a second checkpoint to prevent unregulated HIF-1 α activity. An overall scheme of the HIF-1 α regulatory mechanism is shown in Figure 9.3.

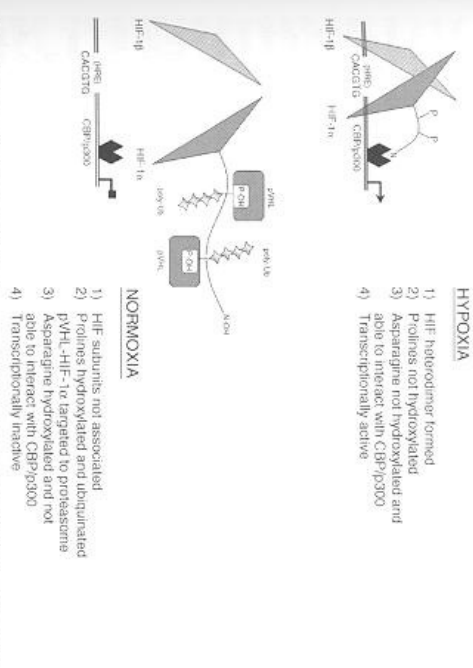


Figure 9.3 Control of HIF-1 α stability in response to oxygen is regulated by proline hydroxylation and polyubiquitination. During hypoxic conditions none of the crucial residues are hydroxylated, allowing for proper binding to the β -subunit of HIF as well as the CBP/p300

9.3 Hypoxia Inducible Factor Target Genes

When the hypoxia inducible factor was first discovered it was thought to be a specific transcriptional activator of erythropoietin. Since that time, it has been shown that HIF plays a role in the upregulation of many genes during hypoxia (Table 9.1). In retrospect, it seems somewhat intuitive that the majority of known HIF target genes encode proteins that are functionally involved with either oxygen delivery or anaerobic metabolism. The HIF heterodimer recognizes a particular DNA sequence to augment its binding (i.e., A/GCGTG), which has been referred to as the hypoxia response element (HRE) [4]. This canonical HRE sequence is found on all of the genes listed in Table 9.1, and has sparked a new wave of research to discover all HIF target genes.

Of the long list of genes known to be regulated by HIF, this chapter will focus on several specific examples. The first such example of an important gene controlled by HIF is the vascular endothelial growth factor (VEGF) [17]. This protein is essential in the process of angiogenesis, or the creation of new blood vessels. It is secreted by hypoxic cells and neighboring endothelial cells react to the signal and start to migrate towards the area of VEGF production. During and after migration to the source of

Box 9.2: Angiogenesis and Cancer

Throughout the book, angiogenesis is mentioned as a major factor in cancer. Angiogenesis is the process of new blood vessel growth. On the face of it, this is a completely normal and benign process, important, for instance, in organ growth and wound healing. Nonetheless, there is also a more sinister side to it. Tumors, for instance, are able to send out chemical signals which stimulate the growth of blood vessels toward the tumor. Once such a blood vessel has reached the tumor, the latter has free access to the body's blood supply with plenty of nutrients and oxygen to grow. Apart from promoting tumor growth, the newly formed vessel also serves as a means for tumor spread, since tumor cells become mobile, can enter the bloodstream and spread throughout the body ("metastasis").

Not surprisingly, there are many attempts to stop the growth and spread of tumors by denying them the lifeline of blood vessels. Within this context, vascular endothelial growth factors (VEGF) and their receptors provide prime targets for anticancer therapy, since they appear to be mostly responsible for blood vessel growth toward tumors. Here, the use of monoclonal antibodies, such as bevacizumab, appears to be a promising avenue which is currently being tested.

There is, of course, a lot more one could say about the various forms of angiogenesis and cancer. As far as this book is concerned, angiogenesis may be seen as yet another issue in signaling, this time between cells, which further "complicates" matters of cell survival, proliferation and death.

VEGF, the endothelial cells proliferate and differentiate to form a fully functional capillary to provide oxygen-rich blood to the ischemic tissue.

The stimulation of VEGF gene expression is controlled directly by HIF and, as would be expected, once new blood vessels are established, more oxygen is present, and thus HIF and VEGF are both downregulated. Thus the overall process of angiogenesis includes the following steps: (1) sensing the deficit of oxygen, (2) HIF protein accumulation, (3) transactivating the proangiogenesis genes, (4) creating new blood vessels to reestablish oxygenation, and (5) shutting down the signaling cascade after the hypoxia is eliminated. The control of the genes necessary for reoxygenation of ischemic tissues by HIF is an elegant cellular adaptive response since no other known transcription factor uses molecular oxygen as a switch to turn the system on, and then as a feedback mechanism to turn the system off when the endpoint is reached.

Other major targets of HIF are many of the enzymes of glycolysis, or anaerobic metabolism. Eukaryotic cells have the advantage of being capable of deriving energy in the form of ATP from both aerobic and anaerobic metabolism. Anaerobic metabolism uses glucose as a source of ATP by substrate level phosphorylation through glycolysis. The endpoint of glycolysis, pyruvate, is able to feed into the mitochondrial tricarboxylic acid cycle to produce reducing equivalents (i.e. NADH and FADH₂) for use as substrates by the electron transport chain in the mitochondria. Here, a proton gradient is formed to create ATP, where molecular oxygen serves as a final electron acceptor to offset the gradient and generate ATP through oxidative

Table 9.1 A partial list of genes transactivated by hypoxia inducible factor transcription factor.

HIF-1 α transactivated gene	Function
6-phosphofructo-2-kinase/fructose 2,6-bisphosphate-3 (PFKFB3)	Anaerobic glucose metabolism
Adenylate kinase-3	
Aldolase-A/C (ALDA/C)	
Carbonic anhydrase-9	
Enolase-1 (ENO1)	
Glucose transporter-1,3 (GLUT1,3)	
Glyceraldehyde phosphate dehydrogenase (GAPDH)	
Hexokinase 1,2 (HK1,2)	
Lactate dehydrogenase-A (LDHA)	
PFKFB3	
Phosphofructokinase-1 (PFKL)	
Phosphoglycerate kinase 1 (PGK1)	
Pyruvate kinase M (PKM)	
Endocrine gland-derived VEGF (EG-VEGF)	Angiogenesis
Fms-related tyrosine kinase 1 (Ftl1)	
Leptin (LEP)	
Plasminogen activator inhibitor 1 (PAI-1)	
Transforming growth factor-beta3 (TGF-beta3)	
Vascular endothelial growth factor (VEGF)	
Bcl-2,adenovirus E1B 19kD-interacting protein 3 (BNIP3)	Apoptosis
Nip3-like protein X (NIX)	
Insulin-like growth factor binding protein 1 (IGFBP-1)	Cell growth, survival
Insulin-like growth factor 2 (IGF-2)	
Transforming growth factor alpha (TGF- α)	
Ceruloplasmin	Copper metabolism
Erythropoietin (EPO)	Erythropoiesis
Collagen prolyl-4-hydroxylase	Extracellular matrix metabolism
Matrix metalloproteinases (MMPs)	
Plasminogen activator receptors and inhibitors (PAIs)	
Heme oxygenase 1	Heme metabolism
p35 α	HIF-1 α feedback
Intestinal trefoil factor	Intestinal tract protection/wound healing
Transferrin (Tf)	Iron metabolism
Transferrin receptor (TfR)	
Nitric oxide synthase (NOS2)	Nitric oxide production (Continued)

Table 9.1 (Continued)

HIF-1 α transactivated gene	Function
Erythropoietin receptor (EPO-R)	Transcription factor
Adrenomedullin (ADM)	Vascular tone
Endothelin 1 (ET1)	
α 1B-Adrenergic receptor	

phosphorylation. The system has evolved to include oxygen only in the final step of the process, and as such cells may metabolize through glycolysis for an extended period of time in the absence of oxygen. Because of this, glycolytic enzymes such as GAPDH, enolase and phosphofructokinase all contain an HRE in their promoter regions that allows them to be upregulated by HIF during hypoxic conditions [4]. This allows the cells to generate more ATP by substrate-level phosphorylation as opposed to oxidative phosphorylation. The downside to this action is that more lactic acid is produced, and if prolonged hypoxia occurs this becomes toxic and overwhelming to the cell.

Interestingly, a recent study has shown that some parts of aerobic metabolism are also under the control of HIF [18]. This study showed that certain members of the electron transport chain are also upregulated by HIF during hypoxia. The argument for this observation is that, by overexpressing members of the electron transport chain, the cells will be able to use more efficiently the little oxygen that may be present to produce ATP by oxidative phosphorylation, and thus reduce the toxic lactic acid build-up seen in pure anaerobic metabolism. This finding opens a door into the potential of HIF transcriptional activation, and with more research many more unexpected targets may be uncovered.

The final topic of HIF transcriptional regulation to be discussed here is the transcriptional regulation of HIF itself. As mentioned previously, both the α - and β -subunits of HIF are constitutively transcribed and translated, but it is the post-translational modification of HIF-1 α that ultimately decides the fate of the transcription factor. It is now known that HIF-1 α can transactivate the prolyl hydroxylase enzymes that control its own degradation. A recent study showed that the PHD3 gene contains an HRE and that PHD3 mRNA expression was upregulated by HIF during hypoxic conditions, while PHD1 and 2 did not respond similarly [19]. Interestingly, it was shown that PHD2 was also upregulated by hypoxia, but in an HIF-independent manner, adding even more complexity to the regulation of these genes.

Another study has displayed an even more upstream level of control for hypoxia-induced gene regulation [20]. Two other ubiquitin ligases, known as Stah1 and 2, have been shown to polyubiquitinate the PHD enzymes during times of hypoxia. The Stah enzymes have also been shown to be upregulated during times of hypoxia, but the mechanism for this has not been fully elucidated. It would appear counterproductive to have the PHD enzymes transcriptionally activated during times of hypoxia, but then degraded by other enzymes at the same time. This is not yet fully understood, but this change in enzyme levels may provide the cells with a graded response to oxygen as opposed to the commonly accepted "ON/OFF" switch of hypoxia-induced

Box 9.3: The Various Forms of RNA and their Roles in Biology – and Research

Our view of the importance and diversity of RNA classes has changed tremendously from the simple system of categorizing RNA into three main groups – mRNA, tRNA and rRNA – that was used and taught until quite recently. Several new types of RNA have been discovered and it is now clear that RNAs are characterized by greater structural and functional diversity and play more diverse roles in cellular processes than previously assumed. Now we can classify RNAs into three major groups: (1) coding RNA or messenger RNAs; (2) stable or non-protein-coding RNAs, such as ribosomal RNA, transfer RNA, small nuclear RNA and small nucleolar RNA; and (3) very small RNA species, such as micro RNA and small heterochromatic RNA.

Messenger RNAs (mRNA) are transcribed from protein-coding genes and build the transcriptome of the cell. mRNAs are characterized by short half-lives (of a few hours), and consequently, a rapid turnover in the cells. This rapid mRNA turnover allows the cell to rapidly change the range of proteins expressed at any one time.

Ribosomal RNAs (rRNAs), as their name indicates, are components of the ribosomes. Transfer RNAs (tRNAs) are responsible for transporting amino acids to the ribosomes for protein synthesis during the process of translation. Small nuclear RNAs (snRNAs) play a role in mRNA processing. Significant advances have been made in the understanding of mRNA processing and splicing in the last few years. Small nuclear RNAs, for example, U1, U2, U3, U4, U5 and U6 cooperate with proteins and form small nuclear ribonucleoprotein complexes, which direct intron splicing. Small nucleolar RNAs (snoRNAs) have a role in rRNA processing. Pre-tRNA undergoes extensive modifications, for example, methylation and conversion of uridine bases to pseudouridine. SnoRNAs are crucial in this modification process. Interestingly, snoRNA coding sequences are often located within the introns of other genes.

The recent discovery of RNA interference has undoubtedly transformed biomedical research and has opened up a new area in RNA-related research. The term very small RNA species refers to RNA molecules of approximately 18–25 bases in length. RNA interference is an evolutionarily conserved mechanism of gene silencing, in the process of which short double-stranded RNA species target and silence homologous mRNAs. Custom-made small interfering double-stranded RNAs (siRNAs) are now extensively used in the technique of *in vitro* RNA interference for the specific silencing of genes. RNA interference thus reduces the need for the use of transgenic animals, as specific gene knock-down can be achieved.

Certain RNA molecules, termed ribozymes, are catalytic and function as enzymes in the absence of proteins and play important roles in crucial cellular processes. Among the various examples of ribozymes are the group I self-splicing introns, such as eukaryotic pre-tRNA introns.

gene regulation. In any event, the field of HIF gene regulation is growing exponentially and in time more of these mechanisms will become fully understood.

9.4

Non-Hypoxia-Induced Activation of Hypoxia Inducible Factor

Although genuine hypoxia is the most commonly considered mechanism involved in the activation of HIF signaling, other non-hypoxia-mediated signaling processes also utilize the HIF transcription factor pathway (Figure 9.4). As discussed, the mechanism of HIF-1 α activation by PHD proteins requires more than just a deficit of molecular oxygen, thus allowing for additional levels of control. These enzymes also require iron, α -ketoglutarate and ascorbate to bring about HIF-1 α hydroxylation, leading to its degradation or lack of transactivating potential. Since iron, α -ketoglutarate and ascorbate are closely related to the production of reactive oxygen species (ROS), including free radicals, it has also been suggested that free radicals or ROS may play a role in the activation of HIF-1 α . The important role for the PHD proteins as sensors of oxygen, and the fact that oxygen is the ultimate source of ROS, taken together with existing observations, strongly suggest that free radicals and/or ROS play a role in the activation of HIF-1 α by non-hypoxic means.

Numerous studies have demonstrated that ROS such as H_2O_2 can stabilize HIF-1 α even in the presence of high oxygen tension [21–23]. In addition, the use of antioxidants that neutralize ROS have been shown to decrease the amount of functional HIF-1 α in an array of different cell types [24–26]. The mechanism behind this phenomenon has yet to be fully elucidated, and different ROS demonstrate specific patterns of regulation upon HIF-1 α at diverse concentrations. For example,

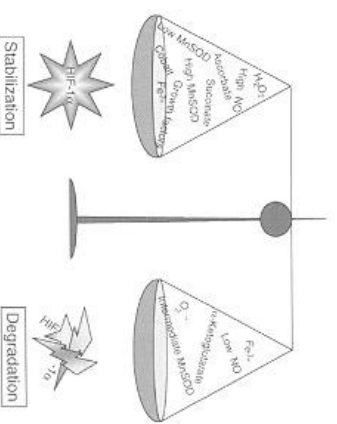


Figure 9.4 An intricate balance is required for the nonhypoxic control of HIF-1 α activity. A number of factors other than molecular oxygen are known to regulate HIF-1 α stability. The list continues to expand and undoubtedly numerous other constituents will be identified that participate in the positive and negative control of this transcription factor.

conflicting results with nitric oxide (NO) have been obtained, in terms of its ability to stabilize or destabilize HIF-1 α . Mateo *et al.* demonstrated that the reason for this discrepancy is due to the concentrations of NO that were sampled [27]. In their study [27], they show that NO displays a biphasic effect on the stabilization of HIF-1 α . HIF-1 α is shown to be activated at high concentrations (500–1000 μM) of NO , but at low ranges (<400 nM) HIF-1 α appears to be degraded and thus inactive. Numerous mechanisms have been hypothesized for this observation, including NO inactivation of the prolyl hydroxylases, NO sequestration of molecular oxygen, and the formation of peroxynitrite ($ONOO^-$). Though all of these hypotheses show potential to explain the observed results, none of them fully explains the phenomenon demonstrated by the biphasic nature of NO .

Another key example of ROS-mediated HIF-1 α regulation is by way of superoxide ($O_2^{\cdot -}$). In an important study to illustrate the effects of free radicals on HIF-1 α activity, Wang *et al.* used the $O_2^{\cdot -}$ scavenging enzyme manganese superoxide dismutase (Mn-SOD) to examine the effect of this specific ROS on HIF-1 α [28]. Their study showed that at low (<2 fold) and high activities (6- to 20-fold) of Mn-SOD (i.e. high and low concentrations of $O_2^{\cdot -}$, respectively), HIF-1 α was stabilized in response to hypoxia, though at intermediate levels of Mn-SOD (2- to 6-fold increase), HIF-1 α was inactivated.

Although in this study $O_2^{\cdot -}$ appears to be the main focus, the products of the Mn-SOD reaction may also play a major role in the regulation of HIF-1 α . Mn-SOD catalyzes the reaction $2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$. The authors proposed that both the production of H_2O_2 as well as O_2 could play a role in the biphasic nature of HIF-1 α stabilization by Mn-SOD activity. Since O_2 is a critical cofactor driving the oxygen-sensing PHD proteins, this hypothesis could not be ruled out. Overall, the mechanism of this control has also yet to be fully understood.

As mentioned previously, the prolyl hydroxylases use iron in its ferrous state at the center of their active site. This makes iron a quintessential component for the proper regulation of HIF-1 α as well. The requirement for iron was demonstrated in an early study using desferrioxamine (an iron-chelating agent) [29]. Use of this chelating agent activated HIF-1 α even in the presence of oxygen, thus mimicking the effect of hypoxia. It was hypothesized that this effect was due to the sequestration of iron from the prolyl hydroxylases. Iron was further shown to be essential in HIF-1 α regulation by the use of cobalt chloride [30]. This metal is assumed to replace iron in the active site of the PHD enzymes, rendering them inactive, and, like desferrioxamine, activates HIF-1 α in a non-hypoxic manner. Another mechanism for cobalt chloride has also been proposed that entails the reduction of intracellular ascorbate pools, thus preventing the reduction of the ferric iron center back to its active ferrous state [31]. Furthermore, ROS stabilization of HIF-1 α has been theorized to inactivate the iron in the PHD enzymes by oxidizing it from the ferrous to the ferric state [28]. This may also explain why the use of certain antioxidants, such as N-acetyl cysteine and ascorbate itself, acts to diminish HIF-1 α activity due to their ability to reduce the iron in the PHD enzymes back to its functioning ferrous state.

Another major cofactor in the hydroxylation reaction of HIF-1 α is α -ketoglutarate. During the reaction, α -ketoglutarate is converted to succinate, both of which are key components of the Krebs cycle, while the latter also plays a role in the electron

transport chain. There may be a role for a critical balance or ratio of these two compounds to be maintained within the cell [32]. When succinate is in high abundance, there is a negative product feedback on the PHD enzymes, allowing HIF-1 α to remain functional. This scenario potentially could happen in the event that the cells become hypoxic. For example, if molecular oxygen is not available to serve as the final acceptor of the electron transport chain, then this pathway of aerobic metabolism is halted. Therefore, succinate would not be properly converted to fumarate by succinate dehydrogenase, and thus succinate would build up in the cell. This accumulation of succinate would offset the homeostatic ratio in a normoxic cell, and would thus be expected to feedback, that is, to inhibit the PHD enzymes due to abundance of product and lack of substrate.

In this manner, the cell has devised a secondary method of sensing oxygen deprivation by product inhibition of the PHD regulatory enzymes. Perhaps not coincidentally, it has been well established that mutations in three out of the four subunits of complex I of the electron transport chain (succinate dehydrogenase (SDH)) can lead to certain types of cancer [33–36]. Though these mutations cause electron leakage and thus ROS production, the build-up of succinate and thus downstream activation of HIF-1 α may also play a role in the transformation of these cells to a malignant phenotype. HIF-1 α and its role in cancer will be discussed in a later section.

Lastly, certain elements that do not directly play a role in the hydroxylation reaction also have an effect on the stabilization of HIF-1 α . A landmark discovery occurred when insulin was found to have a similar stabilization effect on HIF-1 α compared with hypoxia [37]. Zelzer *et al.* [37] demonstrated that numerous target genes of HIF-1 α were upregulated by the addition of insulin. Furthermore the HIF-1 α subunit was posttranslationally stabilized. Though the effect of insulin appears to be very well established, the mechanism behind its action still remains elusive. Another factor that regulates HIF-1 α is epidermal growth factor (EGF). Zhong *et al.* [38] found that EGF has the ability to stabilize HIF-1 α by signaling through the PI3K/PTEN/AKT/FRA pathway and the authors hypothesized that HIF-1 α may be directly phosphorylated, adding yet another level of complexity to the regulation of this transcription factor [38]. Finally, Richard *et al.* showed that angiotensin II, thrombin and PDGF (platelet-derived growth factor) all stabilized HIF [23]. The mechanism behind the last two factors is still unclear, but angiotensin II appears to activate NADPH oxidase, thus creating ROS. The authors showed through the use of antioxidant enzymes that these ROS were essential for the angiotensin stabilization of HIF-1 α .

Taken together, these demonstrations that HIF-1 α can be affected through free radical mechanisms open the field to a whole new route by which these other hormones and signaling factors activate this important transcription factor.

9.5 Diseases Involving Hypoxia Inducible Factor

Cancer is a major cause of death [1], and has been an intense focus of biomedical research in the latter part of the last century. Cancer has been shown to have

numerous causes, and many different cellular pathways may be deficient or mutated in a way that alters the cell cycle and the phenotypic properties of cells as they become increasingly aggressive. In many cancers, HIF-1 α is overexpressed and constitutively active, leading to the idea that it may play a role in tumorigenesis. When the role HIF-1 α plays in cancer is elucidated, it may be found that this transcription factor could be a prime target for cancer therapy.

Most solid tumors originate from one cell that becomes transformed to become tumorigenic. As the cells divide, the neoplastic growth reaches a critical volume such that free oxygen cannot diffuse far enough to reach the cells of the inner mass and they become hypoxic. This critical volume is quite small, only roughly the size of a pinhead, but it is well known that tumors can far surpass this size, thus cancer is able to evade imminent death by hypoxia by the use of HIF-1 α and the induction of blood vessels to the tumor. By producing proangiogenic factors such as VEGF that are crucial in angiogenesis, a tumor provides itself with a means to acquire oxygen and nutrients from the blood. In addition, the accumulation of blood vessels provides a means of systemic dissemination of tumor cells, also known as metastasis. Interestingly, some breast, prostate and renal carcinomas have an abundance of active HIF-1 α even in normoxic conditions [5]. Most renal carcinomas display constitutively activated HIF-1 α due to a mutated VHL protein, allowing for continued HIF-1 α stabilization without degradation. This does not account for the abundance of HIF-1 α in breast or prostate cancer since VHL is usually found in its wild-type form in these cancers.

Some studies have linked HIF-1 α expression and stabilization to hormonal regulation as well. For example, Kazi *et al.* showed that estrogen could induce the stabilization of HIF-1 α in breast cancer [39], while Kimbro *et al.* showed that certain androgens could perform a similar action in prostate [5]. But how do hormones regulate the prolyl and asparaginyl hydroxylase activity in normoxic conditions, or regulate the degradation of HIF-1 α by VHL? These studies are very recent and their findings are not fully understood, but more effort is being put into understanding the role of these hormones in HIF-1 α regulation.

Another interesting debate in the field of hypoxia is the link between HIF-1 α and p53 [40]. p53 is a protein that plays a major role in mediating cellular apoptosis as well as controlling the cell cycle. It is well established that, during periods of severe anoxia, p53 is activated and ultimately triggers cellular apoptosis. How do p53 and HIF-1 α interact to either promote cellular viability in hypoxia versus cellular death? Current thinking proposes a model that explains the issue on the basis of the extent of hypoxia (Figure 9.5). In this depiction, HIF-1 α is only active during transient times of hypoxia and with prolonged insult p53 becomes the dominant regulator of cell fate. As hypoxia continues, p53 begins to compete with HIF-1 α for the coactivators of transcription and thus the effective amount of active HIF-1 α begins to decline as p53 activity becomes dominant. To date, there is no evidence that HIF-1 α upregulates or stabilizes p53 directly, but some new evidence demonstrates that HIF-1 α may play a role in the downregulation of mouse double minute 2 (MDM2), a protein that degrades p53. Eventually, HIF-1 α becomes completely inactive and p53 initiates cellular apoptosis. This concept is particularly important in the field of cancer because p53 is mutated in the majority of human cancers. With the lack of competition by p53

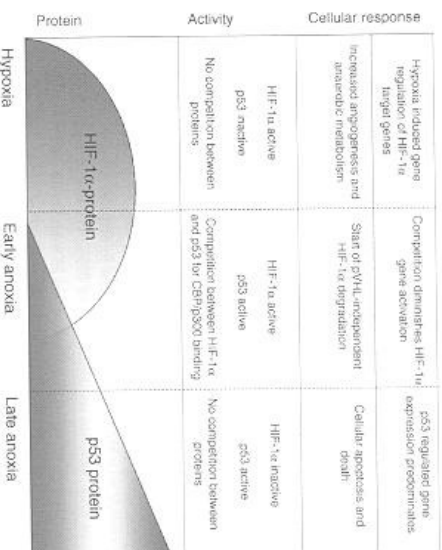


Figure 9.5 HIF-1 α and p53 interact in the cellular response to hypoxia. The current thinking is that during transient times of hypoxia, HIF-1 α transcriptional activation dominates. With prolonged periods of lowered oxygen, p53 becomes upregulated and competes for transcription coactivators. Eventually, p53 outcompetes HIF-1 α , and in addition HIF-1 α is degraded. This leads to p53-mediated apoptosis and cell death.

in cancer cells. HIF-1 α might be allowed to function for a longer period of time, if not indefinitely, providing the tumor with the ability to grow during hypoxia as opposed to dying by apoptosis, as would normally occur if wild-type p53 was present. This finding once again demonstrates the potential value that targeting HIF-1 α may have in the area of cancer therapy.

Another disease that involves the HIF-1 α regulatory pathway is von Hippel-Lindau syndrome. This rare autosomal disease affects approximately 1 in 35,000 humans and manifests itself in numerous ways; patients may develop any combination of the following: renal carcinomas, pheochromocytomas, hemangioblastomas in the central nervous system, pancreatic and renal cysts, as well as angiomatosis in numerous organs including the eye [41]. The disease is caused by a deletion in the long arm of chromosome 3, which includes the gene that encodes for the VHL protein. It is presumed that many of the manifestations of the disease are due to aberrant HIF-1 α activation. This does not fully explain the disease due to the fact that overexpression of HIF-1 α alone in cell models has not been shown to be tumorigenic. In addition, deletion of VHL alone would eliminate the degradation of HIF-1 α , but would not affect the secondary control by FHL-1 (or HIF-1) which is encoded on human chromosome 10. Once again, this disease demonstrates that our understanding of HIF-1 α regulation is by no means complete.

Finally, ischemia is a pathological state that involves hypoxia-induced gene regulation [42]. Ischemia is broadly defined as a reduced amount of blood flow to a specific tissue that ultimately may lead to hypoxia or anoxia. Ischemic events such as myocardial infarction (heart attacks) or cerebrovascular accidents (strokes) are leading causes of death. Studies have demonstrated that in numerous tissues HIF-1 α and VEGF are vastly increased, very rapidly, during times of transient ischemia. Moreover, prolonged periods of ischemia lead to cell death by apoptosis, which supports the idea of an interaction between HIF-1 α and p53 as discussed previously. It is this loss of cells that causes dysfunction of an organ (e.g. brain damage). Because of this observation, research has been targeted to find ways of stabilizing HIF-1 α in tissues so as to prolong cellular viability before death. This could prove very useful in patients with specific clotting disorders that predispose them to numerous transient ischemic attacks in various organs over their lifetime.

9.6 The Promise of Hypoxia Inducible Factor-Targeted Therapies

As discussed previously, HIF-1 α plays a major role during cancer development. HIF-1 α has been shown to be overexpressed in numerous forms of solid tumors, and thus has become a prognostic indicator of poor survival [5]. This active transcription factor enables these tumors to survive under hypoxic environmental conditions, and recruits vasculature which provides a route of emigration for malignant cells from the primary tumor to distant sites of metastasis. Interestingly, the HIF-1 α overexpression noted in the solid tumors does not appear to hold true in the surrounding normal tissue, which argues that HIF-1 α may be an excellent target for cancer therapy.

In vitro studies using siRNA have demonstrated that knocking down HIF-1 α serves to increase treatment efficacy in different cancer models [43]. However, in its current state of development, siRNA is not ready for use in clinical practice, and as such a more practical approach of developing a pharmaceutical inhibitor of HIF-1 α has been proposed [44]. Table 9.2 displays a large variety of drugs that have been shown to decrease HIF-1 α activity, but it should be noted that only one was developed specifically to inhibit HIF-1 α . This area of HIF-1 α research is very much in its infancy, and the potential for rational drug design of pharmaceuticals specific for HIF-1 α is vast. The specific inhibitor of HIF-1 α is known as PX-478, and acts to decrease active amounts of HIF-1 α by increasing its degradation. It has been shown that PX-478 increases the ubiquitination of HIF-1 α , but the mechanism behind this has not yet been elucidated.

Another class of HIF-1 α inhibitors worthy of mention is the topoisomerase inhibitors. The primary mechanism of these drugs is to cause DNA damage to elicit their cytotoxic effects, but recently they have been shown to have a secondary function in reducing the amount of HIF-1 α protein accumulation [44]. Once again, this mechanism is not fully understood, but due to the common use of these drugs a new clinical trial on the efficacy of decreasing HIF-1 α in tumor models might be expected within the next few years.

Table 9.2 A current list of pharmaceutical agents shown to reduce HIF-1 α activity

Pharmaceutical agent	Standard use	Mechanism of HIF-1 α downregulation
2-Methoxyestradiol	Antimicrotubule	Translational
Taxol		
FK228	Histone deacetylase inhibitor	Transcriptional
Geldanamycin	Hsp-90 inhibitor	Direct HIF-1 α breakdown
Radicalol	Hsp-90 inhibitor	Inhibition of DNA binding
PD98 059	MEK1 inhibitor	Transactivational
Carboxamido-triazole (Ca ²⁺ blocker)	Other	Transcriptional
Diphenylene iodonium	Other	Redox inhibition
PX-478	Other	Direct HIF-1 α breakdown
UCNO-1 (ser/thr kinase inhibitor)	Other	Kinase inhibition
Rapamycin	p1-3-kinase pathway inhibitor	Translational
YC-1	Soluble guanylyl cyclase stimulator	Transcriptional
GL331 (topo-1) Topotecan (topo-1)	Topoisomerase inhibitor Topoisomerase inhibitor	Transcriptional Translational

Note that the majority of compounds have been developed for other purposes, but coincidentally display anti-HIF-1 α properties. In addition, many of these drugs have been tested *in vitro* and in an *in vivo* model, which may limit the list to only a few drugs of actual potential in a clinical setting. The topoisomerase inhibitors as well as PX-478 have shown the greatest potential, and appear to be entering clinical trials soon. Adapted from Powis and Kirkpatrick [46].

In cancer it would appear to be beneficial to knock down HIF-1 α . Conversely, however, in other major conditions such as brain or cardiac ischemia it appears that cells would benefit from increased HIF-1 α activity. In addition, patients who have undergone chemotherapy for cancer usually have severe forms of anemia. Increasing the amount of HIF-1 α would upregulate the amount of erythropoietin in these patients, thus reducing recovery time. Most of modern medicine and molecular pharmacology focuses on the inhibition of a particular protein, and as such increasing the activity of HIF-1 α directly provides an additional challenge. Therefore the focus in the future may be targeted to blocking the VHL or prolyl hydroxylase enzymes in patients susceptible to ischemic attacks. This would allow for increased HIF-1 α activity, and potentially increased angiogenesis, reducing the odds of having a hypoxic event.

Unfortunately, targeting of this pathway appears to be a double-edged sword. On one hand, blocking HIF-1 α appears to aid in the cancer therapy but renders patients

more susceptible to cancer. On the other hand, increasing HIF-1 α activity may reduce the chance of a myocardial infarction or cerebrovascular accident, but could predispose these patients to cancer. Currently, there is no clear-cut answer to this issue, but most likely the final determinant will be to aid the disease that poses the greatest imminent threat to the patient, even if another risk may be at hand.

There may be a role as well for other members of the hypoxia-induced gene regulatory family. In von Hippel-Lindau disease, patients lack the crucial VHL protein due to a deletion or mutation of chromosome 3. These patients could serve as prime candidates for delivery of wild-type VHL DNA by gene therapy. Though there have been setbacks in the field of gene therapy, using a delivery vehicle such as a nonreplicative adenovirus into the tumor may greatly improve regression of these tumors. In other cancer patients, the development of small inhibitors toward the Shp proteins may demonstrate a beneficial effect. These enzymes promote the degradation of the PHD enzymes, which ultimately degrade HIF-1 α . If inhibition is achieved at the level of the Shp proteins, then PHD will remain active and able to promote degradation of HIF-1 α .

An entirely new area of study has begun on HIF-1 α and its regulatory elements. In 1998, Wenger *et al.* demonstrated that HIF binding could be abrogated by the methylation of the CpG within the HRE on erythropoietin [45]. With this discovery, the idea that these genes may be under epigenetic control has opened another area of new possibilities on how these proteins are controlled. Understanding that the HRE that HIF-1 α recognizes contains a CpG allows the cells another method of potentially controlling genes that HIF-1 α transactivates. This area is also very much in its infancy, and it is hoped that further studies will unveil another level of understanding of this pathway.

Finally, there may be connections to HIF-1 α in diseases other than those discussed previously. When referring to Table 9.1, it is obvious that HIF-1 α maintains an abundance of enzymes that are crucial for cellular homeostasis. The first gene of interest is known as ceruloplasmin. This protein is essential in maintaining proper copper levels within the blood. Copper in excess is highly toxic, and in the absence of this protein patients develop what is known as Wilson's disease. The cause of this disease is the aberrant expression of ceruloplasmin, which ultimately leads to improper copper balance. A role for HIF-1 α does not appear to be established in this disease, but due to its control over ceruloplasmin it may be essential. Another important gene on the list is heme oxygenase. This enzyme is the first step in the pathway of heme degradation. If heme is not properly degraded, it may cause toxic effects and differing types of jaundice. There may potentially be a role for the HIF-1 α activation of heme oxygenase in certain patients with altered heme metabolism. One of the most important proteins on the list is the well-characterized erythropoietin. Millions of people worldwide are anemic, and in many individuals the reason is not fully understood. HIF-1 α has been established to play a major role in the control of EPO, and could also serve as a potential anti-anemic treatment in the future.

Overall, there appear to be many avenues that have not yet been traversed in hypoxia-induced gene regulation, but this field appears to show great promise in understanding the mechanisms of disease as well as treating them.

9.7

Conclusions

Hypoxia inducible factor was discovered only about 15 years ago. Slow but steady breakthroughs have been made that elucidate more facts about its mechanism, as well as the genes that HIF-1 α controls. The role for HIF-1 α in cancer appears to be very important, and much research has gone into the development of specific inhibitors of this genetic pathway. In addition, numerous other disease states have been shown to involve HIF-1 α in some manner and will require much attention in the future to establish the full role this transcriptional activator may play. In some aspects, our knowledge of hypoxia-induced gene regulation is still in its formative years, and will provide a whole new generation of investigators with new questions, experiments and clinical trials for many years to come.

References

- Heron, M.P. and Smith, B.L. (2007) *National Viol Statistics Reports*, 55 (10). National Center for Health Statistics, Hyattsville, Maryland, pp. 1-92.
- Semeranza, G.L. and Wang, G.L. (1992) *Molecular and Cellular Biology*, 12, 5447-5454.
- Wang, G.L. and Semeranza, G.L. (1993) *Proceedings of the National Academy of Sciences of the United States of America*, 90, 4304-4308.
- Wengert, R.L. (2003) *FASEB Journal*, 16, 1151-1162.
- Kimbro, K.S. and Simons, J.W. (2006) *Endocrine-Related Cancer*, 13, 739-749.
- Wang, G.L., Jiang, B.H., Rue, E.A. and Semeranza, G.L. (1995) *Proceedings of the National Academy of Sciences of the United States of America*, 92, 5910-5914.
- Wood, S.M., Gleadle, J.M., Pugh, C.W., Hankinson, O. and Ratcliffe, P.J. (1996) *The Journal of Biological Chemistry*, 271, 15117-15123.
- Bruck, R.K. (2003) *Cancer and Development*, 17, 2614-2623.
- Maxwell, P.H., Wiesener, M.S., Chang, C.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R. and Ratcliffe, P.J. (1999) *Nature*, 399, 271-275.
- Min, J.H., Yang, H., Ivan, M., Gentile, F., Kadiri, W.G., Jr and Pevsner, N.P. (2002) *Science*, 296, 1886-1889.
- Hutton, J.L., Jr, Tappet, A.L. and Udenfriend, S. (1966) *Biocchemical and Biophysical Research Communications*, 24, 179-184.
- Prockop, D.J. and Irujo, K. (1965) *Proceedings of the National Academy of Sciences of the United States of America*, 53, 661-668.
- Bruck, R.K. and McKnight, S.L. (2001) *Science*, 294, 1337-1340.
- Masson, N., William, C., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) *The EMBO Journal*, 20, 5197-5206.
- Appelhoff, R.J., Tian, Y.M., Raval, R.R., Turley, H., Harris, A.L., Pugh, C.W., Ratcliffe, P.J. and Gleadle, J.M. (2004) *The Journal of Biological Chemistry*, 279, 38458-38465.
- Masson, N. and Ratcliffe, P.J. (2003) *Journal of Cell Science*, 116, 3041-3049.
- Richard, D.E., Berra, E. and Pouyssegur, J. (2000) *The Journal of Biological Chemistry*, 275, 26765-26771.
- Duyndam, M.C., Hulsebos, T.M., Fontijn, D., Pinedo, H.M. and Boven, E. (2001) *The Journal of Biological Chemistry*, 276, 48066-48076.
- Jeikmann, W., Pagel, H., Hellwig, T. and Handberg, J. (1997) *Kidney International*, 51, 497-501.
- Park, J.H., Kim, T.Y., Jong, H.S., Kim, T.Y., Chun, Y.S., Park, J.W., Lee, C.T., Jung, H.C., Kim, N.K. and Baug, Y.I. (2003) *Clinical Cancer Research*, 9, 433-440.
- Mateo, J., Garcia-Lecea, M., Cadenas, S., Hernandez, C. and Morcada, S. (2003) *The Biochemical Journal*, 376, 537-544.
- Wang, M., Kirk, J.S., Venkataratnam, S., Donnan, F.E., Zhang, H.J., Schaler, F.Q., Finnigan, S.W., Weydert, C.J., Spitz, D.R., Brucher, G.R. and Oeverly, L.W. (2003) *Oncogene*, 24, 8154-8166.
- Wang, G.L. and Semeranza, G.L. (1993) *Blood*, 82, 3610-3615.
- Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzzen, E., Wilson, M.L., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Batesoul, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. and Ratcliffe, P.J. (2001) *Cell*, 107, 43-54.
- Sahinkov, K., Donald, S.P., Bruck, R.K., Zhukovitch, A., Phang, J.M. and Kasprzak, K.S. (2004) *The Journal of Biological Chemistry*, 279, 40337-40344.
- Selak, M.A., Duran, R.V. and Corbilly, E. (2006) *Biochimica et Biophysica Acta*, 1757, 567-572.
- Asanti, D., Janif, F., Daliol, A., Dahl, P.L., Douglas, F., George, E., Skoldberg, F., Husebye, E.S., Eng, C. and Maher, E.R. (2001) *American Journal of Human Genetics*, 69, 49-54.
- Bayraktar, B.E., Ferrell, R.E., Willett, Brozick, J.E., Lawrence, E.C., Myssiorok, D., Bosch, A., van der Molen, A., Taschner, P.E., Rubinstein, W.S., Myers, E.N., Richard, C.W., 3rd, Cornelisse, C.J., Deville, P. and Devlin, B. (2000) *Science*, 287, 848-851.
- Niemann, S. and Muller, U. (2000) *Nature Genetics*, 26, 268-270.
- Slane, B.G., Aykin-Burns, N., Smith, B.J., Kalen, A.L., Goswami, P.C., Donnan, F.E. and Spitz, D.R. (2006) *Cancer Research*, 66, 7615-7620.
- Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M. and Cohen, B. (1998) *The EMBO Journal*, 17, 5085-5094.
- Zhong, H., Chiles, K., Faldut, D., Langner, E., Hamada, C., Georgescu, M.M., Simons, J.W. and Semeranza, G.L. (2000) *Cancer Research*, 60, 1541-1545.
- Kazi, A.A. and Koon, R.D. (2007) *Endocrinology*, 148, 2363-2374.
- Schmid, T., Zhou, J. and Brune, B. (2004) *Journal of Cellular and Molecular Medicine*, 8, 423-431.
- Safran, M. and Kadiri, W.G., Jr (2003) *The Journal of Clinical Investigation*, 111, 779-783.
- Semeranza, G.L. (2000) *The Journal of Clinical Investigation*, 106, 809-812.
- Sun, X., Kanwar, J.R., Leung, E., Lehnert, K., Wang, D. and Kirsanov, C.W. (2001) *Gene Therapy*, 8, 638-645.
- Garber, K. (2005) *Journal of the National Cancer Institute*, 97, 1112-1114.
- Wengert, R.L., Kretschke, L., Rolfs, A., Camenisch, G. and Gassmann, M. (1998) *European Journal of Biochemistry*, 253, 771-777.
- Powis, G. and Kirkpatrick, L. (2004) *Molecular Cancer Therapeutics*, 3, 647-654.
- Richard, D.E., Berra, E. and Pouyssegur, J. (2000) *The Journal of Biological Chemistry*, 275, 26765-26771.
- Duyndam, M.C., Hulsebos, T.M., Fontijn, D., Pinedo, H.M. and Boven, E. (2001) *The Journal of Biological Chemistry*, 276, 48066-48076.
- Jeikmann, W., Pagel, H., Hellwig, T. and Handberg, J. (1997) *Kidney International*, 51, 497-501.
- Park, J.H., Kim, T.Y., Jong, H.S., Kim, T.Y., Chun, Y.S., Park, J.W., Lee, C.T., Jung, H.C., Kim, N.K. and Baug, Y.I. (2003) *Clinical Cancer Research*, 9, 433-440.
- Mateo, J., Garcia-Lecea, M., Cadenas, S., Hernandez, C. and Morcada, S. (2003) *The Biochemical Journal*, 376, 537-544.
- Wang, M., Kirk, J.S., Venkataratnam, S., Donnan, F.E., Zhang, H.J., Schaler, F.Q., Finnigan, S.W., Weydert, C.J., Spitz, D.R., Brucher, G.R. and Oeverly, L.W. (2003) *Oncogene*, 24, 8154-8166.
- Wang, G.L. and Semeranza, G.L. (1993) *Blood*, 82, 3610-3615.
- Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzzen, E., Wilson, M.L., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Batesoul, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. and Ratcliffe, P.J. (2001) *Cell*, 107, 43-54.
- Sahinkov, K., Donald, S.P., Bruck, R.K., Zhukovitch, A., Phang, J.M. and Kasprzak, K.S. (2004) *The Journal of Biological Chemistry*, 279, 40337-40344.
- Selak, M.A., Duran, R.V. and Corbilly, E. (2006) *Biochimica et Biophysica Acta*, 1757, 567-572.
- Asanti, D., Janif, F., Daliol, A., Dahl, P.L., Douglas, F., George, E., Skoldberg, F., Husebye, E.S., Eng, C. and Maher, E.R. (2001) *American Journal of Human Genetics*, 69, 49-54.
- Bayraktar, B.E., Ferrell, R.E., Willett, Brozick, J.E., Lawrence, E.C., Myssiorok, D., Bosch, A., van der Molen, A., Taschner, P.E., Rubinstein, W.S., Myers, E.N., Richard, C.W., 3rd, Cornelisse, C.J., Deville, P. and Devlin, B. (2000) *Science*, 287, 848-851.
- Niemann, S. and Muller, U. (2000) *Nature Genetics*, 26, 268-270.
- Slane, B.G., Aykin-Burns, N., Smith, B.J., Kalen, A.L., Goswami, P.C., Donnan, F.E. and Spitz, D.R. (2006) *Cancer Research*, 66, 7615-7620.
- Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M. and Cohen, B. (1998) *The EMBO Journal*, 17, 5085-5094.
- Zhong, H., Chiles, K., Faldut, D., Langner, E., Hamada, C., Georgescu, M.M., Simons, J.W. and Semeranza, G.L. (2000) *Cancer Research*, 60, 1541-1545.
- Kazi, A.A. and Koon, R.D. (2007) *Endocrinology*, 148, 2363-2374.
- Schmid, T., Zhou, J. and Brune, B. (2004) *Journal of Cellular and Molecular Medicine*, 8, 423-431.
- Safran, M. and Kadiri, W.G., Jr (2003) *The Journal of Clinical Investigation*, 111, 779-783.
- Semeranza, G.L. (2000) *The Journal of Clinical Investigation*, 106, 809-812.
- Sun, X., Kanwar, J.R., Leung, E., Lehnert, K., Wang, D. and Kirsanov, C.W. (2001) *Gene Therapy*, 8, 638-645.
- Garber, K. (2005) *Journal of the National Cancer Institute*, 97, 1112-1114.
- Wengert, R.L., Kretschke, L., Rolfs, A., Camenisch, G. and Gassmann, M. (1998) *European Journal of Biochemistry*, 253, 771-777.
- Powis, G. and Kirkpatrick, L. (2004) *Molecular Cancer Therapeutics*, 3, 647-654.