DIFFERENTIAL FUNCTION AND REGULATION OF THE HYPOXIA INDUCIBLE FACTORS IN THE RAT PHEOCHROMOCYTOMA CELL LINE PC12

Submitted for the degree of Doctor of Philosophy

Anthony Olindo Fedele, BBiotech (Hons)

School of Molecular and Biomedical Sciences (Biochemistry), University of Adelaide

December 2004

ADDENDA

1. On page 5, the following two sentences "The regulation of HIF-1α and HIF-2α mRNA levels by hypoxia does not appear to play a major role in their function (Wang *et al.*, 1995b; Kallio *et al.*, 1997; Wiesener *et al.*, 1998). Rather, their levels are chiefly controlled posttranslationally." should be replaced with "There is some controversy with regards to the hypoxic inducibility of HIF-1α and HIF-2α mRNA. There are a limited number of cases where a reduction in oxygen levels does indeed result in an increase in HIF-α transcript. For instance, hypoxia has been shown to cause an increase in HIF-2α mRNA in human neuroblastoma (Jögi *et al.*, 2004) and (albeit moderately) in the liver of rats (Wiesener *et al.*, 2003). However, these cases appear to be exceptions. In the majority of analyses in a range of tissues and cell types, no significant changes are observed in HIF-1α and HIF-2α mRNA levels in response to changes in oxygen tension (Wang *et al.*, 1995b; Kallio *et al.*, 1997; Wiesener *et al.*, 1998). This is particularly evident in the study of whole rats where HIF-2α mRNA was shown to be moderately increased by hypoxia in the liver, but there was no obvious change in HIF-2α mRNA levels in any of the other organs tested (kidney, heart, brain, lung and duodenum) (Wiesener *et al.*, 2003). In short, it appears that HIF-α levels are predominantly controlled posttranslationally."

2. On page 177, insert the following reference "Jögi, A., Vallon-Christersson, J., Holmquist, L., Axelson, H., Borg, Å., and Påhlman, S. (2004). Human neuroblastoma cells exposed to hypoxia: induction of genes associated with growth, survival and aggressive behaviour. Exp. Cell Res. *295*: 469-487."

3. On page 187, insert the following reference "Wiesener, M.S., Jürgensen, J.S., Rosenberger, C., Scholze, C., Hörstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W., Ratcliffe, P.J., Bachmann, S., Maxwell, P.H. and Eckardt, U. (2003). Widespread hypoxia inducible expression of HIF-2α in distinct cell populations of different organs. FASEB J. *17*: 271-273."

TABLE OF CONTENTS

THESIS SUMMARY

Responses to hypoxia in mammals include enhanced erythropoiesis, angiogenesis and expression of enzymes responsible for glucose transport and glycolysis. Hypoxia inducible factor 1α (HIF-1α) and HIF-2α are involved in many of the molecular and physiological responses to low oxygen levels. Both are members of the basic Helix-Loop-Helix/Per-ARNT-Sim (bHLH/PAS) protein family and form DNA binding heterodimers with the aryl hydrocarbon receptor nuclear translocator (ARNT), another bHLH/PAS protein. In normoxia, HIF-1α and HIF-2α are degraded in the cytoplasm by a mechanism involving oxygen dependent hydroxylation of conserved prolines in their respective oxygen dependent degradation domains, which permits the recruitment of the von Hippel-Lindau E3 ligase and targeting of the protein for destruction via the ubiquitin proteasome pathway. Also, oxygen dependent hydroxylation of an asparagine in the carboxy terminal transactivation domain of HIF-1α and HIF-2α results in the repression of their transcriptional activity. During hypoxia, HIF-1α and HIF-2α undergo protein stabilisation, derepression and translocation from the cytoplasm to the nucleus, where they dimerise with the constitutively nuclear ARNT to bind to hypoxia response elements (HREs) and induce transcription of HRE associated genes.

Despite the biochemical similarities between the hypoxic regulation of HIF-1α and HIF-2α, gene targeting experiments show major differences in developmental abnormalities between HIF-1$\alpha^{-/-}$ and HIF-2$\alpha^{-/-}$ mice and thus suggest distinct physiological roles and target gene specificities. A number of HIF-1α specific target genes have been documented, but there are currently none that have been conclusively identified for HIF-2α. The aim of this project was to determine specific gene targets and differential regulation of HIF-1α and HIF-2α in the rat pheochromocytoma cell line PC12.

Both HIF-1α and HIF-2α proteins are known to accumulate during hypoxia in PC12s. Furthermore, PC12s display a hypoxically inducible catecholamine synthesis and release pathway, a disruption of which is believed to contribute to the embryonic lethal phenotype of HIF-2α null mice. Therefore, monoclonal lines were established in which either HIF-1α or HIF-2α could be selectively stabilised and activated. Northern analysis of these lines demonstrated that the mRNA levels of many known targets of HIF-1α are induced by

HIF-1α but not HIF-2α. This suggests that HIF-2α may have its own novel targets. Furthermore, DNA microarray analysis was employed using these inducible cell lines and has uncovered a number of novel putative HIF-2α target genes which may relate to the physiological role of this transcription factor.

A surprising feature of these PC12 cell line derivatives is that the selective upregulation of HIF-1α does not induce HIF-2α, and *vice versa*. This suggests uncharacterised differences in their degradation mechanisms. For HIF-1α, it was demonstrated that destruction is initiated by prolyl-4-hydroxylation, albeit without a requirement for 2-oxoglutarate, which is considered a cosubstrate of the HIF-α prolyl-4-hydroxylases. In contrast, it is shown that HIF-2α levels are not mediated by prolyl-4-hydroxylation. Rather, preliminary data suggest that lysine acetylation is involved in regulating HIF-2α protein stability, but by a mechanism which is independent of the function of the only known HIF-α lysyl acetylase, ARD1. This study clearly shows the differential regulation of endogenous HIF-1α and HIF-2α proteins in the model PC12 cell line and suggests that similar regulation *in vivo* may contribute to the distinct physiological roles of these factors.

CANDIDATE'S DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Anthony Olindo Fedele, December 2004.

ACKNOWLEDGEMENTS

I would like to thank the following people for their involvement in the research described in this thesis. I am aware that my words of thanks are few. However, there is no way to fully quantify my appreciation to all who are mentioned, only to state it.

Firstly, I must thank Associate Professor Murray Whitelaw for being an exceptional supervisor throughout the last few years. From the first day of my Honours year until now, he has always been a source of unlimited help, encouragement, knowledge and the odd good laugh as well. I have learnt more than I could have imagined in his laboratory and I have enjoyed my time there immensely.

I would also like to show gratitude to my cosupervisor Doctor Daniel Peet whose guidance has been invaluable and whose insight has been an inspiration in my learning to be a scientist. He has also highlighted how much of an amateur prankster I truly am.

A mention must be made of all the people with whom I have had the pleasure and distinction of studying throughout the course of my studies in the Whitelaw laboratory, without whom I would have learnt little and would have had enjoyed myself even less: Susi and Jo, who I have known from the first day and who have always been willing to share their wisdom and their technical expertise and to have the odd drink as well; Mike, the now departed patriarch, the source of many helpful words of practical assistance and contributor to even more interesting conversations; Robyn, Sebastian and David who have also left but whose knowledge was many times sought and provided; Anne, Alix, Jodi, Fiona and Lauren, who are all relatively recent arrivals to the laboratory, but who have all already contributed in ways which have only ever been positive; and Saskia, Wiltiana, Christine and Gerwin who flew all the way from Northern Europe to spend nine months with us, to the betterment of the laboratory, and hopefully themselves. I must also thank the inaugural members of the Peet laboratory, some of whom also spent time with me in the Whitelaw laboratory: Karolina and Rachael who were great Honours students and who will both be outstanding postgraduate students; Cameron, who, despite being in his junior years, has always been of assistance and has countless times provided much invaluable advice, no matter how busy he is and despite the fact that I once threw some fruit at him in

high school (according to him, although I do not remember the incident myself); and Sarah whose intelligence and friendliness is matched only by her ability to transform an apparently hopeless situation into something positive, like the mosaicist who turns broken tile into a masterpiece. I would also like to thank the School of Molecular and Biomedical Sciences (Biochemistry) in general, but particularly Jan, Brian, Serge, John, Simon and the Central Services Unit whose assistance has been invaluable and whose work is appreciated beyond measure and the Centre for the Molecular Genetics of Development for their financial assistance.

I must express my gratitude to those who have provided technical assistance to the work in this thesis: Sandy MacIntyre and Alan Bishop at the Hanson Institute, Adelaide, Australia who performed the FACS; Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia who performed the microarray hybridisations and the subsequent statistical analysis; and Wiltiana Barak who performed the *in vitro* hydroxylation assay.

I naturally have to thank all my friends for being exactly that. Our paths may slowly be diverging but in spite of this there are many things that will never change.

Finally, I must express my gratitude to my family: my mother and father for their unconditional support and guidance; Rosa, Susan, Filippo and Max for their encouragement and advice; and Anais, Maxwell and Joshua.

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1. HIF-1α AND HIF-2α

1.1.1. HYPOXIA

Hypoxia is a term used to describe a deficiency in normal oxygen delivery to cells, whether caused by an environmental reduction in oxygen supply, such as that which accompanies an increase in altitude, or a severe localised ischaemia caused by a disruption of blood flow to a given area. In general, responses to hypoxia in mammals serve to increase oxygen delivery to cells and to adapt them metabolically to the deficiency in oxygen (Poellinger and Johnson, 2004). The mechanisms by which these ends are achieved include stimulating the production of red blood cells via erythropoiesis (Goldberg *et al.*, 1988), increasing the supply of blood to the effected areas by angiogenesis (Shweiki *et al.*, 1992) or adapting cells to a greater reliance on glycolysis by increasing glucose transporter and glycolytic enzyme expression (Ebert *et al.*, 1995; Firth *et al*, 1994; Semenza, *et al.*, 1994). It is now evident that many of the molecular and physiological responses to hypoxia are mediated by the gene regulatory proteins hypoxia inducible factor 1α (HIF-1α) and HIF-2α. It is also now apparent that they play a crucial role in mediating the pathophysiology of numerous diseases, including cancer and ischaemia.

1.1.2. DISCOVERY OF HIF-1α AND HIF-2α

The gene for erythropoietin (EPO) (a hormone that stimulates erythrocyte proliferation) was among the first discovered to undergo hypoxia induced transcription and thus became a prototype system to study hypoxic responsive genes and any potential transcription factors regulated by oxygen levels (Goldberg *et al.*, 1988). Discovery of a hypoxia inducible enhancer 3' to the human *EPO* gene resulted in the demarcation of the hypoxia response element (HRE) required for hypoxia mediated transcription (Semenza *et al.*, 1991). This permitted the subsequent isolation (Semenza and Wang, 1992) and cloning (Wang *et al.*, 1995b) of the hypoxia inducible dimer that binds to the HRE, termed HIF-1. This dimer consists of the hypoxically inducible HIF-1α subunit and the constitutively

present aryl hydrocarbon receptor nuclear translocator (ARNT, also referred to as HIF-1β).

It was subsequently observed that HRE mediated expression of a reporter gene and HIF-1 DNA binding activity was induced by hypoxia in both EPO and non EPO producing cells (Maxwell *et al.*, 1993; Wang and Semenza, 1993a; Beck *et al.*, 1993). This suggested that HIF-1 could serve as an activator of genes other than EPO. Other hypoxia inducible genes have subsequently been found to have regulatory sequences with significant homology to the EPO 3' HRE, including the angiogenic mediator vascular endothelial growth factor (VEGF) (Goldberg and Schneider, 1994), glycolytic enzymes such as phosphoglycerate kinase and aldolase A (Firth *et al.*, 1994; Semenza *et al.*, 1994) and glucose transporter 1 (Ebert *et al.*, 1995). HIF-1α thus has a general role in different physiological responses to hypoxia, from processes that provide immediate respite to oxygen deficiency (erythropoiesis and glycolysis), to long term solutions (angiogenesis). As the complement of target genes expands, it is evident that HIF-1α is also involved in processes removed from these classical responses to hypoxia (table 1.1), including B lymphocyte development (Kojima *et al.*, 2002), carotid body function (Kline *et al.*, 2002), adipogenesis (Yun *et al.*, 2002) and apoptosis (Bruick, 2000).

1.1.3. HIF-2α

The related HIF-2α (also referred to as endothelial PAS protein (EPAS), HIF like factor (HLF), HIF related factor (HRF) and MOP2 (Tian *et al.*, 1997; Ema *et al.*, 1997; Flamme *et al.*, 1997; Hogenesch *et al.*, 1997)) was identified and cloned shortly after HIF-1α. Many structural and biochemical similarities are shared between HIF-1α and HIF-2α as best exemplified by the fact that both proteins are potently induced by hypoxia via essentially identical mechanisms. One difference between the two proteins that was noted early on was in their expression patterns. Unlike HIF-1α, which is essentially ubiquitously expressed regardless of tissue or cell type, RNA and protein analysis revealed that HIF-2α is expressed predominantly in selected tissues, such as the lung and carotid body, and also in specific cell types, such as endothelium, epithelium, fibroblasts, macrophages and neurons (Tian *et al.*, 1997; Ema *et al.*, 1997; Flamme *et al.*, 1997; Tian *et al.*, 1998; Wiesener *et al.*, 1998; Talks *et al.*, 2000). In addition, it is clear that their functions are not redundant and, although the complement of HIF-1α target genes has been extensively

Table 1.1. HIF-1α target genes.

Since the discovery of HIF-1α, a number of genes have been found to be direct targets. The products of most of these are involved in the classical responses to reductions in oxygen tension (such as enhanced erythropoiesis and iron metabolism, vascularisation and glucose uptake and glycolysis). However, there has been an emergence in the characterisation of HIF-1α target genes whose function is distanced from these classical hypoxic responses.

HIF-1α target gene	Reference
Erythropoiesis and iron metabolism	
ceruloplasmin	Mukhopadhyay *et al.*, 2000
erythropoietin	Semenza and Wang, 1992
ferrochelatase	Liu *et al.*, 2004
transferrin	Rolfs *et al.*, 1997
transferrin receptor	Bianchi *et al.*, 1999; Lok *et al.*, 1999; Tacchini *et al.*, 1999
Vascularisation and circulation	
α_{1B} adrenergic receptor	Eckhart *et al.*, 1997
adrenomedullin	Cormier-Regard *et al.*, 1998; Nguyen and Claycomb, 1999.
calcitonin receptor like receptor	Nikitenko *et al.*, 2003
CXCR4	Staller *et al.*, 2003
endothelial nitric oxide synthase	Coulet *et al.*, 2003
endothelin	Hu *et al.*, 1998; Minchenko and Caro, 2000; Yamashita *et al.*, 2001
Flt-1	Gerber *et al.*, 1997
haem oxygenase 1	Lee *et al.*, 1997
inducible nitric oxide synthase 2	Melillo *et al.*, 1995; Palmer *et al.*, 1998
intestinal trefoil factor	Furuta *et al.*, 2001
leptin	Grosfeld *et al.*, 2002; Ambrosini *et al.*, 2002
midkine	Reynolds *et al.*, 2004
plasminogen activator inhibitor 1	Kietzmann *et al.*, 1999
stromal cell derived factor 1	Ceradini *et al.*, 2004
tyrosine hydroxylase	Schnell *et al.*, 2003
vascular endothelial growth factor	Levy *et al.*, 1995; Liu *et al.*, 1995; Forsythe *et al.*, 1996
Glucose uptake and glycolysis	
adenylate kinase 3	O'Rourke *et al.*, 1996
aldolase A/C	Semenza *et al.*, 1994; Semenza *et al.*, 1996
autocrine motility factor	Krishnamachary *et al.*, 2003
carbonic anhydrase 9	Wykoff *et al.*, 2000
enolase 1	Semenza *et al.*, 1994; Semenza *et al.*, 1996
glucose transporter 1-3	Ebert *et al.*, 1995
glyceraldehyde-3-phosphate dehydrogenase	Graven *et al.*, 1999
hexokinase 1, 2	Mathupala *et al.*, 2001
lactate dehydrogenase A	Semenza *et al.*, 1994; Firth *et al.*, 1995; Semenza *et al.*, 1996
6-phosphofructo-2-kinase/fructose-2,6-bisphosphate 3	Minchenko *et al.*, 2002
phosphofructokinase L	Semenza *et al.*, 1994
phosphoglycerate kinase 1	Semenza *et al.*, 1994
pyruvate kinase M	Semenza *et al.*, 1994
triosephosphate isomerase	Gess *et al.*, 2004
HIF-α regulation	
HIF-α prolyl-4-hydroxylase 2	del Peso *et al.*, 2003, Metzen *et al.*, 2004
p35srj	Bhattacharya *et al.*, 1999
Transcriptional regulation	
DEC1/2	Miyazaki *et al.*, 2002
ETS-1	Oikawa *et al.*, 2001
Nur77	Choi *et al.*, 2004
retinoic acid receptor related orphan receptor α	Chauvet *et al.*, 2004

Growth and apoptosis

HGTD-P	Park *et al.*, 2004
inhibitor of differentiation 2	Löfstedt *et al.*, 2004
insulin like growth factor 2	Feldser *et al.*, 1999
insulin like growth factor binding protein 1-3	Tazuke *et al.*, 1998; Feldser *et al.*, 1999
NIP-3	Bruick, 2000
NIX	Sowter *et al.*, 2001
presinilin 1, 2	Lukiw *et al.*, 2001; Bazan and Lukiw, 2002
RTP801	Shoshani *et al.*, 2002
telomerase catalytic subunit hTERT	Nishi *et al.*, 2004
TGF-α	Krishnamachary *et al.*, 2003
TGF-β3	Scheid *et al.*, 2002
Wilms' tumour suppressor 1	Wagner *et al.*, 2003

Drug resistance

P glycoprotein	Comerford *et al.*, 2002

Matrix and barrier assembly and function

cathepsin D	Krishnamachary *et al.*, 2003
collagen prolyl-4-hydroxylase α(I)	Takahashi *et al.*, 2000
CD73	Synnestvedt *et al.*, 2002
fibronectin 1	Krishnamachary *et al.*, 2003
keratin 14, 18 and 19	Krishnamachary *et al.*, 2003
matrix metalloprotease 2	Krishnamachary *et al.*, 2003
urokinase plasminogen activator receptor	Krishnamachary *et al.*, 2003
vimentin	Krishnamachary *et al.*, 2003

Redox

ERO1-Lα	Gess *et al.*, 2003
Noxa	Kim *et al.*, 2004

Prostaglandin synthesis

cyclooxygenase 2	Bazan and Lukiw, 2002

Other

VL30	Estes *et al.*, 1995.

studied, it was only recently that an appreciation of the distinct physiological role of HIF-2α has begun to emerge, albeit with much controversy.

There are perhaps two main reasons for any differences in the absolute roles of HIF-1α and HIF-2α *in vivo*. One may be that there are genes which are specifically upregulated by the transcriptional activity of one HIF-α but not the other. Another contributing factor may be that their respective functions are controlled by distinct oxygen dependent mechanisms.

1.1.4. HIF-1α AND HIF-2α ARE bHLH/PAS PROTEINS

HIF-1α and HIF-2α are members of the basic-Helix-Loop-Helix/Per-ARNT-Sim (bHLH/PAS) protein family (Wang *et al.*, 1995b; Tian *et al.*, 1997; Ema *et al.*, 1997). They form DNA binding heterodimers (Wang *et al.*, 1995b; Tian *et al.*, 1997; Ema *et al.*, 1997) with ARNT, another bHLH/PAS protein so called because it was first discovered as the heterodimeric partner of another bHLH/PAS protein known as the aryl hydrocarbon receptor (AHR) or dioxin receptor (DR) (Reyes *et al.*, 1992).

bHLH/PAS proteins are distinguished by the presence of an N terminal bHLH domain and two PAS domains (figure 1.1). The bHLH domain is required for dimerisation and DNA binding whereas the function of the PAS regions can be involved in a variety of processes depending upon the protein (Kewley *et al.*, 2004). In the case of HIF-1α and HIF-2α, the function of the PAS domains remains to be completely characterised, although full DNA binding activity of HIF-1α/ARNT and HIF-2α/ARNT heterodimers is dependent on the presence of the PAS domains (Jiang *et al.*, 1996). Interestingly, the *Drosophila melanogastor* bHLH/PAS proteins trachealess (trh), and single-minded (sim) both heterodimerise with the *Drosophila* equivalent of ARNT and bind the same DNA sequence, but cause the expression of distinct target genes (the former is responsible for the formation of the trachea while the latter is vital for the induction of midline cell fates). A trh-sim chimaera containing the trh bHLH and the sim PAS domains causes it to function as a sim protein (Zelzer *et al.*, 1997). The non DNA binding PAS domains thus confers target gene specificity on these proteins. It is possible that the PAS domains of HIF-1α and HIF-2α contribute to differences in their target gene specificities.

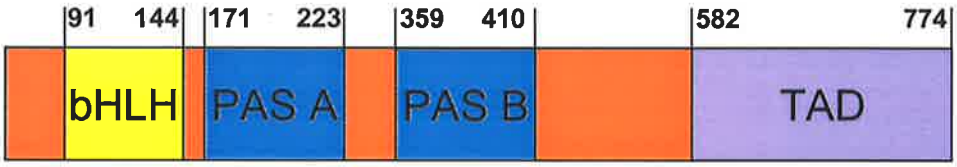
Figure 1.1. Schematic representation of human HIF-1α, HIF-2α and ARNT.

HIF-1α, HIF-2α and ARNT are basic-Helix-Loop-Helix/Per-ARNT-Sim (bHLH/PAS) proteins that contain an NH_2 terminal bHLH domain and two PAS domains. HIF-1α and HIF-2α also contain an oxygen dependent degradation domain (ODD) that mediates its oxygen regulated stability and in which is incorporated an NH_2 terminal transactivation domain. They also contain a COOH terminal transactivation domain (TAD) whose transcriptional repression in normoxia is controlled by the inhibitory domain (ID). ARNT has a TAD that serves no function in the context of HIF-1α activity. Amino acid numbers for each domain of the human protein are indicated.

HIF-1α

HIF-2α

ARNT



1.1.5. TRANSCRIPTION OF HYPOXIA INDUCIBLE GENES BY HIF-α

During hypoxia, HIF-1α and HIF-2α are translocated from the cytoplasm to the nucleus (Kallio *et al.*, 1998). Here they heterodimerise with the constitutively nuclear ARNT to enable binding to the cognate HRE DNA sequence and induce transcription of target genes (figure 1.2). All HIF-1α inducible genes contain at least one copy of the HRE consensus sequence 5' ACGTGC 3' (Guillemin and Krasnow, 1997) and this can function whether it is 5' or 3' of the coding sequence (Beck *et al.*, 1993). Although HIF-2α can activate expression of a luciferase reporter driven by multiple copies of the *EPO* HRE (Tian *et al.*, 1997; Ema *et al.*, 1997), there are no conclusively identified HIF-2α specific target genes and many of those of HIF-1α are not regulated by HIF-2α. It is thus possible that HIF-2α may recognise a different response element from that of HIF-1α.

1.2. STABILISATION OF HIF-α

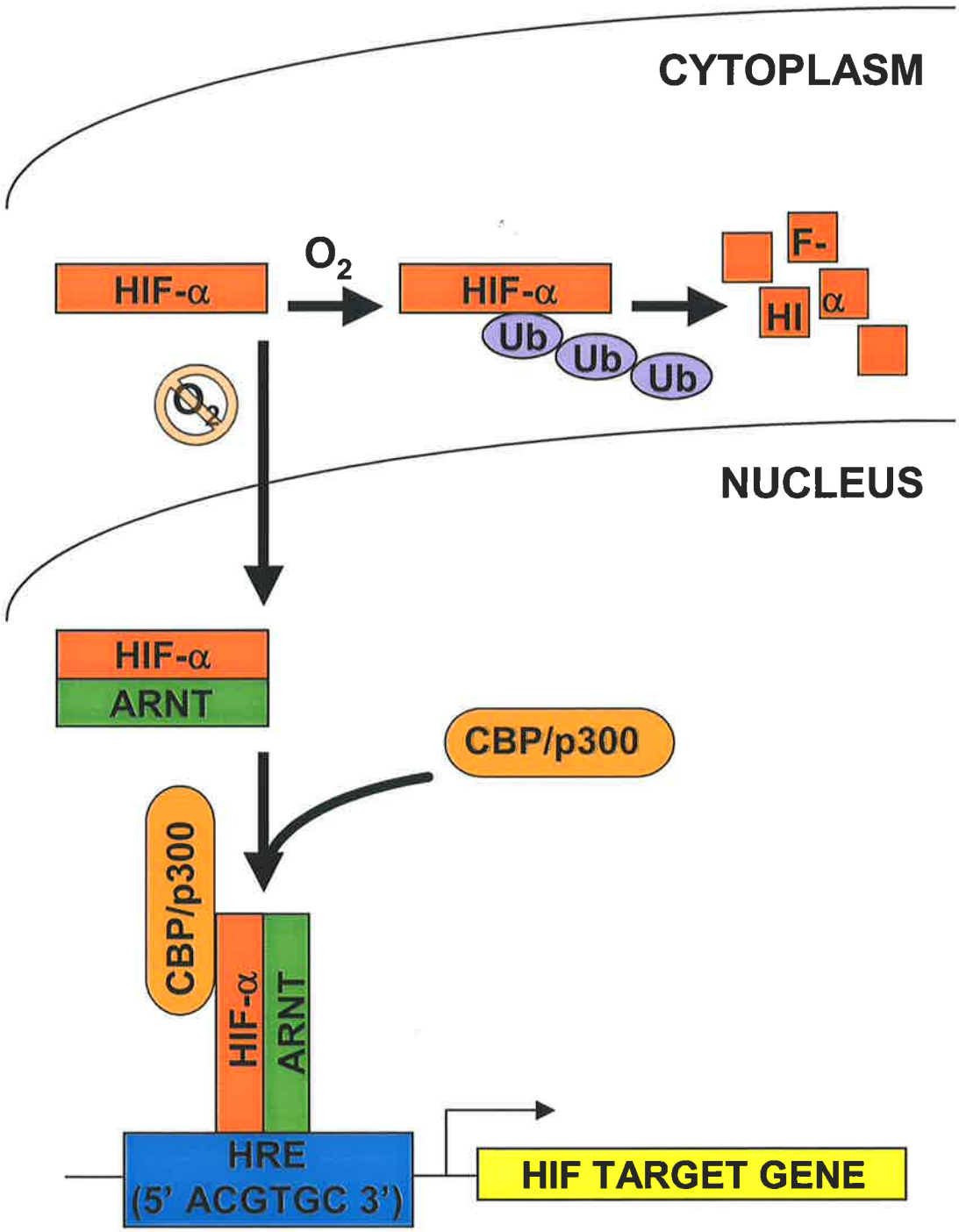
1.2.1. HIF-α LEVELS ARE CONTROLLED POSTTRANSLATIONALLY

ARNT protein is ubiquitously expressed in mammalian cells and is maintained at constant levels regardless of oxygen tension (Kallio *et al.*, 1997). In contrast, HIF-1α and HIF-2α proteins are rapidly induced under hypoxic conditions and thus serve as the hypoxia inducible partners of the constitutive ARNT.

The regulation of HIF-1α and HIF-2α mRNA levels by hypoxia does not appear to play a major role in their function (Wang *et al.*, 1995b; Kallio *et al.*, 1997; Wiesener *et al.*, 1998). Rather, their levels are chiefly controlled posttranslationally. In normoxia, HIF-α protein is virtually undetectable due to degradation via the ubiquitin proteasome pathway (Salceda and Caro, 1997; Huang *et al.*, 1998; Kallio *et al*, 1999) and has a half life in the order of 5 minutes. In hypoxia (1% O_2), its half life is increased to about 30 minutes (Huang *et al.*, 1998). Upon reoxygenation, HIF-α is once again promptly destroyed (Wang *et al.*, 1995b). HIF-1α lability is mediated by the oxygen dependent degradation domain (ODD) whose effects are suppressed by hypoxia and a similar ODD exists in HIF-2α (Huang *et al.*, 1998; O'Rourke et al, 1999). There has been much progress recently with regards to the elucidation of the mechanism of the ODD in regulating the stability of HIF-1α and HIF-2α and specifically how this mediates an interaction with the von Hippel-Lindau tumour suppressor gene product (pVHL).

Figure 1.2. Hypoxia inducible gene regulation by HIF-α.

In normoxia, HIF-α is transcriptionally inactive and is rapidly degraded by the ubiquitin (Ub) proteasome pathway. In hypoxia, HIF-α undergoes protein stabilisation and translocation from the cytoplasm to the nucleus, where it dimerises with ARNT and associates with the transcriptional coactivators (as exemplified by CBP/p300) to induce the transcription of HRE regulated genes.


CYTOPLASM
HIF-α
O2
HIF-α
Ub
Ub
Ub
F-
HI
α
O2
NUCLEUS
HIF-α
ARNT
CBP/p300
CBP/p300
HIF-α
ARNT
HRE
(5' ACGTGC 3')
HIF TARGET GENE

1.2.2. INVOLVEMENT OF pVHL IN THE DEGRADATION OF HIF-α

Mutations in pVHL were originally described as the causative phenomena of an autosomal dominant cancer syndrome termed VHL disease, characterised by the development of multiple tumours, particularly clear cell renal cell carcinomas, pheochromocytomas and haemangioblastomas in the retina and central nervous system (Iliopoulos *et al.*, 1995). It is now known that pVHL functions as a component of a multimeric E3 ubiquitin ligase (Lisztwan *et al.*, 1999; Iwai *et al.*, 1999). A connection between pVHL and HIF-α was established via the observation that the mRNA of hypoxia inducible genes is upregulated in cells defective for pVHL (Gnarra *et al.*, 1996; Iliopoulos *et al.*, 1996). It is now evident that pVHL is involved in regulating HIF-α protein levels. By binding to HIF-1α and HIF-2α in normoxia but not hypoxia, pVHL activates their oxygen dependent degradation (Maxwell *et al.*, 1999). Briefly, HIF-1α and HIF-2α were detected as stable and active in normoxic pVHL defective renal carcinoma cells, while expression of wildtype pVHL restored the hypoxic induction of HIF-α stability. It has since been demonstrated that a portion of the ODD (amino acids 549-572 of human HIF-1α and 517-534 of human HIF-2α) is critical for the binding of HIF-α to the β domain of pVHL and its subsequent ubiquitination and proteasomal degradation (Ohh *et al.*, 2000; Cockman *et al.*, 2000; Tanimoto *et al.*, 2000, Kamura *et al.*, 2000). The structural analysis of a peptide derived from HIF-1α bound to a complex consisting of pVHL and elongins B and C demonstrated the importance of these amino acids in the association with HIF-1α, as the two pVHL contact sites within HIF-1α consist of residues 560-567 and D571-577 with the side chain of D569 also attaching to pVHL (Min *et al.*, 2002; Hon *et al.*, 2002).

Normally, a tumour grows to such a size that O_2 cannot be adequately delivered via diffusion. The consequent intratumoural hypoxia induces HIF-α activity and induction of downstream genes (such as VEGF) and this promotes angiogenesis to service the tumour. One mechanism by which mutations in pVHL cause tumour formation is by permitting the function of HIF-α in normoxia and the subsequent expression of angiogenic factors even prior to the cancer's exposure to hypoxic stress. However, it must be noted that most but not all disease causing pVHL mutations demonstrate a diminished ability to regulate HIF-α levels (Clifford *et al.*, 2001; Hoffman *et al.*, 2001), so the existence of other pVHL substrates and their role in tumour development cannot be ignored. pVHL has also been shown to bind to fibronectin and be involved in extracellular matrix assembly (Ohh *et al.*, 1998). Mutated pVHL that did not regulate HIF-α demonstrated a diminished ability to

associate with fibronectin (Hoffman *et al.*, 2001). Therefore, there may be a role in deregulated fibronectin in this and other VHL disease phenotypes. There are other published targets of pVHL, although the consequences of their ubiquitination remain uncharacterised. These include atypical members of the protein kinase C (PKC) family PKCλ and PKCζ (Okuda *et al.*, 1999; Okuda *et al.*, 2001), VHL interacting deubiquitinating enzyme (VDU) 1 and VDU2 (Li *et al.*, 2002a; Li *et al.*, 2002b), which have since been implicated in thyroid hormone activation (Curcio-Morelli *et al.*, 2003), RNA polymerase II subunit hsRPB7 (Na *et al.*, 2003) and hyperphosphorylated large subunit of RNA polymerase II (Kuznetsova *et al.*, 2003). Identifying other proteins regulated by pVHL may help to explain tumour progression in patients where HIF-α regulation is normal, as well as differences between the types of VHL disease and limited tissue origin of VHL disease tumours.

1.2.3. OXYGEN DEPENDENT PROLYL-4-HYDROXYLATION OF HIF-α

Until recently, the mechanism by which a reduction in O_2 in a cell could translate to enhanced HIF-1α and HIF-2α activity was poorly understood. Studies that served to explain the targeting of HIF-α for association with the pVHL ubiquitination complex perhaps unexpectedly also shed light as to the nature of the cell's oxygen sensors.

These studies demonstrated that hydroxylation of a proline within the ODD is responsible for pVHL's association with HIF-1α and HIF-2α (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001). pVHL bound directly to a short peptide derived from the ODD of HIF-1α only when a specific proline residue was hydroxylated (P564, corresponding to P531 in HIF-2α). Structural analysis of a hydroxylated P564-containing peptide derived from HIF-1α in a complex with pVHL and elongins B and C demonstrated the importance of the hydroxyproline in pVHL's interaction with HIF-1α (Min *et al.*, 2002; Hon *et al.*, 2002). Specifically, the β domain of pVHL contains a single hydroxyproline binding pocket whose recognition of the hydroxyproline group involves hydrogen bonds formed with buried serine and histidine (Min *et al.*, 2002; Hon *et al.*, 2002). Prolyl-4-hydroxylation was previously known to occur in collagen and proteins with collagen like sequences (Kivirikko and Myllharju, 1998). The collagen prolyl-4-hydroxylases are O_2, Fe^{2+}, 2-oxoglutarate and ascorbate dependent (Kivirikko and Myllharju, 1998). Not surprisingly, capture of a HIF-1α derived fragment (including P564) by pVHL after treatment with whole cell extract (believed to contain the hydroxylase activity) was

enhanced by ascorbate and decreased by *N*-oxalylglycine (NOG, which competes with 2-oxoglutarate) or hypoxia (Jaakkola *et al.*, 2001). Furthermore, DMOG (dimethyloxalylglycine, a cell membrane permeable variant of NOG) was able to stabilise HIF-1α in cell culture (Jaakkola *et al.*, 2001). This suggested that HIF-α is hydroxylated by a similar class of prolyl-4-hydroxylases.

In many respects, the HIF-α prolyl-4-hydroxylase activity in these studies reflected characteristics inherent in collagen prolyl-4-hydroxylation. Most strikingly, this proposed O_2 dependent hydroxylase was a candidate for the hypothetical oxygen sensor. It also helped to explain the hypoxia mimicking properties of the transition metal compound $CoCl_2$ (Goldberg *et al.*, 1988) and iron chelators such as desferrioxamine (Wang and Semenza, 1993b) which both increase endogenous HIF-1α protein and activity (Jiang *et al.*, 1997). Originally, the ability of these agents to mimic hypoxia resulted in various proposed models to describe cellular oxygen sensing. Cobalt was believed to substitute for the ferrous iron in a haem protein that serves as a hypothetical oxygen sensor, thus mediating a conformational change to imitate the deoxygenated haem state (Goldberg *et al.*, 1988). In addition to sequestering the iron required by the putative oxygen sensor, it was hypothesised that iron chelation appropriates the free iron that catalyses the conversion of hydrogen peroxide (H_2O_2) to reactive oxygen intermediates (ROIs) which in turn were suggested to serve as signalling molecules to inhibit HIF-1α (Bunn and Poyton, 1996). However, it is now presumed that the mimetics stabilise HIF-α by competing with or sequestering the Fe^{2+} required for prolyl-4-hydroxylase activity and thus inhibiting enzymatic function.

Nonetheless, it was evident from these studies that HIF-1α and HIF-2α are similarly targeted for degradation in normoxia by pVHL in a prolyl-4-hydroxylation dependent manner.

1.2.4. DISCOVERY OF THE HIF-α PROLYL-4-HYDROXYLASES

The collagen prolyl-4-hydroxylases consist of a tetramer of two α and two β units. Despite the similarities between HIF-α and collagen prolyl-4-hydroxylase activity, it was soon clear that different enzymes were responsible as the respective proline containing ODDs of HIF-1α and HIF-2α contain no consensus sequences that resemble those in the collagen prolyl-4-hydroxylase substrates. Also, human collagen prolyl-4-hydroxylases

synthesised in insects were unable to hydroxylate the HIF-1α peptide (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). Therefore, the HIF-α prolyl-4-hydroxylase appeared to be novel, a hypothesis confirmed by the subsequent discovery of the HIF-α prolyl-4-hydroxylases (Epstein *et al.*, 2001; Bruick and McKnight, 2001).

Epstein and coworkers analysed HIF-1α regulation in *Caenorhabditis elegans* to identify three mammalian HIF-α prolyl-4-hydroxylases. The *C. elegans* homologue of HIF-1α (also discovered by Jiang *et al.*, 2001) was shown to be stabilised in hypoxia and possess a pVHL binding region similar to that in mammalian HIF-1α. In normoxia, it interacted with pVHL only upon hydroxylation of a proline homologous to that in mammalian HIF-1α, inferred by inhibition of this interaction by prolyl-4-hydroxylase inhibitors or mutation of the proline residue (Epstein *et al.*, 2001). Putative HIF-α prolyl-4-hydroxylases were then identified by searching in *C. elegans* and mammalian databases for proteins that contained a conserved β-barrel jelly roll motif common to 2-oxoglutarate dependent dioxygenases (as exemplified by the prolyl-4-hydroxylases of collagen). These putative hydroxylases appeared to be closely related to EGL-9 of *C. elegans*, which was also predicted to contain conserved 2-oxoglutarate and iron binding motifs within the β-barrel jelly roll. A mutation in the *egl-9* gene was originally identified as causing an egg laying abnormality, although no function had been attributed to the gene product (Trent *et al.*, 1983). Subsequent analysis demonstrated that the three strains of worm carrying *egl-9* mutations all demonstrated the presence of constitutively high HIF-1α protein levels that were not altered by oxygen tension (Epstein *et al.*, 2001). Furthermore, pVHL could interact with purified HIF-1α after the addition of recombinant EGL-9 without simultaneous treatment with worm extract (which would otherwise contain hydroxylase activity) and HPLC analysis demonstrated that the modification of HIF-1α was indeed prolyl-4-hydroxylation (Epstein *et al.*, 2001). Three EGL-9 human homologues termed prolyl hydroxylase domain containing proteins (PHD) 1, 2 and 3 were demonstrated to enhance capture of human HIF-1α and human HIF-2α by pVHL and (at least PHD1) to be responsible for prolyl-4-hydroxylation of a HIF-1α derived peptide containing P564 (Epstein *et al.*, 2001). A second prolyl-4-hydroxylation site within the HIF-α ODD was also identified (P402 in human HIF-1α and P405 in human HIF-2α) and both sites were shown to be within a conserved LXXLAP motif common to the HIF-α proteins of *C.elegans* and mammals (Epstein *et al.*, 2001; Masson *et al.*, 2001).

In another study, Bruick and McKnight searched GenBank for sequences related to the α subunit (the catalytic component) of the collagen prolyl-4-hydroxylases. Of five candidates, one displayed HIF-α hydroxylase activity (designated HIF prolyl hydroxylase 1 (HPH-1)) and had two highly similarly sequenced and functioning paralogues (HPH-2 and HPH-3). The PHDs and HPHs are indeed identical, with HPH1, HPH2 and HPH3 corresponding to PHD3, PHD2 and PHD1, respectively (Seta *et al.*, 2002). The *Drosophila melanogastor* HPH homologue was identified and an RNA interference (RNAi) experiment that targeted HPH levels in insect cells resulted in an increase in normoxic levels of the *Drosophila* HIF-1α homologue similar a (not to be mistaken with the previously mentioned sim) (Bruick and McKnight, 2001).

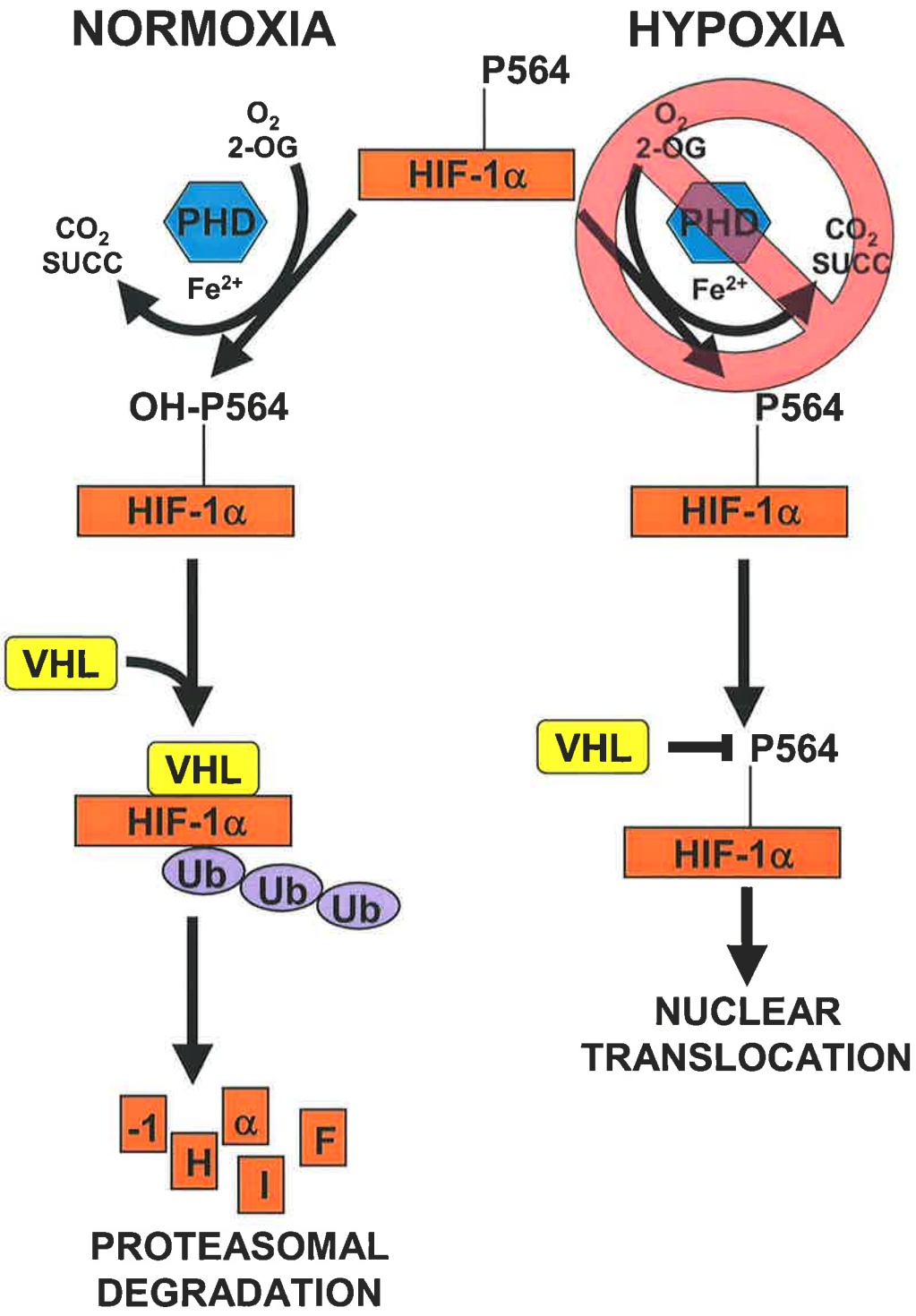
In summary, the three PHD enzymes hydroxylate 2 conserved proline residues within the ODD of both HIF-1α and HIF-2α and target them for binding to pVHL and ultimately degradation by the ubiquitin proteasome pathway (figure 1.3). Furthermore, these proteins were found to be of a previously unknown function and, unlike the collagen prolyl-4-hydroxylases which constitute two α and two β subunits, consist only of the catalytic α unit. In other respects, these hydroxylases share much in common, such as their dependence on Fe^{2+}, 2-oxoglutarate, ascorbate and, most importantly in the context of HIF-α regulation, O_2. However, the K_m values for O_2 of the PHD enzymes (230 to 250 μM or slightly above the concentration of oxygen dissolved in the atmosphere) are much higher than that of collagen prolyl-4-hydroxylases (40 μM) (Hirsilä *et al.*, 2003). As the K_m values for O_2 of the former are similar to atmospheric oxygen levels, small changes in O_2 are predicted to alter their activity, whereas collagen prolyl-4-hydroxylases would function even where oxygen concentrations are minimal (Hirsilä *et al.*, 2003). Therefore, the absolute requirement and its K_m for O_2 suggest that the PHDs may serve as the primary oxygen sensors in cells and can also explain the exponential increase in HIF-1α protein levels as cells are exposed to decreasing pO_2 (Jiang *et al.*, 1996).

1.2.5. SUBSTRATE REQUIREMENTS OF THE HIF-α PROLYL-4-HYDROXYLASES

Of some interest is defining a sequence of amino acids required for recognition by the HIF-α prolyl-4-hydroxylases. This may provide leads to other substrates. The conserved LXXLAP motif is present in both sites of prolyl-4-hydroxylation in HIF-1α and HIF-2α and has been suggested to be required for association with PHD (Masson *et al.*, 2001).

Figure 1.3. Oxygen dependent degradation of HIF-α.

In normoxia, two specific prolines within HIF-1α (P402 and P564, or P405 and P531 in HIF-2α) are hydroxylated by HIF-α prolyl-4-hydroxylases (PHDs) in a reaction that is dependent on 2-oxoglutarate (2-OG), ascorbate and Fe^{2+} and that also produces CO_2 and succinate (SUCC). This targets the HIF-α protein for association with the von Hippel-Lindau protein. This in turn serves as the HIF-α recognition component of an E3 ubiquitin ligase complex (VHL) and results in the degradation of HIF-α protein via the ubiquitin proteasome pathway. In hypoxia, the required O_2 for prolyl-4-hydroxylase activity is deficient. As a result, HIF-α protein is not degraded because the prolyl-4-hydroxylation required for targeting by the VHL complex is inhibited. Only hydroxylation of P564 is shown.


NORMOXIA
HYPOXIA
P564
HIF-1α
O2
2-OG
PHD
Fe2+
CO2
SUCC
OH-P564
HIF-1α
VHL
VHL
HIF-1α
Ub
Ub
Ub
-1
H
α
I
F
PROTEASOMAL
DEGRADATION
P564
HIF-1α
VHL
P564
HIF-1α
NUCLEAR
TRANSLOCATION

Hyperphosphorylated large subunit of RNA polymerase II (Rpb1) has also been shown to associate and be ubiquitinated by pVHL in a hydroxyproline dependent manner and contains this conserved LXXLAP motif (Kuznetsova *et al.*, 2003). However, the necessity for this LXXLAP motif for recognition by the hydroxylases requires further analysis as both *in vivo* and *in vitro* studies have suggested that the only amino acid within this sequence that is absolutely necessary for prolyl-4-hydroxylation is the actual proline that accepts the hydroxyl group (Yu *et al.*, 2001; Huang *et al.*, 2002). However, it has been demonstrated that L574 of human HIF-1α (conserved as L542 in human HIF-2α) is an obligate requirement for PHD mediated prolyl-4-hydroxylation (Kageyama *et al.*, 2004). That other sequence or structural determinants may dictate the specificity of PHD activity to specific proline residues is exemplified by the fact that long substrates are required for efficient *in vitro* hydroxylation. When compared with a 19 residue synthetic peptide corresponding to the region around P564 of HIF-1α, its hydroxylation by any of the PHD enzymes purified from insect cells was significantly inhibited by removing four NH_2 or five COOH terminal residues or by reducing the peptide at both ends to one of 11 amino acids (Hirsilä *et al.*, 2003).

1.2.6. CELLULAR LOCALISATION OF THE HIF-α PROLYL-4-HYDROXYLASES

Details are emerging as to how the HIF-α prolyl-4-hydroxylases themselves are regulated. It is becoming evident that there are peculiarities in where they are distributed in a cellular context and how their levels are controlled. This implies differential functions of each and serves to justify the existence of more than one PHD.

As mentioned above, a significant difference is in their cellular localisation. Overexpressed Flag/PHD1 in COS-1 cells (Huang *et al.*, 2002) and PHD1 fused to enhanced green fluorescent protein (EGFP) in U2OS cells (Metzen *et al.*, 2003a) were shown to be predominantly nuclear and PHD2 mainly cytoplasmic. However, in the former study, PHD3 was also shown to be primarily cytoplasmic, although in the latter paper it was shown to be evenly distributed between the cytoplasm and nucleus.

Nonetheless, the existence of multiple PHD enzymes and their distribution to different locales hints at a tight regulation of the HIF1-α protein where a lack of prolyl-4-hydroxylation of HIF-α by one PHD in normoxia is compensated by the recognition by

another. Thus, accounting for their localisation, it is possible to begin to assign functions for PHD1, PHD2 and PHD3. The latter two may be the master regulators of HIF-α levels and immediately recognise and hydroxylate HIF-α in normoxia in the cytoplasm and target it for destruction upon translation. The nuclear localisation of PHD1 suggests a secondary role whereby any HIF-α which has escaped destruction in the cytoplasm and that has entered the nucleus can be still be hydroxylated.

A fourth enzyme (PH-4, Oehme *et al.*, 2002) has been discovered and is distinguished by its localisation in the endoplasmic reticulum (ER). Although classed as a HIF-1α prolyl-4-hydroxylase, that HIF-1α is a substrate is unlikely due to its absence in the ER.

1.2.7. REGULATION OF THE HIF-α PROLYL-4-HYDROXYLASES

Also of interest is the hypoxia inducibility of the respective HIF-α prolyl-4-hydroxylases. HIF-α degradation in normoxia is enhanced by prior exposure to hypoxia and this is dependent on HIF-1α activity (Berra *et al.*, 2001). Presumably, this reduces the time required for cells to adapt to a return to normoxia and thus any deleterious effects of extended HIF-α activity. Therefore, it is perhaps not surprising that PHD enzymes demonstrate hypoxia inducible expression. PHD2 and PHD3 transcripts were first shown to be upregulated in HeLa cells (Epstein *et al.*, 2001) and U2OS cells (Metzen *et al.*, 2003a) and ultimately in a number of other lines (del Peso *et al.*, 2003). Another study involving rat C6 glioma cells shows a hypoxia inducibility of PHD2 only (D'Angelo *et al.*, 2003). Nonetheless, a common feature of all these studies is the limited or absence of PHD1 upregulation in hypoxia. Coupled to its nuclear localisation, this suggests a secondary role of PHD1 in HIF-α prolyl-4-hydroxylation.

Naturally, given the hypoxia inducibility of at least some of the HIF-α prolyl-4-hydroxylases, the next question relates to whether they are targets as well as regulators of HIF-α. In a study of pVHL deficient renal clear cell carcinoma lines (where HIF-α stability and activity is therefore not regulated by hypoxia), it was shown that the stable introduction of pVHL expression reduced PHD2 but not PHD1 or PHD3 mRNA levels, thus forming a correlation between HIF-α and PHD2 regulation (del Peso *et al.*, 2003). This was somewhat surprising given the demonstration of the hypoxia inducibility of PHD3 in some studies. Still, it is possible that PHD2 is a HIF-α target whereas PHD3 may require signals in addition to HIF-α transcriptional activity. Indeed, del Peso and

coworkers demonstrated that introduction of a constitutively stable HIF-1α (that mutated at P402 and P564) into 786-O cells where pVHL had been introduced was sufficient to increase PHD2 but not PHD3 levels (del Peso *et al.*, 2003). Furthermore, PHD2 has been shown to contain a functional HRE in its promoter (Metzen *et al.*, 2004), although there is no published data with respect to whether this is also the case for PHD3. In short, PHD1 appears not to be a hypoxically inducible gene. PHD2 is in all likelihood a direct target of HIF-1α. So too may be PHD3, but other factors generated in hypoxia could be necessary. To confirm this hypothesis, it is imperative that the presence or absence of HREs associated with the PHD3 gene is demonstrated.

1.2.8. DIFFERENTIAL ACTIVITIES OF THE HIF-α PROLYL-4-HYDROXYLASES

It was originally assumed that all three PHD enzymes are HIF-α prolyl-4-hydroxylases based on their ability to hydroxylate HIF-α *in vitro* (Epstein *et al.*, 2001; Bruick and McKnight *et al.*, 2001). However, it has recently been proposed that PHD2 is the master oxygen sensor and PHD enzyme of HIF-α (Berra *et al.*, 2003). RNAi was employed to independently reduce the expression of PHD1, 2 and 3. Silencing of PHD2 alone was sufficient to induce HIF-1α protein and activity in a number of cell lines, whereas this was not the case with PHD1 or 3 (Berra *et al.*, 2003). This raises questions with regards to the role of these other enzymes in HIF-α regulation. One possibility is that they are not involved at all *in vivo* and so this supports the notion that the PHD proteins may have other targets. It may also be a consequence of the relative amounts of the three PHD enzymes. Across a range of cell lines tested, the normoxic levels of PHD1 and PHD3 were relatively low, with PHD2 as clearly the most abundant protein (Appelhoff *et al.*, 2004). It is thus not surprising that PHD2 downregulation by RNAi can upregulate HIF-α in normoxia. However, the same study demonstrated that the hypoxia inducibility of PHD3 protein was markedly greater than that of PHD2, so it is possible that the main task of PHD3 is to increase HIF-α destruction after reoxygenation, which is consistent with the prolonged half life of HIF-α when hypoxic cells treated with PHD3 RNAi were restored to normoxic conditions (Appelhoff *et al.*, 2004). A third possibility is that the role of each in relation to HIF-α prolyl-4-hydroxylation is contextual and may depend on the nature, severity or duration of the hypoxic stress. Indeed, Berra and coworkers showed that in cells permanently exposed to RNAi of PHD2 and thus continual depletion of PHD2 protein, levels of HIF-1α, although initially high, eventually declined (Berra *et al.*, 2003).

However, HIF-1α could be stabilised when PHD1 was silenced by RNAi in these circumstances (Berra *et al.*, 2003). This suggests that the accumulation of a target of HIF-1α may be required to activate PHD1's ability to hydroxylate HIF-α. Finally, there may be a differential regulation of HIF-1α and HIF-2α by the PHD enzymes. Given the conservation of the targeted prolines, it has been generally assumed that are identically regulated by PHD activity. Indeed, it has been shown that the PHD enzymes contribute to the regulation of HIF-1α and HIF-2α protein, especially with regards to enhancing normoxic levels via the RNAi of PHD2 in normoxia and decreasing the rate of degradation after reoxygenation via the RNAi of PHD3 (Appelhoff *et al.*, 2004). However, using the breast carcinoma MCF7 as an example, the RNAi of PHD2 had a greater effect on increasing HIF-1α than HIF-2α protein in normoxia, whereas that of PHD3 had a greater effect on slowing the decay of HIF-2α than HIF-1α protein after reoxygenation and also contributed to enhancing HIF-2α levels in hypoxia (Appelhoff *et al.*, 2004).

1.3. TRANSCRIPTIONAL ACTIVATION OF HIF-α

1.3.1. HIF-α ACTIVITY IS CONTROLLED POSTTRANSLATIONALLY

Despite the crucial role of proline-4-hydroxylation and pVHL in regulating HIF-1α and HIF-2α protein levels, their stabilisation alone is not sufficient to permit their full transcriptional activation. Both HIF-1α and HIF-2α proteins contain two hypoxia inducible transactivation domains separated by an inhibitory domain (ID) that inhibits transcriptional activity in normoxia (Jiang *et al.*, 1997). One transactivation domain is encompassed within the ODD of HIF-α and the regulation of its activity appears to be integrated with protein stability (Jiang *et al.*, 1997; Pugh *et al.*, 1997; O'Rourke *et al.*, 1999). The dominant transactivation domain consists of the COOH terminal 40 amino acids. In isolation it is constitutively active, but in conjunction with the ID is repressed at normoxia and induced by hypoxia independent of protein stability (Jiang *et al.*, 1997; Pugh *et al.*, 1997; O' Rourke *et al.*, 1999). Recent studies have shed light on how activity in HIF-1α and HIF-2α is regulated. They also confirm the two part model of HIF-α regulation whereby hypoxia promotes both protein stabilisation and transcriptional activity by different molecular mechanisms.

1.3.2. OXYGEN DEPENDENT ASPARAGINYL HYDROXYLATION OF HIF-α

The final 100 amino acids of HIF-1α and HIF-2α (the COOH terminal transactivation domain plus the COOH terminus of the ID, hereafter referred to as the CAD) when fused to the Gal4 DNA binding domain (Gal4DBD) cause an increase in Gal4 response element (GRE) mediated reporter activity in hypoxia without altering protein stability (Jiang *et al.*, 1997; O'Rourke *et al.*, 1999). Lando and coworkers established a stable mammalian cell line expressing the final 100 amino acids of mouse HIF-2α, exposed it to normoxia or hypoxia and searched for posttranslational modifications using mass spectroscopy of the purified protein (Lando *et al.*, 2002a). HIF-2α protein purified from normoxic cells contained a specific hydroxylated asparagine (N851) that was essentially unmodified in protein from hypoxic cells. As this asparagine is present in human HIF-1α (N803), it was hypothesised that failure to hydroxylate this amino acid was the mechanism by which hypoxia modulates CAD activity. It also suggested that the enzymes that target HIF-α for normoxic degradation were different to those that inhibit assembly of the transcriptional machinery by the CAD. However, like prolyl-4-hydroxylation, HIF-α aparaginyl hydroxylation was shown to be dependent on 2-oxoglutarate, Fe^{2+} and O_2. Treatment of cells with the 2-oxoglutarate inhibitor DMOG activated Gal4DBD/HIF-1α CAD and Gal4DBD/HIF-2α CAD to an extent comparable with hypoxia and mutation of the crucial asparagine in HIF-1α and HIF-2α resulted in maximum transcriptional activity that was not enhanced by hypoxia or DMOG (Lando *et al.*, 2002a), confirming the importance of this residue in regulating CAD activity.

Regarding the full length protein, it was shown that full transcriptional activity of HIF-1α and HIF-2α in normoxia could be obtained only through mutation of both the critical proline in the ODD and the asparagine in the CAD. A mutation in the former permitted a constitutively stable protein with low transcriptional activity, whereas in the latter, although presumably more active, the protein was labile (Lando *et al.*, 2002a). Finally, asparaginyl hydroxylation of the CAD inhibited its recruitment of the CH1 domain of the transcriptional coactivator CBP and suggested that asparaginyl hydroxylation in normoxia inhibits the previously observed hypoxic recruitment of CBP/p300 (Arany *et al.*, 1996; Kallio *et al.*, 1998; Ebert and Bunn, 1998; Ema *et al.*, 1999; Gu *et al.*, 2001) and subsequently other coactivators such as SRC-1 (Carrero *et al.*, 2000). Subsequent structural analysis of the HIF-1α CAD when associated with CBP/p300 demonstrated that N803 is deeply buried within the complex and it is postulated that hydroxylation of this

asparagine would render the recruitment of CBP/p300 by the HIF-1α CAD unlikely (Dames *et al.*, 2002; Freedman *et al.*, 2002).

It has therefore been confirmed that there are two major steps required for the hypoxic induction of HIF-1α and HIF-2α function: inhibition of the oxygen dependent prolyl-4-hydroxylation in the ODD to prevent the association with the pVHL ubiquitin ligase complex and subsequent destruction by the ubiquitin proteasome pathway, and inhibition of the oxygen dependent asparaginyl hydroxylation in the CAD to permit association with CBP/p300 and assembly of the transcriptional coactivator complex.

1.3.3. DISCOVERY OF THE HIF-α ASPARAGINYL HYDROXYLASE

The identification of HIF-α asparaginyl hydroxylases was then of primary interest. A β asparaginyl/aspartyl hydroxylase had been previously identified that catalyses hydroxylation of specific asparagine or aspartic acid residues of epidermal growth factor like domains (Stenflo *et al.*, 1989; Wang *et al.*, 1991). It displays no obvious biological function in hydroxylating its substrate, although studies involving null mice implicate a role for the hydroxylase in tumour suppression and facial and limb development (Dinchuk *et al.*, 2002). This known β asparaginyl/aspartyl hydroxylase functions on the consensus sequence CXN/DXXXXP/YXCXC (Stenflo *et al.*, 1989; Wang *et al.*, 1991). As this sequence is not found in HIF-1α, it was likely that the HIF-α asparaginyl hydroxylase was a novel enzyme.

The first protein shown to cause inhibition of CAD activity in normoxia was factor inhibiting HIF-1 (FIH-1). This was identified by a yeast two-hybrid screen using HIF-1α 576-826 as bait and shown to reduce the activity of cotransfected HIF-1α and form a ternary complex with pVHL and HIF-1α *in vitro* (Mahon *et al.*, 2001). This suggested that prolyl-4-hydroxylation of the ODD permits HIF-α/pVHL complex formation and in turn draws FIH-1 to the CAD for transcriptional inhibition, thus indirectly coupling protein instability and CAD inhibition. However, there is evidence to distance pVHL from reduced CAD activity in normoxia. Firstly, the hydroxylation dependent regulation of the CAD occurs independently of pVHL. A Gal4DBD/HIF-1α 740–826 chimaera displays hypoxia inducible reporter expression in pVHL deficient cells to essentially the same degree as when pVHL is reintroduced (Sang *et al.*, 2002). There was also evidence to suggest that HIF-α asparaginyl hydroxylase activity was pVHL independent. The CADs

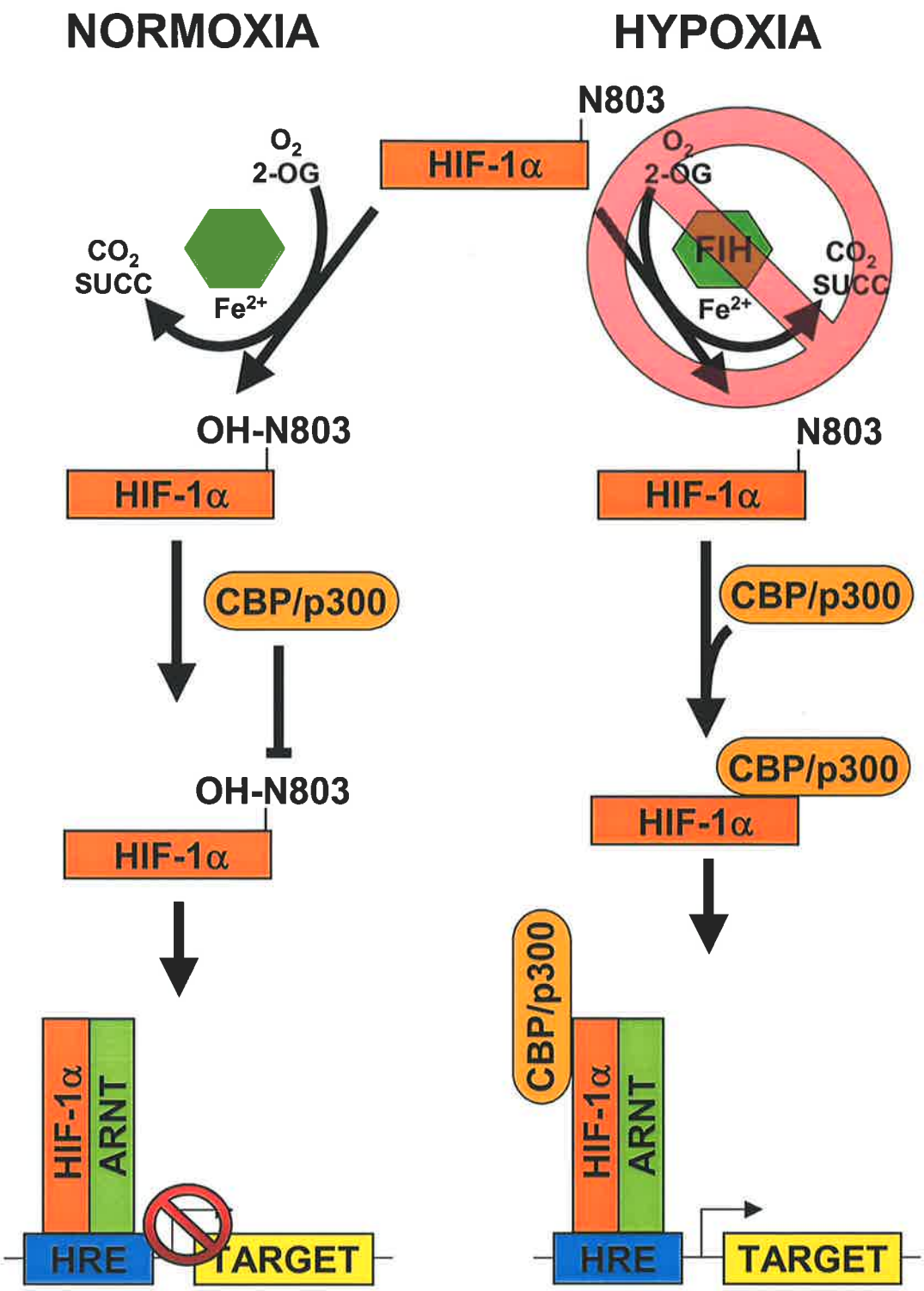
first purified and shown to contain a hydroxylated asparagine in normoxia contained only the final 100 amino acids of HIF-1α and HIF-2α and thus no pVHL binding site (Lando *et al.*, 2002a). Finally, recent work shows that FIH-1 is a 2-oxoglutarate, Fe^{2+} and O_2 dependent asparaginyl hydroxylase that hydroxylates the β carbon of N803 of HIF-1α and N851 of HIF-2α and inhibits their association with the CH1 domain of CBP (Lando *et al.*, 2002b; Hewitson *et al.*, 2002; McNeill *et al.*, 2002). All observed results in these studies occurred independently of pVHL.

Initially, it was shown that FIH-1 repression of the HIF-α CADs could be ablated with DMOG treatment, suggesting that FIH-1 activity requires 2-oxoglutarate (Lando *et al.*, 2002b). Most importantly, mass spectroscopy of recombinant mouse HIF-2α 774-874 treated with FIH-1, Fe^{2+}, ascorbate and 2-oxoglutarate resulted in the hydroxylation of N851, although this was unmodified when the inhibitor DMOG was included (Lando *et al.*, 2002b). Also, FIH-1 treatment inhibited the interaction between ^{35}S labelled HIF-2α 774-874 and the CH1 domain of CBP unless DMOG had been added or N851 had been mutated to alanine (Lando *et al.*, 2002b). In an almost identical study, mass spectroscopy of recombinant human HIF-1α 775-826 treated with FIH-1 showed an increase in mass of 16 Da, suggesting hydroxylation of the substrate (Hewitson *et al.*, 2002). The hydroxylation of N803 and its ability to inhibit HIF-α CAD association with CBP/p300 was demonstrated by no interaction between ^{35}S labelled CH1 domain of CBP and HIF-1α 775-826 when the latter was preincubated with FIH-1, although this interaction was permitted when N803 was mutated to A (Hewitson *et al.*, 2002).

These experiments confirmed that FIH-1 is a 2-oxoglutarate, O_2 and Fe^{2+} dependent enzyme that hydroxylates a conserved asparagine in the CAD of HIF-α and inhibits its association with CBP/p300 and subsequent assembly of the transcriptional coactivator complex. Hypoxia presumably ablates this inhibition by depriving the enzyme of the required O_2, ultimately permitting the potentiation of HIF-α CAD activity (figure 1.4). Interestingly, although it has been shown that PHD mediated inhibition of HIF-α activity is reduced in hypoxia (Bruick and McKnight, 2001), FIH-1 is able to repress Gal4DBD/HIF-1α 727-826 activity in hypoxia when overexpressed in cell culture (Lando *et al.*, 2002b). Also, not only does the downregulation of FIH-1 via RNAi allow for the induction of HIF-1α target genes in a number of cell lines in normoxia (despite the absence of any additional reduction of PHD enzyme), it causes a further increase in these

Figure 1.4. Oxygen dependent transcriptional inactivation of HIF-α.

In normoxia, a specific asparagine within HIF-1α (N803, or N847 in HIF-2α) is hydroxylated by a HIF-α asparaginyl hydroxylase (FIH-1) in a reaction that is dependent on 2-oxoglutarate (2-OG), ascorbate and Fe^{2+} and that also produces CO_2 and succinate (SUCC). This inhibits the direct interaction of the COOH terminal transactivation domain with CBP/p300 and ultimately the assembly of the transcriptional coactivator complex. In hypoxia, the required O_2 for asparaginyl hydroxylase is deficient. As a result, CBP/p300 can associate with the unhydroxylated CAD and potentiate transcriptional activity.


NORMOXIA
HYPOXIA
N803
HIF-1α
O2
2-OG
CO2
SUCC
Fe2+
FIH
OH-N803
CBP/p300
HIF-1α
ARNT
HRE
TARGET

transcripts in hypoxia (Stolze *et al.*, 2004). Therefore, the prospect that FIH-1 function is mediated by factors in addition to affinity for dioxygen cannot be discounted. For example, FIH-1 may compete with CBP/p300 for binding to the HIF-α CADs even in hypoxia. However, there is evidence to suggest that its behaviour in hypoxia is a manifestation of enzymatic activity. That is, although the overexpression of FIH-1 causes the downregulation of the normoxic levels of HIF-1α target gene transcripts and of those otherwise enhanced by hypoxia, this was not the case when an inactive mutant of FIH-1 (FIH-1 D201A) is used or when there is no oxygen (anoxia) (Stolze *et al.*, 2004). This means that hydroxylation activity is essential for any inhibitory effect that FIH-1 has on HIF-α transactivation. That FIH-1 activity is less sensitive to reductions in O_2 is perhaps best explained by the fact that, in *in vitro* kinetic assays using recombinant proteins, FIH-1 has a K_m for O_2 of 90 μM and an apparent higher affinity for oxygen than the HIF-α prolyl-4-hydroxylases which have a K_m for O_2 in the range of 230-250 μM (Hirsilä *et al.*, 2003; Koivunen *et al.*, 2004).

1.3.4. CHARACTERISATION OF THE HIF-α ASPARAGINYL HYDROXYLASE

For now, it seems that FIH-1 is the only mammalian HIF-α asparaginyl hydroxylase and that HIF-α is the only characterised substrate. Determining its biochemical features may provide leads to novel substrates and therefore to other functions of FIH-1.

Of interest is the complement of amino acids required for hydroxylation of the crucial asparagine of HIF-α as this should provide a consensus sequence with which to screen for potential FIH-1 substrates. By using bacterially expressed proteins where amino acids 798-808 had been individually substituted for alanine, it was shown that *in vitro* hydroxylation of human HIF-1α 737-826 was only inhibited when V802 and abolished when N803 were mutated to alanine (Linke *et al.*, 2004). Transposing this alanine scan to a cell based activity assay showed that Gal4DBD/HIF-1α 737-826 displayed constitutive GRE mediated reporter activity with the V802A or N803A mutations (Linke *et al.*, 2004). The experiments demonstrate that at least with regards to amino acids in the vicinity of the hydroxylated asparagine, the only other crucial residue for FIH-1 activity is the preceding valine. Still, as with the PHD enzymes, relatively long substrates are required for effective hydroxylation. Using as a starting point a synthetically prepared 35 amino acid HIF-1α 788-822 peptide, the ability of substrates to be *in vitro* hydroxylated by an FIH-1 synthesised and purified from insects cells drastically decreased upon removal of even

only a few amino acids (Koivunen *et al.*, 2004). Indeed, the crystal structure of FIH-1 when complexed with the HIF-1α CAD shows two sites of interaction with respect to the latter, namely residues 795-806 (which contains the crucial asparagine) and 813-822 (Elkins *et al.*, 2003). In addition, at least with respect to the ability of the synthetic peptide mentioned above to be hydroxylated, it has also been shown that 788-794 are important (Koivunen *et al.*, 2004).

Of particular interest also is the apparent difference between the relative affinities of FIH-1 for the HIF-1α CAD and HIF-2α CAD. A 35 amino acid HIF-2α 832-866 peptide demonstrated only 7% of the ability to be hydroxylated by purified FIH-1 when compared to the corresponding HIF-1α peptide described above (Koivunen *et al.*, 2004). In other words, FIH-1 may have a drastically reduced ability to hydroxylate asparagine within and inhibit the association of HIF-2α with CBP/p300 in contrast with its effect on HIF-1α. It has been postulated therefore that an ability to downregulate FIH-1 may have a greater effect on the regulation of HIF-1α than HIF-2α target genes (Koivunen *et al.*, 2004). The exact residues responsible for this discrepancy and the significance of this to the function of the full length proteins remains to be determined, although it may contribute to the differential activation and consequently distinct physiological roles of HIF-1α and HIF-2α.

1.3.5. LOCALISATION OF THE HIF-α ASPARAGINYL HYDROXYLASE

Whether overexpressed as an EGFP fused protein in U2OS cells (Metzen *et al.*, 2003a) or recognised via immunofluorescence of endogenous or overexpressed protein in 293T or U2OS cells (Linke *et al.*, 2004; Stolze *et al.*, 2004), FIH-1 is located predominantly in the cytoplasm. Localisation and protein levels of FIH-1 are not altered by treatment with the hypoxia mimetic 2, 2'-dipyridyl (Linke *et al.*, 2004) or by hypoxia itself (Stolze *et al.*, 2004). Also, the mRNA of FIH-1 is not altered by hypoxia (Metzen *et al.*, 2003a). It may thus be possible to assign FIH-1 a secondary, albeit still crucial role in mediating HIF-α function. Any residual HIF-α that has escaped prolyl-4-hydroxylation in the cytoplasm can undergo asparaginyl hydroxylation of the CAD and be inactivated. Furthermore, the role of enhancing HIF-α inhibition after reoxygenation and readapting cells to normoxia may only require hypoxic induction of PHD activity.

1.3.6. REGULATION OF THE HIF-α ASPARAGINYL HYDROXYLASE

Despite an apparent lack of oxygen dependent regulation, there is some evidence to suggest that other pathways may regulate FIH-1 function. The renal cell carcinoma (RCC) 786-O line expresses a non functioning truncated pVHL, so HIF-2α expression (HIF-1α is absent) is not regulated by hypoxia (Maxwell *et al.*, 1999). Given that the targeting of HIF-α for asparaginyl hydroxylation by FIH-1 and thus the downregulation of the former's transactivation does not require pVHL (Sang *et al.*, 2002; Lando *et al.*, 2002a; Lando *et al.*, 2002b; Hewitson *et al.*, 2002), it is not clear how the stabilised protein also displays essentially maximum activity.

It was first shown that the inhibition of protein kinase PKCζ in RCC cells resulted in reduced VEGF expression, suggesting a role for PKCζ activity in the constitutively high levels of hypoxia inducible transcripts in pVHL deficient cells, possibly via promoting the activity of the stable HIF-α protein (Pal *et al.*, 1997; Pal *et al.*, 2001). Indeed, expression of a dominant negative form (DN) or RNAi of PKCζ in 786-O cells inhibited coimmunoprecipitation of HIF-2α with p300 (Datta *et al.*, 2004). It was also shown that DN expression in 786-O cells enhanced FIH-1 levels (Datta *et al.*, 2004). In other words, it has been demonstrated that pVHL deficient RCC cells have increased PKCζ activity, which in turn downregulates FIH-1 levels and asparaginyl hydroxylase activity and enhances the association of HIF-α with CBP/p300 (Datta *et al.*, 2004). As mentioned above, PKCζ can interact with pVHL (Okuda *et al.*, 1999). However, although activated PKCλ has been shown to be ubiquitinated by the pVHL E3 ubiquitin ligase (Okuda *et al.*, 2001), it is not known whether pVHL is involved in the processing of PKCζ. Could the lack of pVHL function in 786-O cells be responsible for enhanced PKCζ function? For instance, pVHL mediated ubiquitination may inactivate PKCζ, thus upregulating FIH-1 and ultimately ensuring repressed HIF-α activity in normoxia. A mutation in pVHL may then permit constitutive activation of PKCζ and enhanced transcriptional activity of the now high levels of HIF-α protein. However, it is difficult to reconcile this with the fact that a Gal4DBD/HIF-1α 740–826 chimaera displays hypoxia inducible reporter expression in 786-O cells to essentially the same degree as when pVHL is reintroduced (Sang *et al.*, 2002), suggesting little change in FIH-1 levels or activity in 786-O cells as a consequence of pVHL expression. PKCζ activity in 786-O cells must therefore be due to mutational events other than those in pVHL.

1.4. SUMMARY OF THE HIF-α HYDROXYLASES

The discovery of the HIF-α prolyl-4- and asparaginyl hydroxylases and their respective independent involvement in controlling protein stability and transcriptional activity supports the two step model of HIF-α function. Furthermore, the evidence generally supports the conclusions that the oxygen dependent regulation of the protein levels and function of both HIF-1α and HIF-2α is mediated by the concerted effort of the same hydroxylases. In other words, HIF-1α and HIF-2α appear to share similar fundamental mechanisms of regulation.

Future studies will presumably concentrate on PHD and FIH-1 expression patterns and their specific roles in regulating HIF-α activity in the context of organisms. Other substrates of the HIF-α prolyl-4- and asparaginyl hydroxylases and their fates post hydroxylation are no doubt also being actively sought. These may be proteins whose expression in hypoxia is crucial but whose transcription is not enhanced by HIF-α and instead are controlled posttranslationally, much like the HIF-α proteins themselves. Hydroxylation could even confer a novel functional switch to these substrates.

1.5. OTHER FORMS OF OXYGEN DEPENDENT HIF-α REGULATION

Although it is now clear that both classes of hydroxylases are vital oxygen sensors in HIF-α regulation, there is, albeit less clear, evidence to suggest that oxygen levels may be sensed via other means and that these lead to novel posttranslational modifications in addition to the prolyl-4- and asparaginyl hydroxylation and/or promotion or inhibition of the interactions with proteins other than the well substantiated pVHL and CBP/p300.

1.5.1. REACTIVE OXYGEN SPECIES AND HIF-α

Prior to the discovery and characterisation of the hydroxylases, it had been suggested that reactive oxygen species (ROS) are involved in mediating HIF-α function. As with molecular oxygen, it could be expected that ROS would inhibit HIF-α accumulation, and some data suggests this. For instance, treatment of HeLa cells with H_2O_2 prior to hypoxic treatment inhibited HIF-1α protein accumulation (Huang *et al.*, 1996). However, other studies suggest that ROS contribute to the upregulation of HIF-α in hypoxia. For instance, it has been demonstrated that Hep3B cells increase ROS generation in hypoxia and

treatment with antioxidants or removal of mitochondrial DNA abolishes HIF-1α stability and activity, suggesting a role for ROS in the hypoxic induction of HIF-α (Chandel *et al.*, 1998; Chandel *et al.*, 2000). This is coupled with the observation that H_2O_2 treatment of cells in normoxia inhibits the degradation and repression of HIF-1α (Chandel *et al.*, 2000). It is nevertheless difficult to reconcile how ROS can contribute to HIF-α stabilisation and how this can be attenuated by the inhibition of mitochondrial respiration given what is known about PHD activity.

1.5.2. NITRIC OXIDE AND HIF-α

Nitric oxide (NO) has been shown to compete with O_2 for binding to the iron at the active site of 2-oxoglutarate dependent dioxygenases and render them inactive (Zhang *et al.*, 2002). As such, NO should in theory enhance HIF-α levels and activity. Indeed, various NO donors have been shown to promote the accumulation and activity of HIF-α in normoxia in cell culture (Sandau *et al.*, 2001). This phenomenon has been attributed to decreased PHD hydroxylase activity. Synthetic HIF-1α 556-574 where P564 was a hydroxyproline could interact with ^{35}S labelled pVHL regardless of *S*-nitrosoglutathione (GSNO, an NO donor) presence, suggesting that NO does not interact with the pVHL/HIF-α complex (Metzen *et al.*, 2003b). However, a Gal4DBD/HIF-1α 540-582 column mixed with PHD activity in the presence of GSNO displayed reduced binding to ^{35}S labelled pVHL (Metzen *et al.*, 2003b). This demonstrates NO inhibition of HIF-α prolyl-4-hydroxylase activity.

However, in some studies, NO appears to inhibit HIF-α accumulation and activity in hypoxia. NO donors have been shown to inhibit the ability of HIF-α to bind to the HRE and promote HRE reporter activity (Sogawa *et al.*, 1998, Huang *et al.*, 1999). Furthermore, NO has been displayed to inhibit the hypoxia inducibility of HIF-1α targets (Sogawa *et al.*, 1998; Huang *et al.*, 1999). NO prevents both endogenous HIF-α accumulation and the activation of its CAD (as measured by the ability of Gal4DBD/HIF-1α CAD to promoted GRE mediated reporter expression) in hypoxic cell culture (Huang *et al.*, 1999). If PHD and FIH-1 activity are indeed being enhanced in hypoxia by NO, how could a supposed inhibitor of 2-oxoglutarate dioxygenases increase their activity in hypoxia when oxygen levels are limiting? Interestingly, it has now been proposed that the little O_2 available in hypoxia is distributed to oxygen dependent processes, such as mitochondrial respiration, and if these pathways are interfered with, it is made available to

other oxygen dependent reactions, such as prolyl-4-hydroxylation of HIF-α. To explain the work leading to this hypothesis, it has been shown that inhibition of mitochondrial respiration by low concentrations of NO, the endogenous inhibitor of complex IV of the respiratory chain (specifically cytochrome c oxidase), leads to the inhibition of HIF-1α protein accumulation in hypoxia (Mateo *et al.*, 2003). Further studies by Hagen and coworkers discounted any involvement in changes in ROS levels as antioxidants failed to reverse destabilisation or inhibit accumulation of HIF-α in hypoxia (Hagen *et al.*, 2003). Rather, these observations appear to result from a change in intracellular oxygen allocation. A substrate of renilla luciferase is O_2, so this enzyme's activity can be reduced if the assay is performed in hypoxia. It was shown that the activity of a mitochondria targeted renilla luciferase is limited by oxygen availability in a dose dependent manner (Hagen *et al.*, 2003). However, when similarly transfected cells were treated with an NO donor (DETA-NO), and therefore inhibitor of mitochondrial respiration, the activity of this luciferase was enhanced in hypoxia relative to untreated cells (Hagen *et al.*, 2003). This was most likely due to a decrease in oxygen expenditure by respiration and therefore an increase in its availability for other processes, even if in this case an albeit artificial one. When the activity of cytosolic renilla luciferase was observed in hypoxia, this was also enhanced by treatment with the DETA-NO (Hagen *et al.*, 2003). In other words, general intracellular O_2 is increased by repressors of mitochondrial respiration resulting in increased activity in other pathways, such as prolyl-4-hydroxylation of HIF-α.

1.5.3. PHOSPHORYLATION OF HIF-α

Determination of HIF-1α mass by comparison with molecular weight markers during electrophoresis suggests that it is of a larger size than that predicted from its amino acid sequence. Treatment of HIF-1α (immunoprecipitated from hypoxically treated HeLa cells with anti HIF-1α antibodies) with a phosphatase results in a HIF-1α protein that is now electrophoresed equivalent to its predicted size, thus suggesting that phosphorylation is responsible for its higher than expected apparent molecular weight (Richard *et al.*, 1999). Furthermore, phosphorylated HIF-2α can be immunoprecipitated from hypoxic PC12 cells grown in ^{32}P labelled orthophosphate (Conrad *et al.*, 1999). Little is known with regards to which residues are phosphorylated and the significance of any of these post translational modifications to HIF-α function but it does appear that both HIF-1α and HIF-2α are normally phosphorylated.

Only the phosphorylation of threonine 796 of HIF-1α (844 of HIF-2α) to HIF-α function has been denoted some significance. The ability of Gal4DBD/HIF-2α 824-874 to enhance a GRE mediated reporter in hypoxia was abolished when T844 was mutated to an alanine, although maintained when T844 was mutated to an aspartic acid (which mimics a constitutively phosphorylated residue) or serine (a phosphorylatable amino acid), all independent of protein levels (Gradin *et al.*, 2002). ^{35}S labelled HIF-2α T844A also showed a marked reduction in ability to interact with GST/CBP 1-452 when compared to wildtype HIF-2α, suggesting a role for the phosphorylation of T844 in the transcriptional activity of HIF-2α by permitting an interaction with CBP/p300 (Gradin *et al.*, 2002). Finally, overall *in vivo* phosphorylation of GST/HIF-2α 828-874 was not altered by hypoxia but reduced by T844 mutation (Gradin *et al.*, 2002). Therefore, threonine phosphorylation may be of structural importance and is thus a prerequisite for maximum HIF-α association with CBP/p300 without being hypoxically inducible itself. However, it has also been shown that, in contrast with its non phosphorylated variant, synthetic HIF-1α 788-806 which is phosphorylated at T796 could not become hydroxylated by purified FIH-1 and this is consistent with the fact that a phosphothreonine at this position disrupts a HIF-1α/FIH-1 interaction (Lancaster *et al.*, 2004). T phosphorylation could therefore be important to HIF-α activation by both promoting the CBP/p300 interaction and inhibiting FIH-1 association. The signals that result in the phosphorylation thus require further investigation.

1.5.4. p53 AND HIF-α

HIF-1α has been shown to interact with the p53 tumour suppressor gene *in vivo* (An *et al.*, 1998) and to promote the ubiquitin proteasome pathway destruction of HIF-1α via subsequent association with the Mdm-2 E3 ubiquitin ligase and the formation of a ternary complex (Ravi *et al.*, 2000; Chen *et al.*, 2003). It is difficult to comprehend, how HIF-1α instability conferred by p53 can be inhibited by hypoxia, given that p53 is a hypoxia inducible gene (An *et al.*, 1998). However, it could be proposed that, while pVHL performs a role in normoxia by promoting the degradation of the prolyl-4-hydroxylated HIF-α subunit, p53 may serve to maintain HIF-α levels in hypoxia. This is particularly likely given that angiogenesis of tumours derived from $p53^{-/-}$ lines was greater than in $p53^{+/+}$ lines and more specifically, $p53^{-/-}$ lines showed greater HIF-1α mediated VEGF activity in hypoxia than its $p53^{+/+}$ counterparts (Ravi *et al.*, 2000). Elucidating where p53

interacts with HIF-1α and HIF-2α and what modifications if any permit or inhibit it to do so may shed light on its role in controlling HIF-α function.

Also, hypoxically upregulated p53 may inhibit HIF-α function by other means. Indeed, overexpression of p53 mutated in its DNA binding domain, but not one mutated in both its DNA binding and p300 binding regions, inhibits the activity of HIF-1α (Blagosklonny *et al.*, 2001). Also, the interaction between the CH1 domain of CBP/p300 and ^{35}S labelled HIF-1α generated via *in vitro* translation can be outcompeted by increasing amounts of similarly generated p53 (Schmid *et al.*, 2004). Therefore, p53 which has accumulated in hypoxia may inhibit HIF-α by competing with the CBP/p300, whose necessity for transcriptional activity is common to both.

1.5.5. ARD1 AND HIF-α

A yeast two hybrid screen by Jeong and coworkers identified the N-acetyltransferase ARD1 as a protein that interacted with HIF-1α 401-603 (its ODD), and this was confirmed both *in vitro* (via an interaction between ^{35}S labelled HIF-1α 401-603 and GST/ARD1) and *in vivo* (coimmunoprecipitation of Flag/ARD1 with GFP/HIF-1α overexpressed in 293 cells) (Jeong *et al.*, 2002). It was thus proposed that it is involved in the regulation of HIF-1α stability. Indeed, overexpression of ARD1 in hypoxia resulted in a downregulation of endogenous HIF-1α without any change in the latter's mRNA (Jeong *et al.*, 2002). This suggests that ARD1 has a role in downregulation of HIF-1α protein. Cotreatment with MG132 (an agent that stabilises HIF-1α via an inhibition of the ubiquitin proteasome but still permits pVHL association) restored the HIF-1α protein levels, suggesting a role for ARD1 in recruiting the pVHL E3 ubiquitin ligase complex, which itself was confirmed via an increased capacity of MG132 stabilised HIF-1α in normoxic cells to be coimmunoprecipitated with pVHL upon coexpression of ARD1 (Jeong *et al.*, 2002). Ultimately, it was shown via treatment of recombinant human HIF-1α ODD fragments mutated at various lysines to arginines with ARD1 and their immunoblotting with acetylated lysine antibodies that the former is acetylated except when mutated at K532 (corresponding to K497 in human HIF-2α) and acetylation of this residue was confirmed by mass spectroscopy (Jeong *et al.*, 2002). When expressed in 293 cells HIF-1α K532R was more stable than the wildtype protein (as shown by pulse chase experiments), showed no hypoxia inducibility and demonstrated a decreased capacity to coimmunoprecipitate with pVHL (Jeong *et al.*, 2002). Furthermore, ARD1 treatment of

recombinant HIF-1α ODD caused an increase in coprecipitation with ^{35}S labelled pVHL, but not when the K532R mutation was present. (Jeong *et al.*, 2002). As pVHL only interacts with HIF-α after prolyl-4-hydroxylation, it appears that acetylation of the crucial lysine by ARD1 enhances pVHL's interaction with the hydroxylated ODD. One method by which acetylation of HIF-1α is reduced in hypoxia is the downregulation of the transcript and subsequently protein of ARD1 upon a reduction in O_2 levels (Jeong *et al.*, 2002). But the accumulation of HIF-1α upon exposure of cells to hypoxia occurs rapidly (Jewell *et al.*, 2001), perhaps more rapidly than would be expected if oxygen dependent downregulation of ARD1 transcript was significant to HIF-α stability. However it was also observed that hypoxia reduced the coimmunoprecipitation of Flag/ARD1 with GFP/HIF-1α when overexpressed in 293 cells, despite no change in the levels of the former, and there is no reason to suggest that the signalling required to inhibit this interaction cannot occur almost instantaneously (Jeong *et al.*, 2002). The exact cause of the inhibition of the ARD1/HIF-1α interaction requires further investigation.

Evidence to support a function of lysyl acetylation in controlling HIF-α protein levels has involved the use of berberine, a chemical present in the herb *Coptis chinensis*, itself a traditional Chinese therapeutic applied to the treatment of inflammatory diseases such as gastroenteritis (Lin *et al.*, 2004). Berberine was shown to downregulate the expression of HIF-1α protein (but not mRNA) in response to hypoxia in the gastric cancer cell line SC-M1 (Lin *et al.*, 2004). HIF-1α coimmunoprecipitated with anti acetyl lysine antibody from SC-M1 cells in normoxia or hypoxia when cells had been treated with berberine (where HIF-1α was not admittedly detected in either case in whole cell extracts) but not in untreated hypoxic cells (where HIF-1α was expectedly abundant) (Lin *et al.*, 2004). Berberine may thus inhibit the hypoxic accumulation of HIF-α via promoting lysyl acetylation (or inhibiting deacetylation?) of K532 in human HIF-1α or K497 in human HIF-2α, possibly via activation of ARD1.

1.6. THE ROLE OF HIF-α IN DISEASE

It now evident that VHL disease is manifested as a consequence of mutations in pVHL and that in most cases deregulated HIF-α has a crucial involvement in VHL disease tumour progression. However, it was also evident prior to the appreciation of pVHL's function that HIF-α plays a role in non VHL associated cancers. As it progresses, a solid tumour ultimately requires vascularisation in order to supply its cells with oxygen and to overcome the deficiencies in diffusion. Hypoxic regions within the tumour can cause HIF-

α stability and transcriptional activity, VEGF expression and ultimately angiogenesis and it has been demonstrated on a number of occasions that HIF-1α and HIF-2α are overexpressed in many tumours presumably, as a result of intratumoural hypoxia (Semenza, 2002). Indeed, studies over 12 years ago demonstrated the hypoxia inducible nature of VEGF expression in both cell culture and vascularising tumours (Shweiki *et al*, 1992; Plate *et al.*, 1992). The identification of VEGF as a HIF-1α target implicated the latter in tumour angiogenesis (Forsythe *et al.*, 1996). This was confirmed with the use of HIF-1$\alpha^{-/-}$ ES cells which displayed a reduced ability to form tumours in immunocompromised mice when compared with wildtype cells, showing a reduced size and decreased vascularisation, presumably due to compromised VEGF expression (Ryan *et al.*, 1998; Carmeliet *et al.*, 1998). Interestingly, it was also shown that hypoxia induced apoptosis in HIF-1$\alpha^{+/+}$ ES cells was increased in comparison to HIF-1$\alpha^{-/-}$ cells, suggesting that HIF-1α may also play a role in inhibiting cell growth in hypoxia in some tumours while promoting growth in others (Carmeliet *et al.*, 1998).

HIF-1α has also been implicated in the Warburg effect, a phenomenon whereby cancer cells display a high rate of aerobic glycolysis (Warburg, 1956). It has been proposed that the observed increase in glycolytic enzyme expression is caused by constitutive HIF-1α activity (Minchenko *et al.*, 2002), which is the most obvious explanation given the role of HIF-1α in the enhanced transcription of glycolytic enzymes and glucose transporters (Firth *et al.*, 1994; Semenza *et al.*, 1994; Ebert *et al.*, 1995). Conversely, it has also been suggested that the glycolytic end products lactate and pyruvate may induce HIF-1α accumulation (Lu *et al.*, 2002). It has been shown that a transfected GFP/pVHL fusion protein translocates to the nucleolus of cell culture lines under physiological acidosis (Mekhail *et al.*, 2004). This was correlated with a prolonged period of HIF-α stabilisation in acidotic cells after reoxygenation of cell lines exposed to hypoxia when compared with cells exposed to physiologically neutral pH, as well as the stabilisation of HIF-α in normoxic acidosis (Mekhail *et al.*, 2004). In other words, it appears that HIF-α is upregulated in acidic conditions (which is itself a product of hypoxia induced HIF-α activity) as the pVHL which should associate with it and promote its destruction is retained in the nucleolus. Either way, in addition to an important role in tumour angiogenesis, HIF-α is also implicated in regulating other major biochemical processes involved in tumour formation and progression.

The development of a potential antiangiogenic therapy has stemmed from studies using a peptide derived from the last 41 amino acids of HIF-1α, which can bind to and sequester the CH1 domain of CBP (Kung *et al.*, 2000). It was shown that this peptide reduced hypoxia inducible reporter activity in cell culture as well as VEGF mRNA levels in normoxia and hypoxia (Kung *et al.*, 2000). Furthermore, expression of this peptide in tumour cell lines reduced tumour growth in mouse models with diminished vessel density. This suggests that the HIF-α/CBP interaction may be a good therapeutic target (Kung *et al.*, 2000). Similar results were recently seen with a chemical known as chetomin, which was shown to disrupt the interaction between the final 41 amino acids of HIF-1α and the CH1 domain of CBP/p300 (Kung *et al.*, 2004). However, specificity may be a concern as the CH1 domain associates with numerous transcription factors and any inhibitor may serve as a negative regulator of these proteins as well, as was the case with STAT2 in the first study (Kung *et al.*, 2000).

An interesting finding is the identification of inhibitory PAS protein (IPAS) that consists of a bHLH and a PAS domain but no transactivation domain (Makino *et al.*, 2001). *In vitro* binding studies demonstrate that IPAS binds to HIF-1α but the resultant heterodimer does not associate with an HRE, suggesting that IPAS is a dominant negative regulator of HIF-1α (Makino *et al.*, 2001). IPAS mRNA was observed predominantly in the eye, suggesting a role in inhibiting angiogenesis in the cornea (Makino *et al.*, 2001). Also, not only did tumours derived from cells engineered to express IPAS display a slower growth rate and decreased vascular density when compared to those from wildtype cells, but primary corneal cells transfected with an antisense IPAS expression vector displayed enhanced hypoxic inducibility of VEGF mRNA (Makino *et al.*, 2001). Furthermore, expression of antisense IPAS in the cornea of mice induced angiogenesis of the cornea (Makino *et al.*, 2001). It is imperative that the cornea experiences little vascularisation as vessels would inhibit the passage of light to the retina. The identification of IPAS primarily in the eye suggests a mechanism by which HIF-1α induced angiogenesis is reduced in periods of localised hypoxia, such as during closure of the eyelids. Interestingly, IPAS is a splice variant of the poorly characterised HIF-3α paralogue (Gu *et al.*, 1998; Makino *et al.*, 2002). Accounting for this, it is odd that IPAS would associate with HIF-1α and not ARNT (Makino *et al.*, 2001), although this would seem preferable, given the involvement of ARNT in the function of other transcription factors. Nonetheless, if the association of IPAS binding capacity is limited to interactions with

HIF-α, IPAS or a similarly functioning variant could be administered as an antiangiogenic agent in newly established tumours.

The application of HIF-α upregulation also holds promise for the treatment of certain diseases. Strokes and heart attacks are caused by localised hypoxia manifested as cerebral and myocardial ischaemia, respectively. A preventative therapy for these conditions might involve HIF-α activated VEGF expression to induce neovascularisation of the target area (Semenza, 1998). This would provide increased blood flow and oxygen supply to disease prone areas of the brain and heart (where there has been any cerebrovascular or coronary occlusion, respectively) and would enhance protective responses to ischaemia. The exposure of animals to systemic hypoxia has been shown to protect them from the subsequent effects of induced cerebral and coronary ischaemia (Gidday *et al.*, 1994; Tajima *et al.*, 1994). VEGF mediated angiogenesis stimulated by activated HIF-α is the likely explanation for this. Recent studies have indeed shown that overexpressing HIF-1α in mice promoted enhanced vascularisation with few of the defects seen when VEGF alone is overexpressed, such as vascular leakage and inflammation (Elson *et al.*, 2001). Therefore, the administration of drugs that activate HIF-1α and induce VEGF controlled vascularisation of the effected area is a viable option in the treatment of ischaemic diseases. The recent discovery of hydroxylases as key oxygen sensors make them attractive targets as disruption of their activity should collectively stabilise and activate HIF-α thus inducing angiogenesis. The importance of two classes of hydroxylases and multiple genes also suggests that specific targeting may be able to achieve differential results.

1.7. DIFFERENTIAL FUNCTIONS OF HIF-1α AND HIF-2α

1.7.1. HIF-α TARGET GENES

HIF-1α and HIF-2α share 48% amino acid identity (Ema *et al.*, 1997). Both are stabilised and transcriptionally activated by hypoxia in a hydroxylase dependent manner and bind to HREs. However, there is strong evidence to suggest each protein possesses unique qualities and distinct biological roles and although the complement of direct HIF-1α target genes has been widely studied there are have been none conclusively identified for HIF-2α.

1.7.2. HIF-1α AND HIF-2α ARE NOT FUNCTIONALLY REDUNDANT

The hypothesis that HIF-1α and HIF-2α possess distinct functions is particularly evident in light of studies on HIF-1$\alpha^{-/-}$ or HIF-2$\alpha^{-/-}$ mice. HIF-1$\alpha^{-/-}$ mouse embryos die by E11 as a consequence of multiple defects of cardiovascular development as well as heart malformation and incomplete neural tube closure (Iyer *et al.*, 1998; Ryan *et al.*, 1998). Furthermore, in HIF-1$\alpha^{-/-}$ embryonic stem (ES) cells exposed to 20% O_2 (normoxia), the mRNA levels of glucose transporters and glycolytic enzymes and the rate of cell division are decreased compared to those of wild type ES cells (Iyer *et al.*, 1998). When exposed to 1% O_2 (hypoxia) mRNA levels of VEGF are lower than those of similarly treated wildtype cells (Iyer *et al.*, 1998). In contrast, a number of studies into HIF-2α null mice have been performed, all of which have resulted in the observation of different developmental phenotypes. In the first study, embryos lacking HIF-2α developed a normal circulatory system, though died as a result of blood congestion caused by bradycardia by E16.5 (Tian *et al.*, 1998). A role for HIF-2α in the synthesis and/or release of the heart rate mediating catecholamines has been hypothesised given that HIF-2α is highly expressed in the organ of Zuckerkandl (the primary source of embryonic catecholamine production) and that HIF-2α deficient embryos contain a significantly reduced level of catecholamines (Tian *et al.*, 1998). Embryonic lethality was rescued by the addition of DOPS (a precursor to norepinephrine) to the mother's diet, although the mice died within 24 hours of birth due to the discontinued supply of DOPS (Tian *et al.*, 1998). However, a second study implicates HIF-2α in vascular remodelling (Peng *et al.*, 2000). Here, HIF-2$\alpha^{-/-}$ mice died by E12.5. Although vascularisation of the embryo had taken place, there was inadequate fusion or subsequent vascular assembly (Peng *et al.*, 2000). A third report has associated HIF-2α loss in mice with an impediment in foetal lung maturation (Compernolle *et al.*, 2002). Here, the HIF-2$\alpha^{-/-}$ mice that did not die due to cardiac failure before E13.5 died shortly after birth due to respiratory distress syndrome. This was shown to be due to decreased VEGF expression in alveolar epithelia, resulting in insufficient lung surfactant production, and thus suggested that VEGF is a HIF-2α target gene. Recently, a further two reports into HIF-2$\alpha^{-/-}$ mice have been published. Here, HIF-2$\alpha^{-/-}$ adult mice were generated as a consequence of the partial survival to this point in development after the crossing of two different strains of HIF-2α heterozygous mice (Scortegagna *et al.*, 2003a; Scortegagna *et al.*, 2003b). One observed pathology in these mice was pancytopaenia (a reduction of all circulating blood cell types), thus implicating a role for HIF-2α in haemopoietic development (Scortegagna *et*

al., 2003a). Another was a mitochondrial dysfunction resulting from increased oxidative stress because of increased ROS, which in turn may explain the increased expression of genes induced by oxidative stress (such as *Ddit3* and metallothionein II) (Scortegagna *et al.*, 2003b). This was correlated with a downregulation of antioxidant enzyme (AOE) transcript levels (such as catalase, glutathione peroxidase and copper/zinc and manganese superoxide dismutase) (Scortegagna *et al.*, 2003b). Furthermore, it was shown that HIF-2α overexpressed in 293T cells from a mammalian expression construct enhanced the activity of a luciferase reporter fused downstream of the promoters of the aforementioned AOEs (Scortegagna *et al.*, 2003b). The difference in phenotypes and subsequently the postulated roles for HIF-2α in the studies may be a consequence of utilising different ES cell and mouse strains. Alternatively, it may be due to varying degrees of compensation by HIF-1α in the HIF-2α null mice. Nonetheless, the ubiquitous HIF-1α does not completely compensate for the lack of HIF-2α in any of the reports, and thus HIF-2α appears to perform a distinct role and be differentially regulated during embryogenesis. This may also be the case in the developed organism.

1.7.3. DIFFERENTIAL DNA BINDING ACTIVITY OF HIF-1α AND HIF-2α

It has been shown that the DNA binding activity of HIF-2α but not HIF-1α is regulated by reducing conditions. A cysteine in the DNA binding basic region of HIF-2α requires reduction, possibly by the redox factor Ref-1, prior to the activation of HIF-2α's DNA binding ability (Lando *et al.*, 2000). This is not so for HIF-1α, where serine is present in the equivalent position (the only difference between the amino acid sequences of their basic regions) and DNA binding is constitutive (Lando *et al.*, 2000). This identifies one mechanism of differential regulation of HIF-1α and HIF-2α activity.

1.7.4. HIF-2α AS A TUMOUR PROMOTER

The renal cell carcinoma 786-O line produces detectable levels of HIF-2α but not HIF-1α. As it expresses a non functioning truncated pVHL, this HIF-2α expression is not regulated by hypoxia (Maxwell *et al.*, 1999). Studies using the 786-O line have helped unravel HIF-2α's function *in vivo*. These demonstrate that the pVHL function as a tumour suppressor protein is linked at least partly to its ability to regulate HIF-α (Maranchie *et al.*, 2002; Kondo *et al.*, 2002). A fascinating observation was that expression of a HIF-1α P564A mutant in 786-O cells where wildtype pVHL had been stably reintroduced, was sufficient to recover the morphological phenotype observed in the originally pVHL-

deficient cells. However, the deregulated HIF-1α P564A could not increase the rate of tumour growth when these were injected into mice (Maranchie *et al.*, 2002). This is in contrast with wildtype 786-O cells (which display constitutive HIF-2α expression) or 786-O cells stably transfected with wildtype pVHL but also expressing a mutant of HIF-2α that cannot be prolyl-4-hydroxylated (HIF-2α P405/531A), both of which could cause the formation of tumours when injected into mice (Maranchie *et al.*, 2002; Kondo *et al.*, 2003). Furthermore, downregulation of HIF-2α via RNAi allowed for the inhibition of tumour formation that results from the introduction of 786-O cells into mice (Kondo *et al.*, 2003). At the most, this suggests a tumour promoting role for HIF-2α and not HIF-1α in the context of 786-O cells, given the implication of HIF-1α in tumour progression in previous studies. At the very least, this hints that HIF-1α transcriptional activity requires additional cofactors that are neither expressed in 786-Os nor necessary for HIF-2α function.

1.7.5. CYTOPLASMIC ENTRAPMENT OF ENDOGENOUS HIF-2α

A recent study proposes a novel signalling pathway for HIF-2α activation. In MEFs, it was shown that HIF-1α protein levels were undetectable at normoxia but were enhanced in hypoxia and the protein was entirely nuclear, whereas HIF-2α was easily detectable regardless of O_2 levels or pVHL presence and was primarily cytoplasmic (Park *et al.*, 2003). Furthermore, hypoxic upregulation of some classical HIF-α targets (PGK, Glut-1 and VEGF) and an HRE reporter was shown to occur in wildtype MEFs (where HIF-1α and HIF-2α are present), but not HIF-1α$^{-/-}$ (where only HIF-2α is expressed) (Park *et al.*, 2003). These observations are difficult to reconcile with all other studies. However, Park and coworkers do demonstrate that endogenous HIF-2α can function as a hypoxia inducible transcription factor in the human embryonic kidney 293 cell line and HIF-2α overexpressed in MEFs is also active, suggesting that the cytoplasmic entrapment of endogenous HIF-2α in MEFs controls its activity as a transcription factor (Park *et al.*, 2003). So what is the purpose of endogenous HIF-2α in MEFs? Maybe it is a transcription factor but requires an as yet unknown mechanism to translocate to the nucleus and upregulate levels of target transcripts, a hypothesis that can be clearly tested once genes directly mediated by HIF-2α have been identified. Interestingly, a nuclear localisation sequence of HIF-2α is present within its ID (Hara *et al.*, 1999; Luo *et al.*, 2001) and overexpression of this ID increases HIF-1α transactivation (Sang *et al.*, 2002). It is possible that a function of the ID is to recruit a protein that inhibits nuclear translocation

(and as a consequence transactivation) by concealing the required sequence of amino acids and that this protein has a specific or increased affinity for HIF-2α over HIF-1α at least in MEFs (Park *et al.*, 2003). What is certain is that many qualities of HIF-2α, and for that matter HIF-1α, are different and depend on cellular context.

1.7.6. PROPOSED HIF-2α TARGET GENES

As mentioned previously, there are no conclusively identified HIF-2α specific target genes. This is not to say however that HIF-2α has not been proposed to function as a transactivator of a selection of genes (table 1.2). These will be discussed in more detail in chapter 3. However, it will suffice to mention here that in each case either HIF-2α has not been convincingly established as the direct transcription factor responsible for mediating mRNA expression, the gene described is also a characterised HIF-1α target or, in situations where both HIF-1α and HIF-2α are expressed, HIF-2α cannot be classified as the specific transactivator responsible for their hypoxic upregulation.

1.7.7. SELECTIVE EXPRESSION OF HIF-1α AND HIF-2α AS A MODEL TO DETERMINE THEIR DIFFERENTIAL REGULATION AND ROLES

Although all of these studies provide clues as to the differential roles of HIF-1α and HIF-2α, more work is required to understand the mechanisms of differential activation, specific target genes and their pre and post natal roles. In particular, the distinct physiological roles of HIF-1α and HIF-2α suggest that HIF-2α has a separate set of target genes to HIF-1α. Therefore, to entirely understand the significance of these factors in the regulation of oxygen homeostasis, especially in the developed organism and in the context of disease, it is imperative to identify and characterise these target genes. This may be achieved by employing a model system in which HIF-1α or HIF-2α can be selectively expressed and where their respective downstream effects can be analysed and compared. The oxygen sensing rat pheochromocytoma PC12 cell line is ideal to form the basis of such a system.

1.8. PC12 CELLS

1.8.1. SIMILARITY OF PC12 CELLS TO THE TYPE I CAROTID BODY

As exemplified by the ubiquitous presence of HIF-1α, all cells are sensitive to changes in oxygen levels and can respond accordingly. In addition to responding to hypoxia by an increase in glycolysis and glucose transportation, some cell types serve to adapt some or

Table 1.2. Proposed HIF-2α target genes.

There are no conclusively identified HIF-2α target genes, although a number have been proposed. However, in each case, either HIF-2α has not been conclusively identified as the direct transactivator, the gene described is also a characterised HIF-1α target or, where both HIF-1α and HIF-2α are expressed in the system analysed, HIF-2α cannot be classified as the specific transactivator responsible for their hypoxic upregulation.

Proposed HIF-2α target gene	Reference
adrenomedullin	Tanaka *et al.*, 2002; Takeda *et al.*, 2004
α_{1H} gene of the T type voltage gated calcium channel	Del Toro *et al.*, 2003
erythropoietin	Morita *et al.*, 2003; Warnecke *et al.*, 2004
Flk-1	Kappel *et al.*, 1999
Flt-1	Takeda *et al.*, 2004
HIF-α prolyl-4-hydroxylase 2	del Peso *et al.*, 2003
HIF-α prolyl-4-hydroxylase 3	Aprelikova *et al.*, 2004
plasminogen activator inhibitor 1	Sato *et al.*, 2004
Tie-2	Tian *et al.*, 1997
tyrosine hydroxylase	Schnell *et al.*, 2003
vascular endothelial growth factor	Wiesener *et al.*, 1998; Compernolle *et al.*, 2002; Hu *et al.*, 2004

all of the rest of the body to hypoxia by improving the efficiency of oxygen delivery to the effected areas. For instance, the liver and kidneys generate and secrete erythropoietin in response to reduced oxygen, which in turn increases red blood cells and the capacity to circulate O_2 throughout an organism (Goldberg *et al.*, 1988). The type I cells of the carotid body detect changes in arterial O_2 levels and ultimately signal the central nervous system to increase or decrease ventilation of the lungs and cardiac output via oxygen sensitive catecholamine synthesis and release (Paulding *et al.*, 2002).

Studies into type I carotid body cells are hindered by the absence of immortalised lines of this type. The PC12 cell line derived from a rat pheochromocytoma (Greene and Tischler, 1976) has long been established as a model for type I carotid body cells, specifically in studies into their physiological responses to changes in O_2 levels, as they share a number of significant characteristics (Seta *et al.*, 2002). For instance, their immediate response to hypoxia is a rapid depolarisation caused by the inhibition of oxygen sensitive K^+ channels (Conforti and Millhorn, 1997; López-Barneo *et al.*, 1988), followed by an increase in intracellular Ca^{2+} (Zhu *et al.*, 1996; Buckler and Vaughan-Jones, 1994). Most significantly in the context of examining differences between HIF-1α and HIF-2α, both cell types synthesise and release catecholamines in response to hypoxia (Taylor and Peers, 1998; Kumar *et al.*, 1998; Fishman *et al.*, 1985).

Of the enzymes of the catecholamine synthesis and release pathway in both PC12 and type I carotid body cells, TH is involved in the rate limiting step (the conversion of tyrosine to dopamine) and is thus considered to be a marker of dopaminergic cells (Kumer and Vrana, 1996). TH mRNA is present and hypoxically inducible in both PC12 cells (Czyzyk-Krzeska *et al.*, 1994a) and the type I carotid body (Czyzyk-Krzeska *et al.*, 1992). This further emphasises the similarity between the two systems and justifies the use of the former as a model for the latter. Indeed, PC12 cells have been used to determine the biochemical characteristics of TH with the inference that it is similarly regulated in the carotid body. There is evidence to suggest that hypoxia serves to enhance the stability of the TH transcript. Specifically, a pyrimidine rich tract in the 3' untranslated region of the mRNA was found to bind poly C binding protein (PCBP) in a hypoxically inducible manner (Czyzyk-Krzeska *et al.*, 1994b; Czyzyk-Krzeska and Beresh, 1996; Paulding and Czyzyk-Krzeska, 1999), mutations of which ablate PCBP binding and result in decreased steady state TH mRNA levels and an abolishment of hypoxia inducible TH mRNA

stability (Paulding and Czyzyk-Krzeska, 1999). TH upregulation upon reduced oxygen levels also appears to involve an increase in its transcription. The TH gene contains an HRE which confers O_2 responsiveness and displays hypoxically inducible protein binding (Norris and Millhorn, 1995). Subsequent studies demonstrated HIF-1α and HIF-2α binding to and mediating transcription from this HRE and thus their involvement in hypoxia inducible TH transcription (Schnell *et al.*, 2003). This latter observation is of relevance to the research described in this thesis as it implies that HIF-2α may target TH for transcriptional regulation, but to what extent when compared to HIF-1α is uncertain. Still, HIF-α mediated transcription appears at least partly responsible for the hypoxia induciblity of tyrosine hydroxylase in PC12 cells and may regulate catecholamine production and secretion in the type I carotid body.

1.8.2. PC12 CELLS AS A MODEL FOR DETERMINING DIFFERENTIAL ROLES OF HIF-1α AND HIF-2α

The emergence of the role of HIF-α in catecholamine production and secretion perhaps became most evident upon the publication of the results of the first HIF-2α null mouse, where the embryonic lethal phenotype is correlated with a significantly reduced level of catecholamines (Tian *et al.*, 1998). Even the two subsequent studies into HIF-$2\alpha^{-/-}$ mice where different phenotypes were observed do not discount the developmental importance of HIF-2α with regards to the catecholamine pathway. In the first instance, (Peng *et al.*, 2000), some of the mice whose mothers' diets were supplemented with DOPS survived until birth. Specifically, where mothers were not receiving DOPS, all mutant embryos that survived did so for several weeks with no death after 24 hours. However, three of the eleven live born mice whose mothers were acquiring an intake of DOPS died within 24 hours – a phenotype observed during the study into the initial null mice (Tian *et al.*, 1998). In another study (Compernolle *et al.*, 2002), where many mice die postnatally, it was observed that a number of mice still die prenatally due to cardiac failure. Even in the study into the fourth HIF-2α null mouse, it is proposed that the increase in ROS due to HIF-2α elimination may be the cause of the reduction in catecholamine levels and subsequent bradycardia in the first HIF-2α null mouse (Tian *et al.*, 1998), given that ROS have been shown to directly inhibit tyrosine hydroxylase activity and general catecholamine synthesis (Ischiropoulos *et al.*, 1995; Kuhn *et al.*, 1999). Collectively, the studies into the null mice of both HIF-1α and HIF-2α serve as perhaps the best evidence

that they have differential functions and that most at least hint at the involvement of HIF-2α in catecholamine synthesis and release.

It would therefore seem logical to use a catecholaminergic cell line to determine HIF-2α specific modes of control and target genes. PC12 cells express both HIF-1α and HIF-2α and their respective protein products are upregulated in hypoxia (Conrad *et al.*, 1999; Agani *et al.*, 2000), although little is known with regards to differential regulation and function in the context of this cell line. In short, it is proposed that PC12 cells would serve as a physiologically and biochemically relevant cell line with which to study differences in HIF-1α and HIF-2α given this line's ability to express both these proteins and its similarity to the organ where HIF-2α is suggested to perform its most crucial function.

1.9. AIMS AND APPROACH

The fundamental aim of the research described in this thesis was to determine the differential function and regulation of HIF-1α and HIF-2α using the PC12 cell line. This was intended to be collectively satisfied via the completion of two separate investigations.

The first aim was to use PC12 cells to identify HIF-2α specific target genes. This would involve a study into the complement of genes regulated by HIF-1α and/or HIF-2α in PC12 cells and entailed the generation of monoclonal derivatives in which HIF-1α or HIF-2α could be selectively stabilised and activated. Northern analysis of these lines for the expression of some known HIF-1α target genes was employed to examine whether these could be also regulated by HIF-2α. Also, DNA microarray analysis was employed using lines to identify novel putative HIF-2α target genes which may relate to the physiological role of this transcription factor.

The second aim was to identify and characterise the differences in the mechanisms of regulation HIF-1α and HIF-2α in PC12 cells. Chemicals known to interfere with the regulatory mechanisms of both HIF-1α and HIF-2α were used. In addition, functional studies employing RNAi directed towards or the introduction and subsequent overexpression of the regulatory enzymes were employed to show that the stabilisation of HIF-1α and HIF-2α are mediated by reactions that are not necessarily mutual.

CHAPTER 2

METHODS AND MATERIALS

CHAPTER 2

METHODS AND MATERIALS

2.1. ABBREVIATIONS

AMV	avian myeloblastosis virus	M	molar
APS	ammonium persulphate	µg	microgram
ATP	adenosine triphosphate	µL	microlitre
BSA	bovine serum albumin	µM	micromolar
bp	base pair	mL	millilitre
cDNA	complementary DNA	MOPS	3-(N-Morpholino)propanesulfonic acid
Ci	curie	mRNA	messenger RNA
cpm	counts per minute	ng	nanogram
DEPC	diethylpyrocarbonate	OD_{600}	optical density at 600 nanometres
DMEM	Dulbecco's modified Eagle's medium	PAGE	polyacrylamide gel electrophoresis
DMSO	dimethylsulphoxide	PBS	phosphate buffered saline
DNA	deoxyribonucleic acid	PCR	polymerase chain reaction
dNTP	deoxyribonucleoside triphosphate	PMSF	phenylmethyl sulphonyl fluoride
DTT	dithiothreitol	RNA	ribonucleic acid
EDTA	ethylene diamine tetraacetic acid	RT	room temperature
EMSA	electrophoretic mobility shift assay	RTPCR	reverse transcription PCR
FCS	foetal calf serum	RPM	revolutions per minute
g	gram	SDS	sodium dodecyl sulphate
HRP	horse radish peroxidase	TBE	Tris/borate/EDTA
HS	horse serum	TE	Tris/EDTA
IPTG	isopropyl-β-D-thiogalactopyranoside	TEMED	N,N,N',N'-tetramethylethylenediamine
kb	kilobase	TES	tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
kDa	kilodalton	Tris	tris(hydroxymethyl)aminomethane
L	litre	UV	ultraviolet
LB	luria broth	X gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

2.2. MATERIALS

2.2.1 GENERAL MATERIALS

3MM chromatography (Whatman) paper	Whatman

Anaero*Gen* sachet	Oxoid
Corex tubes	Corex
bottle top filters (0.22 or 0.45 μM pore)	Corning
Cryotube vials	Nunc
disposable pipettes (2, 10 and 25 mL)	Falcon
glass pipettes	Chase Instruments
Minisart syringe top 0.2 or 0.45 μM filters	Sartorius
needles (various gauges)	Terumo
Nytran N nylon transfer membrane	Schleicher and Schuell
ProbeQuant G-50 Micro Columns	Amersham Pharmacia
Protran nitrocellulose transfer membrane	Schleicher and Schuell
scalpel blades	Swann Morton
sealed plastic container	ClickClack
syringes (various volumes)	Becton Dickinson
tissue culture vessels (various volumes)	Falcon
X ray film	Agfa

2.2.2 CHEMICALS AND REAGENTS

Accugel 40% acrylamide	National Diagnostics
ATP	Sigma-Aldrich
agarose (DNA grade)	Sigma-Aldrich
ampicillin	Sigma-Aldrich
apoprotinin	Sigma-Aldrich
APS	BDH Chemicals
Bacto-agar	Difco Labs
Bacto-tryptone	Difco Labs
Benchmark prestained protein markers	Invitrogen
bestatin	Sigma-Aldrich
BigDye Version 3	Amersham
BSA	Sigma-Aldrich
Bradford protein assay reagent	Bio-Rad
bromophenol blue	Sigma-Aldrich
coumaric acid	Sigma-Aldrich
desferrioxamine	Sigma-Aldrich
DMEM	Invitrogen/Gibco BRL
DMSO	Sigma-Aldrich
1kb DNA Plus Ladder	Life Technologies
dNTPs	Finnzymes
2, 2'-dipyridyl	Sigma-Aldrich
DTT	Sigma-Aldrich
EDTA	Sigma-Aldrich

ethidium bromide	Sigma-Aldrich
formaldehyde	BDH AnalaR
FCS	JRH Biosciences
formamide	BDH AnalaR
L-glutamine	Sigma
herring sperm DNA	Roche
HS	CSL
	JRH Biosciences
IGEPAL	Sigma-Aldrich
IPTG	Sigma-Aldrich
leupeptin	Sigma-Aldrich
Lipofectamine 2000	Gibco BRL
luminol	Sigma-Aldrich
methylene blue	Sigma-Aldrich
MG132	BioMol
oligo dT cellulose	Roche/Ambion
Optiscint Scintillation Fluid	Wallace UK
pepstatin	Sigma-Aldrich
paraformaldehyde	BDH AnalaR
10 X Pfu Turbo polymerase buffer	Stratagene
PMSF	Sigma-Aldrich
Rapid Hyb	Amersham Pharmacia
Restriction enzyme buffers	New England Biolabs
RNaseZAP	Ambion
RNAZol B	Tel-Test
Sigmacote	Sigma-Aldrich
SDS	Sigma-Aldrich
skim milk powder	Diploma
sodium azide	Sigma-Aldrich
SuperasIN RNase inhibitor	Ambion
10 X T4 DNA ligase buffer	New England Biolabs
TEMED	Sigma-Aldrich
tRNA	Roche
Trypan Blue	Sigma-Aldrich
trypsin-EDTA	Gibco BRL
Triton X 100	Sigma-Aldrich
Tween 20	Sigma-Aldrich
X gal	BioVectra
Yeast extract	Difco Labs

2.2.3 KITS

BRESAclean DNA Purification Kit	Geneworks
Dual Luciferase Reporter Assay System	Promega
pGEMTEASY vector system	Promega
RNeasy Midi Kit	Qiagen

2.2.4 ENZYMES

AMV reverse transcriptase	Sigma-Aldrich
Klenow fragment	Geneworks
lysozyme	Sigma-Aldrich
Pfu Turbo	Stratagene
proteinase K	Roche
restriction endonucleases	New England Biolabs
	Geneworks
shrimp alkaline phosphatase	USB
Taq polymerase	Geneworks
	New England Biolabs
T4 DNA ligase	Geneworks
	New England Biolabs
T4 polynucleotide kinase	Geneworks

2.2.5. RADIOCHEMICALS

^{32}P-dATP	Geneworks
	Perkin Elmer

2.2.6. ANTIBODIES

HRP conjugated goat anti rabbit immunoglobulins	DAKO
	Pierce
monoclonal mouse anti Myc (9E10)	Institute of Medical and Veterinary Sciences
polyclonal rabbit anti ARD1 amino acids 216-235	Rebecca Bilton
polyclonal rabbit anti ARNT amino acids 666-774	Murray Whitelaw
polyclonal rabbit anti HIF-1α amino acids 727-826	Dan Peet
polyclonal rabbit anti HIF-2α amino acids 612-823	Dan Peet
polyclonal rabbit anti HIF-2α amino acids 632-646	Novus Biologicals
polyclonal rabbit anti PHD1	Novus Biologicals
polyclonal rabbit anti PHD2	Richard Bruick
polyclonal rabbit anti PHD3	Novus Biologicals

2.2.7. BUFFERS AND SOLUTIONS

1st strand buffer. 500 mM Tris (pH 8.5), 500 mM potassium chloride, 100 mM magnesium chloride, 1 mM DTT, 10 μg/mL BSA.

Blocking buffer. 10% skim milk powder in milli Q H_2O.

100 X Denhardt's. 20 g Ficoll 400, 20 g gelatin, 20 g polyvinylpyrrolidine to 1 L with milli Q H_2O.

Enhanced chemiluminescence reagent 1. 100 mM Tris (pH 8.5), 2.5 mM luminol, 400 μM coumaric acid.

Enhanced chemiluminescence reagent 2. 100 mM Tris (pH 8.5), 0.2% hydrogen peroxide.

6 X loading buffer. 0.5 X TBE, 40% glycerol, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol.

Loading buffer for dried RNA. 48% formamide, 6.4% formaldehyde, 1 X MOPS, 5.3% glycerol, 0.05% methylene blue.

Lysis buffer. 200 mM sodium hydroxide, 0.1% SDS.

oligo dT binding buffer. 500 mM sodium chloride, 10 mM Tris pH (pH 7.2), 0.1 mM EDTA, 0.2% SDS.

oligo dT elution buffer. 10 mM Tris (pH 7.2), 0.1 mM EDTA, 0.2% SDS.

oligo dT lysis buffer. 500 mM sodium chloride, 10 mM Tris (pH 7.2), 10 mM EDTA, 1% SDS, 200 μg/mL proteinase K.

oligo dT wash buffer. 100 mM sodium chloride, 10 mM Tris (pH 7.2), 0.1 mM EDTA, 0.2% SDS.

PBS. 20 mM sodium phosphate (pH 7.6), 137 mM sodium chloride.

PBT. PBS, 0.1% Tween 20.

3 M/5 M potassium acetate. 3 M potassium, 5 M acetate.

Prehybridisation buffer. 50% formamide, 5 X SSC, 50 mM potassium phosphate pH 6.5, 5 X Denhardt's solution, 0.1% SDS, 0.2 mg/mL tRNA, 0.1 mg/mL herring sperm DNA.

100 X protease inhibitor cocktail. 200 μg/mL apoprotinin, 400 μg/mL of bestatin, 500 μg/mL of leupeptin, 100 μg/mL of pepstatin, 150 mM EDTA.

Resuspension buffer. 25 mM Tris (pH 7.5), 10 mM EDTA, 0.9% glucose, 2 mg/mL lysozyme.

10 X SDS running buffer. 250 mM Tris (pH 8.3), 2 M glycine, 1% SDS.

2 X SDS sample buffer. 62.5 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 2 mM EDTA, 1.6 mM DTT, 50 µg/mL bromophenol blue.

4 X SDS separation buffer. 1.5 M Tris (pH 8.8), 0.4% SDS.

20 X SSC. 175.3 g sodium chloride, 88.23 g trisodium citrate, to 1 L with milli Q H_2O. Adjusted to pH 7.2 with citric acid.

4 X SDS stacking buffer. 0.5 M Tris (pH 6.8), 0.4% SDS.

10 X Taq polymerase buffer. 200 mM Tris (pH 8.5), 500 mM potassium chloride.

TEN. 40 mM Tris (pH 7.5), 10 mM EDTA, 150 mM sodium chloride.

10 X TM buffer. 100 mM Tris (pH 8.0), 100 mM magnesium chloride.

TFBI. 30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15 % glycerol. Adjusted to pH 5.8 with acetic acid and filter sterilised.

TFBII. 10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15 % glycerol. Adjusted to pH 6.5 with potassium hydroxide and filter sterilised.

Tris tricine running buffer. 50 mM Tris base, 100 mM tricine, 0.1% SDS.

Western transfer buffer (for semi dry transfer). 380 mM glycine, 50 mM Tris base, 20% methanol.

Western transfer buffer (for wet transfer). 380 mM glycine, 50 mM Tris base.

Whole cell extract buffer. 20 mM HEPES (pH 7.8), 0.42 M sodium chloride, 0.5% IGEPAL CA-630, 25% glycerol, 0.2 mM EDTA, 1.5 mM magnesium chloride, 1mM DTT, 1 mM PMSF, 1 X protease inhibitor cocktail.

2.2.8. PLASMIDS

2.2.8.1. CLONING VECTORS

pET-32a/HIF-1α. From Anthony Fedele, University of Adelaide, Australia.

pHIF-1α/Bluescript. From Murray Whitelaw, University of Adelaide, Australia.

pHIF-1αP564A/Bluescript. From Sarah Linke, University of Adelaide, Australia.

pHLF/SL301. From David Lando, University of Adelaide, Australia.

pGEMTEASY. Supplied by Promega. This plasmid is prelinearised with *Eco*RV. The addition of 3' terminal thymidines permits its direct ligation with the product of a Taq PCR.

2.2.8.2. MAMMALIAN EXPRESSION VECTORS

pcDNA3.1/HPH1. From Richard Bruick, University of Texas Southwestern Medical Center, U.S.A.

pcDNA3.1/HPH2. From Richard Bruick, University of Texas Southwestern Medical Center, U.S.A.

pcDNA3.1/HPH3. From Richard Bruick, University of Texas Southwestern Medical Center, U.S.A.

pEFBOS. From Jerome Langer, Robert Wood Johnson Medical School, U.S.A.

pEFBOS/HIF-1α. From Sarah Linke, University of Adelaide, Australia.

pEF/HisMycHIF-1α/IRES/PURO. From Daniel Peet, University of Adelaide, Australia.

pEFBOS/HIF-1αN803A. From Sarah Linke, University of Adelaide, Australia.

pEFBOS/HIF-2α. From Anthony Fedele, University of Adelaide, Australia.

pEF/IRES/NEO. From Michael Lees, University of Adelaide, Australia.

pEF/IRES/PURO. From Steve Hobbs, Institute of Cancer Research, Surrey, U.K.

pEF/IRES/PURO5. From Daniel Peet, University of Adelaide, Australia.

pEF/IRES/PURO6. From Daniel Peet, University of Adelaide, Australia.

pEF/TetON/IRES/PURO. From Michael Lees, University of Adelaide, Australia.

pGal4DBD. From George Muscat, University of Queensland, Australia.

pGal4DBD/HIF-1αCAD. From Dan Peet, University of Adelaide, Australia

pGal4DBD/HIF-1αCADN803A. From Sarah Linke, University of Adelaide, Australia

pSUPER. Supplied by Oligoengine. Allows for an introduced oligonucleotide to be transcribed under the control of the H1 RNA promoter.

pTR/DC/EYFP. From Steven Finney, University of Adelaide, Australia.

2.2.8.3. MAMMALIAN REPORTER VECTORS

pBI/GL. Supplied by Clontech (now BD Biosciences). Contains the tetracycline response element (that is, seven copies of the tet operator sequence) between two minimal CMV promoters, one of which controls the expression of β galactosidase in one direction and the other mediates that of *Photinius pyralus* (North American firefly) luciferase expression in the opposite direction.

pGRELUC. From George Muscat, University of Queensland, Australia. Contains five Gal 4 response elements upstream of an E1b promoter, which in turn drives the transcription of the North American firefly luciferase reporter gene.

pGL3. Supplied by Promega. Contains an SV40 promoter upstream of the North American firefly luciferase reporter gene.

pHRE$_4$GL3. From Yoshiaki Fujii-Kuriyama, Tohoku University, Japan. Identical to the pGL3 vector, except for the insertion of four hypoxic response elements immediately upstream of the SV40 promoter.

pRLTK. Supplied by Promega. Contains the herpes simplex virus thymidine kinase promoter upstream of the *Renilla reniformis* (sea pansy) luciferase reporter gene.

2.2.9. OLIGONUCLEOTIDES

2.2.9.1. OLIGONUCLEOTIDES FOR GENERATION OF PROBES FOR NORTHERN ANALYSIS

aldo a sense. 5' GTC ATC CTC TTC CAT GAG AC 3'. A forward primer designed to amplify nucleotides 1236-1678 of human aldolase a (GenBank accession number NM_000034).

aldo a antisense. 5' AGG TAG ATG TGG TGG TCA CT 3'. A reverse primer designed to amplify nucleotides 1236-1678 of human aldolase a (GenBank accession number NM_000034).

β actin upper. 5' CTG GCA CCA CAC CTT CTA C 3'. A forward primer designed to amplify nucleotides 327-564 of human β actin (GenBank accession number BC013835).

β actin lower. 5' GGG CAC AGT GTG GGT GAC 3'. A reverse primer designed to amplify nucleotides 327-564 of human β actin (GenBank accession number BC013835).

BARD1 upper. 5' GGA GAA ATC CGT GAG CCA AC 3'. A forward primer designed to amplify nucleotides 1019-1220 of rat BARD1 (GenBank accession number AF182946).

BARD1 lower. 5' AGG TAG ATG TGG TCA CT 3'. A reverse primer designed to amplify nucleotides 1019-1220 of rat BARD1 (GenBank accession number AF182946).

Ca^+ upper. 5' ACA GAT CTA AAG CCC TGG TGC 3'. A forward primer designed to amplify nucleotides 561-1221 of rat voltage gated calcium channel $\alpha_2\delta$-1 subunit (GenBank accession number AF286488).

Ca^+ lower. 5' ACA TTC GCT TGA ACC AGG TGC 3'. A reverse primer designed to amplify nucleotides 561-1221 of rat voltage gated calcium channel $\alpha_2\delta$-1 subunit (GenBank accession number AF286488).

C/ACP upper. 5' ACC ATC GAC TGT TTC CGG AAG 3'. A forward primer designed to amplify nucleotides 220-840 of rat carnitine/acylcarnitine carrier protein (GenBank accession number X97831).

C/ACP lower. 5' TCC TTC TTC TCG GAT CAG CTC 3'. A reverse primer designed to amplify nucleotides 220-840 of rat carnitine/acylcarnitine carrier protein (GenBank accession number X97831).

CPT1 upper. 5' CTG GAA TCT GTA AGG CCA CTG 3'. A forward primer designed to amplify nucleotides 661-1080 of rat carnitine palmitoyltransferase 1 (GenBank accession number L07736).

CPT1 lower. 5' TTG GAT GGT GTC TGT CTC CTC 3'. A reverse primer designed to amplify nucleotides 661-1080 of rat carnitine palmitoyltransferase 1 (GenBank accession number L07736).

GIIG11 upper. 5' GAG TTG TGT TTG AGA AGC TG 3'. A forward primer designed to amplify nucleotides 202-430 of rat global ischaemia inducible protein 11 (GenBank accession number AF324255).

GIIG11 lower. 5' AAT AAG CTC TAA GAG AAT TC 3'. A reverse primer designed to amplify nucleotides 202-430 of rat global ischaemia inducible protein 11 (GenBank accession number AF324255).

hENO1 upper. 5' GTC TCA AAG GCT GTT GAG CAC 3'. A forward primer designed to amplify nucleotides 335-724 of human enolase 1 (GenBank accession number NM_001428).

hENO1 lower. 5' GTT GTG GTA AAC CTC TGC TCC 3'. A reverse primer designed to amplify nucleotides 335-724 of human enolase 1 (GenBank accession number NM_001428).

hGT1 upper. 5' CAC CAC GCT CAC CAC GCT CTG 3'. A forward primer designed to amplify nucleotides 353-852 of human glucose transporter 1 (GenBank accession number K03195).

hGT1 lower. 5' TGG CCC GGT TCT CCT CGT TAC 3'. A reverse primer designed to amplify nucleotides 353-852 of human glucose transporter 1 (GenBank accession number K03195).

hTH upper. 5' GTC CCC TGG TTC CCA AGA AAA 3'. A forward primer designed to amplify nucleotides 589-1436 of human tyrosine hydroxylase (GenBank accession number NM_199292).

hTH lower. 5' CTG AGC TTG TCC TTG GCG TCA 3'. A reverse primer designed to amplify nucleotides 589-1436 of human tyrosine hydroxylase (GenBank accession number NM_199292).

hVEGF upper. 5' GCC TTG CTG CTC TAC CTC CAC 3'. A forward primer designed to amplify nucleotides 37-513 of human VEGF (GenBank accession number AJ010438).

hVEGF lower. 5' CAA ATG CTT TCT CCG CTC TGA 3'. A reverse primer designed to amplify nucleotides 37-513 of human VEGF (GenBank accession number AJ010438).

INrf2 upper. 5' ATG CAG CCC GAA CCC AAG CCT 3'. A forward primer designed to amplify nucleotides 78-580 of rat cytosolic inhibitor of Nrf2 (GenBank accession number AF304364).

INrf2 lower. 5' ACC ACG CTG TCA ATC TGG TAC 3'. A reverse primer designed to amplify nucleotides 78-580 of rat cytosolic inhibitor of Nrf2 (GenBank accession number AF304364).

Na^+ upper. 5' ATG CCT GCC TTC AAC AGA TTG 3'. A forward primer designed to amplify nucleotides 363-1010 of rat voltage gated sodium channel β3 subunit (GenBank accession number AJ243395).

Na^+ lower. 5' TTA TTC CTC CAC AGG TAC CAC 3'. A reverse primer designed to amplify nucleotides 363-1010 of rat voltage gated sodium channel β3 subunit (GenBank accession number AJ243395).

NETa/b upper. 5' CCT GTA CTG GAG ACT GTG TTG 3'. A forward primer designed to amplify nucleotides 1584-1932 of rat norepinephrine transporter a (GenBank accession number AB021970) and 1584-2112 rat norepinephrine transporter b (GenBank accession number AB021971).

NETa/b lower. 5' ACA GTA GAG CAA GGA AGG CAC 3'. A reverse primer designed to amplify nucleotides 1584-1932 of rat norepinephrine transporter a (GenBank accession number AB021970) and 1584-2112 rat norepinephrine transporter b (GenBank accession number AB021971).

PGHα upper. 5' TAT GCG GCT GTC ATT CTG GTC 3'. A forward primer designed to amplify nucleotides 80-406 of rat pituitary glycoprotein hormone α subunit precursor (GenBank accession number D00575).

PGHα lower. 5' ACA AGT GCT ACA GTG GCA GTC 3'. A reverse primer designed to amplify nucleotides 80-406 of rat pituitary glycoprotein hormone α subunit precursor (GenBank accession number D00575).

PTTG upper. 5' GTA AAA CCC CTG CAA TCG AAA 3'. A forward primer designed to amplify nucleotides 515-715 of rat pituitary tumour transforming gene (GenBank accession number U73030).

PTTG lower. 5' CAC TCC ATT CAA GGG GAG AA 3'. A reverse primer designed to amplify nucleotides 515-715 of rat pituitary tumour transforming gene (GenBank accession number U73030).

rARD1 upper. 5' ATG AAC ATC CGC AAT GCT AGG 3'. A forward primer designed to amplify the entire coding sequence of rat ARD1 (GenBank accession number XM_343842).

rARD1 lower. 5' CTA GGA GGC AGA ATC AGA GGC 3'. A reverse primer designed to amplify the entire coding sequence of rat ARD1 (GenBank accession number XM_343842).

Rgs4 upper. 5' ATG TGC AAA GGA CTC GCT GGT 3'. A forward primer designed to amplify nucleotides 110-727 of rat regulator of G protein signalling 4 (GenBank accession number NM_017214).

Rgs4 lower. 5' TTA GGC ACA CTG AGG GAC TAG 3'. A reverse primer designed to amplify nucleotides 110-727 of rat regulator of G protein signalling 4 (GenBank accession number NM_017214).

rLDHA upper. 5' ACA GTT GTT GGG GTT GGT GCT 3'. A forward primer designed to amplify nucleotides 173-552 of rat lactate dehydrogenase A (GenBank accession number NM_017025).

rLDHA lower. 5' ATC TTC CAA GCC ACG TAG GTC 3'. A reverse primer designed to amplify nucleotides 173-552 of rat lactate dehydrogenase A (GenBank accession number NM_017025).

rPHD1 upper. 5' ATC AAG CTC TCC CTC AGT TGC 3'. A forward primer designed to amplify nucleotides 32-591 of rat PHD1 (GenBank accession number AY229997).

rPHD1 lower. 5' ATA GTA CCG CAT GCA AGG CAC 3'. A reverse primer designed to amplify nucleotides 32-591 of rat PHD1 (GenBank accession number AY229997).

rPHD2 upper. 5' AAC TGG TCA GCC AGA AGA GTG 3'. A forward primer designed to amplify nucleotides 113-666 of rat PHD2 (GenBank accession number AY228140).

rPHD2 lower. 5' GCT GAC TGA ATT GGG CTT GAG 3'. A reverse primer designed to amplify nucleotides 113-666 of rat PHD2 (GenBank accession number AY228140).

rPHD3 upper. 5' ATG ACG TTG AGG TCA AGG CGG 3'. A forward primer designed to amplify nucleotides 190-879 of rat PHD3 (GenBank accession number NM_019371).

rPHD3 lower. 5' ACA TAG TAT TTG CCC AGG CGG 3'. A reverse primer designed to amplify nucleotides 190-879 of rat PHD3 (GenBank accession number NM_019371).

TAGE4 upper. 5' AAC TTG AGG TGA TGG TGC TGG 3'. A forward primer designed to amplify nucleotides 65-551 of rat tumour associated glycoprotein E4 (GenBank accession number AF125562).

TAGE4 lower. 5' TCG GTC TAG CTT TGG CTA CAC 3'. A reverse primer designed to amplify nucleotides 65-551 of rat tumour associated glycoprotein E4 (GenBank accession number AF125562).

VN upper. 5' ATG GCA TCT CTG AGG CCC TTT 3'. A forward primer designed to amplify nucleotides 70-708 of rat vitronectin (GenBank accession number U44845).

VN lower. 5' ACA GTT GAT GCG AGT GAA GGC 3'. A reverse primer designed to amplify nucleotides 70-708 of rat vitronectin (GenBank accession number U44845).

2.2.9.2. OLIGONUCLEOTIDES FOR QUICKCHANGE MUTATION

HIF P402A up. 5' GCT TTA ACT TTG CTA GCC GCC GCC GCT GGA GAC 3'. An upper primer designed to mutate P402 of human HIF-1α to alanine. It also introduces a silent mutation resulting in the generation of an *Nhe*I site.

HIF P402A lo. 5' GTC TTC AGC GGC GGC GGC TAG CAA AGT TAA AGC 3'. A lower primer designed to mutate P402 of human HIF-1α to alanine. It also introduces a silent mutation resulting in the generation of an *Nhe*I site.

2.2.9.3. OLIGONUCLEOTIDES FOR ANNEALING AND DIRECT CLONING INTO MAMMALIAN EXPRESSION VECTORS FOR RNA INTERFERENCE

ARD1 144 upper. 5' GAT CCC CTG GGA AGA TTG TGG GAT ACT TCA AGA GAG TAT CCC ACA ATC TTC CCA TTT TTG GAA A 3'. An upper primer designed to be annealed with ARD1 144 lower for direct cloning into pSUPER. It is specifically directed towards bases 144-164 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 144 lower. 5' AGC TTT TCC AAA AAT GGG AAG ATT GTG GGA TAC TCT CTT GAA GTA TCC CAC AAT CTT CCC AGG G 3'. A lower primer designed to be annealed with ARD1 144 upper for direct cloning into pSUPER. It is specifically directed towards bases 144-164 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 150 upper. 5' GAT CCC CGA TTG TGG GAT ACG TCT TGT TCA AGA GAC AAG ACG TAT CCC ACA ATC TTT TTG GAA A 3'. An upper primer designed to be annealed with ARD1 150 lower for direct cloning into pSUPER. It is specifically directed towards bases 150-170 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 150 lower. 5' AGC TTT TCC AAA AAG ATT GTG GGA TAC GTC TTG TCT CTT GAA CAA GAC GTA TCC CAC AAT CGG G 3'. A lower primer designed to be annealed with ARD1 150 upper for direct cloning into pSUPER. It is specifically directed towards bases 150-170 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 552 upper. 5' GAT CCC CCG TGC TTC TGA GCT CAG GAT TCA AGA GAT CCT GAG CTC AGA AGC ACG TTT TTG GAA A 3'. An upper primer designed to be annealed with ARD1 552 lower for direct cloning into pSUPER. It is specifically directed towards bases 552-572 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 552 lower. 5' AGC TTT TCC AAA AAC GTG CTT CTG AGC TCA GGA TCT CTT GAA TCC TGA GCT CAG AAG CAC GGG G 3'. A lower primer designed to be annealed with ARD1 552 upper for direct cloning into pSUPER. It is specifically directed towards bases 552-572 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 627 upper. 5' GAT CCC CGG ACC TCA GTG AGG TCA GTT TCA AGA GAA CTG ACC TCA CTG AGG TCC TTT TTG GAA A 3'. An upper primer designed to be annealed with ARD1 627 lower for direct cloning into pSUPER. It is specifically directed towards bases 627-647 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

ARD1 627 lower. 5' AGC TTT TCC AAA AAG GAC CTC AGT GAG GTC AGT TCT CTT GAA ACT GAC CTC ACT GAG GTC CGG G 3'. A lower primer designed to be annealed with ARD1 627 upper for direct cloning into pSUPER. It is specifically directed towards bases 627-647 of the rat ARD1 coding sequence (derived from GenBank accession number XM_343842).

PHD2 112 upper. 5' GAT CCC CCT GGT CAG CCA GAA GAG TGT TCA AGA GAC ACT CTT CTG GCT GAC CAG TTT TTG GAA A 3'. An upper primer designed to be annealed with PHD2 112 lower for direct cloning into pSUPER. It is specifically directed towards bases 112-132 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 112 lower. 5' AGC TTT TCC AAA AAC TGG TCA GCC AGA AGA GTG TCT CTT GAA CAC TCT TCT GGC TGA CCA GGG G 3'. A lower primer designed to be annealed with PHD2 112 upper for direct cloning into pSUPER. It is specifically directed towards bases 112-132 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 126 upper. 5' GAT CCC CGA GTG ACT CTT CCA AGG ACT TCA AGA GAG TCC TTG GAA GAG TCA CTC TTT TTG GAA A 3'. An upper primer designed to be annealed with PHD2 126 lower for direct cloning into pSUPER. It is specifically directed towards bases 126-146 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 126 lower. 5' AGC TTT TCC AAA AAG AGT GAC TCT TCC AAG GAC TCT CTT GAA GTC CTT GGA AGA GTC ACT CGG G 3'. A lower primer designed to be annealed with PHD2 126 upper for direct cloning into pSUPER. It is specifically directed towards bases 126-146 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 542 upper. 5' GAT CCC CCG TAC GCA ATA ACC GTT TGT TCA AGA GAC AAA CGG TTA TTG CGT ACC TTT TTG GAA A 3'. An upper primer designed to be annealed with PHD2 542 lower for direct cloning into pSUPER. It is specifically directed towards bases 542-562 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 542 lower. 5' AGC TTT TCC AAA AAG GTA CGC AAT AAC CGT TTG TCT CTT GAA CAA ACG GTT ATT GCG TAC CGG G 3'. A lower primer designed to be annealed with PHD2 542 upper for direct cloning into pSUPER. It is specifically directed towards bases 542-562 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 554 upper. 5' GAT CCC CCC GTT TGG TAT TTC GAT GCT TCA AGA GAG CAT CGA AAT ACC AAA CGG TTT TTG GAA A 3'. An upper primer designed to be annealed with PHD2 554 lower for direct cloning into pSUPER. It is specifically directed towards bases 554-574 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

PHD2 554 lower. 5' AGC TTT TCC AAA AAC CGT TTG GTA TTT CGA TGC TCT CTT GAA GCA TCG AAA TAC CAA ACG GGG G 3'. A lower primer designed to be annealed with PHD2 554 upper for direct cloning into pSUPER. It is specifically directed towards bases 554-574 of the rat PHD2 coding sequence (derived from GenBank accession number AY228140).

scrambled upper. 5' GAT CCC CAC TAC CGT TGT TAT AGG TGT TCA AGA GAC ACC TAT AAC AAC GGT AGT TTT TTG GAA A 3'. An upper primer designed to be annealed with scrambled lower for direct cloning into pSUPER. It is directed towards a succession of 21 bases, which has no significant sequence homology to any sequence in the human, mouse or rat genome.

scrambled lower. 5' AGC TTT TCC AAA AAA CTA CCG TTG TTA TAG GTG TCT CTT GAA CAC CTA TAA CAA CGG TAG TGG G 3'. A lower primer designed to be annealed with scrambled upper for direct cloning into pSUPER. It is directed towards a succession of 21 bases, which has no significant sequence homology to any sequence in the human, mouse or rat genome.

2.2.9.4. OLIGONUCLEOTIDES FOR SEQUENCING

BS M13-20 primer. 5' GTA AAA CGA CGG CCA GT 3'. A primer designed for the sequencing of inserts cloned into pBluescript.

BS reverse primer. 5' AAC AGC TAT GAC CAT G 3'. A primer designed for the sequencing of inserts cloned into pBluescript.

2.2.10. PROKARYOTIC STRAINS

E. coli* DH5α *endA1 hsdR17*($r_K^- m_K^-$) sup*E44 thi-1 recA1 gyrA* (Nal^r) *relA1 Δ(lacZYA-argF)*$_{U169}$*(m80lacZΔM15). A strain commonly used for subcloning.

2.2.11. PROKARYOTIC MEDIA

LA 100. LB supplemented with 100 μg/mL of ampicillin.

LA 100 agar. LA 100 supplemented with 1.5% agar.

LB. 1% tryptone, 0.5% yeast extract, 1% sodium chloride. Adjusted to pH 7 with sodium hydroxide.

LB agar. LB supplemented with 1.5% agar.

2.2.12. EUKARYOTIC LINES

PC12. A rat adrenergic pheochromocytoma derived cell line.

2.2.13. EUKARYOTIC MEDIA

PC12 medium. DMEM with 10% HS, 5% FCS, 100 U/mL penicillin and 100 U/mL streptomycin.

2.2.14. MISCELLANEOUS

5417C benchtop centrifuge	Eppendorf
Agarose gel system	Bio-Rad
Curix 60 developer	Agfa
FX molecular imager	Bio-Rad
Imaging plate	Fuji
Mini 2D wet transfer system	Bio-Rad
SDS PAGE Mini 2D gel system	Bio-Rad
Stratalinker 1800 UV crosslinker	Stratagene
TD 20/20 luminometer	Turner Designs
Transblot SD semi dry transfer cell	Bio-Rad

2.3. GENERAL METHODS

2.3.1. PREPARATION OF COMPETENT BACTERIA

A tube of competent bacteria stored at -80°C was thawed on ice and streaked onto an LB agar plate and incubated for 16 hours at 37°C. A loopful of colonies was then used to inoculate 500 mL of LB. This was incubated at 37°C until the OD_{600} of the culture was 0.4 to 0.5 and placed for 30 minutes on ice. The suspension was centrifuged at 4000 RPM and 4°C for 10 minutes, the supernatant discarded and the pellet resuspended in 200 mL of TFBI. After incubation for 5 minutes on ice, the suspension was centrifuged at 4000 RPM and 4°C for 10 minutes, the supernatant discarded and the pellet resuspended in 30 mL of TFBII. After incubation for 20 minutes on ice, the suspension was distributed among tubes (200 µL to each), snap frozen in a dry ice/ethanol bath and stored at -80°C.

2.3.2. DNA MANIPULATION

2.3.2.1. TRANSFORMATION OF BACTERIA WITH INTACT PLASMID

100 µL of competent bacteria were mixed with approximately 100 ng of plasmid DNA and incubated on ice for 5 minutes. This mixture was plated onto LA 100 agar plates and incubated for 16 hours at 37°C.

2.3.2.2. TRANSFORMATION OF BACTERIA WITH A LIGATION MIXTURE

100 µL of competent bacteria were mixed with 10 µL of a ligation mixture and incubated on ice for 20 minutes. This mixture was incubated at 42°C for 2 minutes prior to 10 minutes on ice. 100 µL of LB was added followed by incubation at 37°C for 1 hour. The mixture was plated onto LA100 plates and incubated for 16 hours at 37°C.

2.3.2.3. ALKALINE LYSIS PROTOCOL FOR PLASMID PREPARATION

4 mL of LA 100 was inoculated with a single bacterial colony from an LA 100 agar plate and incubated for 16 hours at 37°C. 1 mL of the culture was centrifuged at 6500 RPM for 1 minute. The supernatant was discarded and the cells resuspended in 100 µL of TES supplemented with 500 µg/mL RNase A. 200 µL of lysis buffer was added to the suspension and mixed and the tube was incubated for 5 minutes at RT. 150 µL of ice cold 3 M/5 M potassium acetate solution was added to precipitate chromosomal DNA and cellular debris. Incubation of this mixture for 30 minutes at 37°C was followed by

centrifugation at 14000 RPM for 5 minutes. The supernatant was transferred to a new tube, mixed with 1 mL of ice cold 95% ethanol and incubated at -20°C for 20 minutes. After centrifugation at 14000 RPM for 10 minutes, the 95% ethanol was removed and replaced with 70% ethanol. After a centrifugation at 10000 RPM for 3 minutes, the ethanol was discarded and the plasmid DNA dried. The pellet was resuspended in 1 X TE and its concentration and purity estimated on a 1% agarose/1 X TBE/ethidium bromide electrophoresis gel.

2.3.2.4. AMMONIUM ACETATE PROTOCOL FOR PLASMID PREPARATION

50 mL of LA100 was inoculated with a single bacterial colony from an LA 100 agar plate and grown for 16 hours at 37°C. The culture was transferred to an Oakridge tube and centrifuged at 10000 RPM and 4°C for 5 minutes. After removal of the supernatant, the pellet was dispersed in 1.2 mL of resuspension buffer and incubated for 20 minutes on ice. 2.4 mL of lysis buffer (containing 20 instead of 200 mM sodium hydroxide) was added followed by incubation for 10 minutes on ice. 1.8 mL of ice cold 7.5 M ammonium acetate (pH 7.6) was added to the mixture and after shaking there was 10 minutes incubation on ice. The sample was centrifuged at 10000 RPM and 4°C for 10 minutes. The supernatant was transferred to another Oakridge tube containing 3.25 mL of isopropanol, mixed by inversion, incubated at RT for 10 minutes and centrifuged at 10000 RPM and 4°C for 10 minutes. The supernatant was removed and the crude plasmid DNA was resuspended in 700 µL of 2 M ammonium acetate (pH 7.4) and transferred to a new tube. After incubation for 10 minutes on ice, the mixture was centrifuged at 10000 RPM for 10 minutes and the supernatant transferred to a new tube containing 700 µL of isopropanol. This mixture was incubated for 10 minutes at RT and centrifuged at 10000 RPM for 10 minutes. The supernatant was removed and the pellet dispersed in 400 µL of milli Q H_2O. 1 µL of 5 mg/mL RNase A was added and the mixture was incubated for 20 minutes at 37°C, after which 200 µL of 7.5 M ammonium acetate (pH 7.6) was aliquotted. The mixture was incubated for 5 minutes at RT and centrifuged at 10000 RPM for 10 minutes. The supernatant was transferred to a new tube containing 600 µL of isopropanol and incubated for 10 minutes at RT. Centrifugation of at 10000 RPM for 10 minutes pelleted the plasmid DNA, which was washed with 500 µL of 70% ethanol. The DNA was dried, resuspended in 1 X TE and its purity and concentration estimated on a 1% agarose/1 X TBE/ethidium bromide electrophoresis gel.

2.3.2.5. RESTRICTION DIGESTION OF PLASMID DNA

1-5 µg of plasmid DNA was digested with 5-10 units of the chosen restriction enzyme in the buffer as recommended by the manufacturer. For a double digestion, the recommended buffer conditions for the more sensitive of the two enzymes (such as that which required BSA) were applied. Reactions were incubated overnight at 37°C. With respect to the destination vector, 1 U of shrimp alkaline phosphatase was then added to the reaction and incubated at 37°C for 45 minutes prior to purification.

2.3.2.6. PURIFICATION OF REQUIRED DIGESTION FRAGMENTS

The entire digestion reaction mixture was electrophoresed on a 1% agarose/1 X TBE/ethidium bromide gel. The portion of the gel containing the fragment of interest was excised and DNA was recovered via the use of the BRESAclean DNA Purification Kit and according to the manufacturer's instructions.

2.3.2.7. GENERATION OF INSERT DNA VIA PCR WITH PFU POLYMERASE

100 ng of plasmid DNA was added to 1.25 units of Pfu polymerase, 1 X Pfu polymerase buffer, 2 µM of each primer and 0.2 mM dNTPs in a mixture made up to 25 µL with H_2O. PCR was performed with the following cycle: 92°C for 30 seconds, 35 X (92°C for 30 seconds, 55°C for 1 minute, 72°C for 4 minutes), 72°C for 5 minutes.

2.3.2.8. GENERATION OF INSERT DNA VIA PCR WITH TAQ POLYMERASE

DNA was incubated with 2-4 units of Taq polymerase, 1 X Taq polymerase buffer, 2 µM of each primer, 0.2 mM dNTPs, 1 mM magnesium chloride and 3% DMSO in a mixture made up to 25 µL with H_2O. PCR was performed with the following cycle: 92°C for 30 seconds, 35 X (92°C for 30 seconds, 50°C for 1 minute, 72°C for 4 minutes), 72°C for 5 minutes.

2.3.2.9. QUICKCHANGE MUTAGENESIS

50 ng of template plasmid DNA was incubated with 1.25 units of Pfu polymerase, 1 X Pfu polymerase buffer, 1.25 ng of each primer and 0.2 mM dNTPs in a reaction mixture

made up to 25 µL with H_2O. PCR was then performed with the following cycle: 95°C for 30 seconds, 22 X (95°C for 30 seconds, 55°C for 1 minute, 68°C for 20 minutes).

2.3.2.10. SEQUENCING

500 ng of DNA was incubated with 8 µL of BigDye Version 3 and 2 µM primer in a mixture made up to 20 µL with H_2O. PCR was performed with the following cycle: 96°C for 3 minutes, 25 X (96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes).

2.3.2.11. PHOSPHORYLATION AND ANNEALING OF OLIGONUCLEOTIDES FOR DIRECT SUBCLONING

100 pmol of either the upper or lower oligonucleotide was incubated in 100 mM Tris (pH 7.5), 100 mM magnesium chloride, 5 mM DTT, 1 mM ATP and 5 units of T4 polynucleotide kinase in a mixture made up to 30 µL with H_2O. This was incubated for 1 hour at 37°C, 15 minutes at 75°C and 15 minutes at RT. 20 µL of each phosphorylated oligonucleotide was mixed with 20 µL of phosphorylated lower oligonucleotide in 50 mM sodium chloride in a mixture made up to 50 µL with H_2O. This was placed in a 95°C heating block. The block with the tube was removed from the heating element and allowed to cool to RT. The annealed oligonucleotides were serially diluted by a factor of 10 until a dilution of 10^{-3} had been attained prior to ligation with the vector.

2.3.2.12. LIGATION OF INSERT DNA WITH VECTOR

Approximately 150-300 ng of insert DNA was incubated with approximately 50-100 ng of linearised vector DNA overnight at 4°C or for 3-5 hours at RT in 1 X ligase buffer and 5 units of T4 DNA ligase in a 20 µL reaction volume.

2.3.2.13. LABELLING OF DNA PROBES WITH ^{32}P-dATP

DNA suitable for use in Northern analysis was generated by *Eco*RI digestion of the pGEMTEASY derivative into which the sequence to serve as the probe had been subcloned and purification of the required fragment. 25-100 ng of the DNA was mixed with 1 µL of 100 µM random dNTP hexamer and made up to 14 µL with milli Q H_2O. This was incubated at 95°C for 5 minutes and then cooled on ice. To this was added 2.5 µL of 5 mM dNTP mixture without dATP, 2.5 µL 10 X TM, 5 µL of ^{32}P-dATP and 1 µL

of 10 U/μL Klenow fragment and the mixture was incubated at 37°C for 1 hour. The labelled fragment was purified from unincorporated dNTP via the use of a ProbeQuant G-50 Micro Column. The 50 μL of purified probe was diluted in 350 μL of 1 X TE and 2 μL was counted with 1 mL of scintillation fluid in a scintillation counter.

2.3.3. RNA MANIPULATION

2.3.3.1. GENERAL INFORMATION

Any of the following protocols involving the preparation, manipulation or analysis of RNA employed the use of reagents and equipment designated specifically for RNA. RNase free H_2O was prepared by incubating milli Q H_2O with 1% DEPC for at least 16 hours prior to autoclaving to inactivate the DEPC. Any equipment not solely assigned for RNA was cleaned with RNaseZAP prior to use.

2.3.3.2. TOTAL RNA

Total RNA was isolated from mammalian cells using RNAZolB. Cells were grown in 6 cm dishes, the medium removed and 1 mL of RNAZolB added to the adherent cells. The lysate was transferred to a tube. 100 μL of chloroform was added and the tubes shaken for 15 seconds, followed by an incubation for 5 minutes on ice. Tubes were centrifuged for 14000 RPM and 4°C for 15 minutes. The aqueous phase was removed to a new tube. An equal volume of isopropanol was added and the tubes were incubated 4°C for 15 minutes. Tubes were centrifuged at 14000 RPM and 4C for 15 minutes. The supernatant was removed and 75% ethanol added to the pellet, prior to centrifugation at 14000 RPM and 4°C for 8 minutes. After removal of the supernatant and the drying of the pellet, the RNA was resuspended in 50 μL of milli Q H_2O. Evaluation of RNA concentration and purity was performed by measurement of OD_{260} and OD_{280} with a spectrophotometer and visualisation on a 1% agarose/1 X TBE/ethidium bromide gel.

2.3.3.3. POLY A RNA

On the first day, cells were grown in 4 X T175 flasks per experimental treatment, the medium removed and 20 mL of oligo dT lysis buffer added direct to the cells (5 mL/flask). Lysates were collected from flasks with the aid of a cell scraper and those of the same treatment pooled into a 20 mL tube. Lysates were passed 3 X through a 20

gauge and 2 X through a 26 gauge needle. Proteinase K concentration was adjusted to 300 µg/mL and the tubes were incubated at 37°C for 3-5 hours. 1 mL of oligo dT slurry (a 1:1 mixture of oligo dT cellulose and oligo dT binding buffer) was added to the cell lysate and left to incubate overnight at 25°C with slow end over end mixing.

On the second day, an oligo dT cellulose column was prepared by placing sufficient glass wool in the neck of a Pasteur pipette such that cellulose flow was impeded. Both the columns and collection tubes (30 mL Corex) were siliconised by coating with Sigmacote. The mixture of slurry and cell lysate from the previous day was centrifuged at 2500 RPM for 3 minutes. The pellet was resuspended in 10 mL of oligo dT binding buffer, centrifuged at 2500 RPM for 3 minutes and the supernatant removed. This was repeated a further 2 X. The final pellet was resuspended in 10 mL of oligo dT binding buffer and loaded onto the column, with the flowthrough discarded. The column was washed 5 X with 2 mL of oligo dT wash buffer. Poly A RNA was removed from the column with 2 mL oligo dT elution buffer into the siliconised Corex tube. To this was added 5 mL of ethanol and 200 µL of 3 M sodium acetate (pH 5.2). This mixture was incubated at -70°C for 30 minutes prior to centrifugation at 10000 RPM and 4°C for 20 minutes. The supernatant was discarded, the pellet dried and resuspended in 100-200 µL of milli Q H_2O. Evaluation of RNA concentration and purity was performed by measurement of OD_{260} and OD_{280} with a spectrophotometer and by visualisation on a 1% agarose/1 X TBE/ethidium bromide gel.

2.3.3.4. RNA FOR MICROARRAY STUDIES

On the first day, cells were grown in 4 X T175 flasks per experimental treatment, the medium removed and 4 mL of RNAZolB added to the adherent cells in the flasks (1 mL/flask). Lysates were collected from flasks with the aid of a cell scraper and transferred to tubes. 100 µL of chloroform was added to the lysate and the tubes shaken vigorously for 15 seconds, followed by an incubation for 5 minutes on ice. Tubes were centrifuged at 14000 RPM and 4°C for 15 minutes. The aqueous phase was removed, pooled with those originating from the same experimental treatment, and mixed with an equal volume of 70% ethanol. This was then loaded onto an RNeasy Midi Kit column placed in a 15 mL tube. The column was centrifuged at 3500 RPM for 5 minutes in a swinging bucket rotor and the flow through discarded. To the column was added 4 mL of RW1 buffer. The column was centrifuged at 3500 RPM for 5 minutes and the flow through discarded. To

the column was added 2.5 mL of RBE buffer. The column was centrifuged at 3500 RPM for 5 minutes and the flow through discarded. This step was repeated. Finally, 150 µL of milli Q H_2O was added to the column. The column was centrifuged at 3500 RPM for 5 minutes and the flow through collected. This was repeated and the two aliquots of collected RNA pooled. To this was added 600 µL of ethanol and 30 µL of 3 M sodium acetate (pH 5.2), after which it was incubated overnight at -20°C. The following day, the tubes were centrifuged at 14000 RPM and 4°C for 30 minutes. The supernatant was removed and replaced with 500 µL of 70% ethanol. The tubes were then centrifuged at 14000 RPM and 4°C for 15 minutes. The supernatant was discarded, the pellet dried and resuspended in 100-400 µL of milli Q H_2O. Evaluation of RNA concentration and purity was performed by measurement of OD_{260} and OD_{280} with a spectrophotometer and by visualisation on a 1% agarose/1 X TBE/ethidium bromide gel.

2.3.3.5. cDNA SYNTHESIS FROM RNA

1-2 µg of total RNA or 100-200 ng of poly A RNA was mixed with 0.5 µL of 100 µM random dNTP hexamer and to 8 µL with milli Q H_2O. This was incubated at 70°C for 5 minutes and cooled on ice. Also, a mixture consisting of 3.5 U/µL of AMV reverse transcriptase, 10% glycerol, 10 mM potassium phosphate (pH 7.4), 0.2% Triton X and 2 mM DTT was incubated on ice for 30 minutes. 3 µL was added to the annealed RNA, plus 4.2 µL of 10 X 1st strand buffer, 4 µL of 5 mM dNTP mixture and 0.5 µL of SuperasIN. This was incubated at 37°C for 2 hours and then 95°C for 5 minutes.

2.3.4. AGAROSE GEL ELECTROPHORESIS FOR DNA ANALYSIS

2.3.4.1. AGAROSE/TBE GEL ELECTROPHORESIS

Analysis of DNA preparations and the separation of DNA fragments were performed via agarose/TBE gel electrophoresis. The gel was prepared by melting 1-2% agarose in 1 X TBE, adding ethidium bromide to 1 µg/mL and pouring into a moulding apparatus. Prior to loading into the wells, all DNA samples were mixed with 1 X loading buffer. The samples were electrophoresed in 1 X TBE for 30 minutes to 2 hours at 90 to 120 volts, depending on the size of DNA and the degree of separation desired.

2.3.5. AGAROSE GEL ELECTROPHORESIS FOR RNA ANALYSIS

2.3.5.1. GENERAL INFORMATION

Any of the following protocols involving the analysis of RNA employed the use of reagents and equipment designated specifically for RNA. RNase free H_2O was prepared by incubating milli Q H_2O with 1% DEPC for at least 16 hours prior to autoclaving to inactivate the DEPC. Any equipment not solely assigned for RNA was cleaned with RNaseZAP prior to use.

2.3.5.2. AGAROSE/TBE GEL ELECTROPHORESIS

Analysis of RNA was performed via agarose/TBE gel electrophoresis as per DNA.

2.3.5.3. AGAROSE/MOPS/FORMALDEHYDE GEL ELECTROPHORESIS

For Northern analysis of RNA, agarose/MOPS/formaldehyde electrophoresis was performed. The gel was prepared by melting 1% agarose in 84 mL of milli Q H_2O and then cooling to about 60°C. 10 mL of 10 X MOPS and 5.4 mL of 37% formaldehyde were added and the mixture was poured into a moulding apparatus. Prior to loading, all RNA samples were dried, resuspended in 20 µL of loading buffer for dried RNA, heated to 95°C for 2 minutes and cooled on ice. The samples were electrophoresed in 1 X MOPS for 1.5-2.5 hours at 60 volts.

2.3.5.4. NORTHERN TRANSFER ANALYSIS

Upon the completion of agarose/MOPS/formaldehyde gel electrophoresis of RNA (when the dye had electrophoresed approximately 75% of the length of the gel), it was removed and placed in milli Q H_2O for 40 minutes. The gel was incubated in 20 X SSC for 20 minutes. A moulding apparatus of the same size as that used to prepare the gel was placed upside down in a tray of a sufficient volume of 20 X SSC to perform the transfer. Over this was placed in this order: 3 X pieces of Whatman paper of the dimensions 25 X 10 centimetres (the ends of which were in contact with the reservoir of 20 X SSC), the agarose/MOPS/formaldehyde gel with the wells facing downwards, Nytran N nylon transfer membrane of the dimensions 15 X 10 centimetres (which was presoaked in milli Q H_2O), 3 X pieces of Whatman paper of the dimensions 15 X 10 centimetres, a stack of absorbent paper towelling of the dimensions 15 X 10 X 10 centimetres and a glass plate.

This was covered in plastic wrap and a half full 500 mL bottle placed on top of the plate as a weight. The transfer occurred overnight.

The following day, the nylon membrane was removed from the stack, placed RNA side up on damp Whatman paper and the RNA was immediately crosslinked. To observe RNA loading and transfer efficiency, the membrane was stained for 5 minutes in 72 mL of milli Q H_2O, 8 mL of 3M sodium acetate (pH 5.2) and 240 µL of 10% methylene blue and rinsed in 2 X in milli Q H_2O to remove background staining and photographed. Destaining was performed via 2 X washes in 2 X SSC/0.1% SDS.

Initially (figure 3.12), membranes were prehybridised in Rapid Hyb according to the manufacturer's instructions. Due to inadequacies in the ability of membranes to be stripped and reprobed, an alternative technique was adopted for the remainder of the thesis. This entailed incubating the membrane in 20 mL of prehybridisation buffer for 5 hours at 42°C, followed by the addition of the relevant ^{32}P-dATP labelled probe and continued incubation overnight at 42°C. The probe was heated for 5 minutes at 95°C and chilled on ice prior to addition of to the prehybridisation solution.

The following day, the membrane was washed twice in 2 X SSC/0.1 % SDS for 20 minutes at RT and twice in 2 X SSC/0.1% SDS at 52°C before sealing in plastic and exposure to a phosphorimager screen for 1-24 hours before scanning

Northern blots were stripped was follows. H_2O was boiled and SDS was added to 0.1% once boiling had ceased. The blots were then shaken in this solution for at least 30 minutes prior to rinsing in 2 X SSC/0.1% SDS.

2.3.6. POLYACRYLAMIDE GEL ELECTROPHORESIS FOR PROTEIN ANALYSIS

2.3.6.1. DENATURING SDS PAGE

Separation of proteins for immunoblotting was performed using 7.5-15% acrylamide gels. The separating gel was prepared by mixing the appropriate volume of 40% acrylamide, 5 mL of 4 X separation buffer and H_2O to allow for a total of 20 mL. 16 µL of TEMED and 160 µL of 30% APS was added and the mixture poured into a moulding apparatus. Once set, the stacking gel was prepared by mixing 6.25 mL of H_2O, 1.25 mL of 40%

acrylamide, 2.5 mL of 4 X stacking buffer, 8 µL of TEMED and 80 µL of 30% APS. Prior to loading into wells, all samples were mixed with an equal volume of 2 X SDS sample buffer and incubated at 95°C for 5 minutes. Gels were electrophoresed at 160 volts in 1 X SDS running buffer.

2.3.6.2. DENATURING TRIS TRICINE PAGE

The separating gel was prepared by mixing the appropriate volume of 40% acrylamide, 4 mL of 4 mL of 3 M Tris (pH 8.3) and H_2O to allow for a total of 12 mL. 16 µL of TEMED and 160 µL of 30% APS was added and the mixture poured into a moulding apparatus. Once set, the stacking gel was prepared by mixing 4.4 mL of H_2O, 1 mL of 40% acrylamide, 2.6 mL of 3 M Tris (pH 8.3), 8 µL of TEMED and 80 µL of 30% APS. Prior to loading into wells, all protein samples were mixed with an equal volume of 2 X SDS sample buffer and incubated at 95°C for 5 minutes. Gels were then electrophoresed at 160 volts in 1 X Tris tricine running buffer.

2.3.6.3. IMMUNOBLOTTING

Following PAGE, proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour in blocking buffer at RT. After blocking, the membrane was incubated ON at 4°C in PBT with the appropriate concentration of antibody and skim milk powder: 1/500 antibody in 10% skim milk powder (polyclonal rabbit anti ARNT amino acids 666-774, polyclonal rabbit anti HIF-1α amino acids 727-826, polyclonal rabbit anti HIF-2α amino acids 612-823), 1/500 antibody in 1% skim milk powder (monoclonal mouse anti Myc (9E10)), 1/5000 antibody in 1% skim milk powder (polyclonal rabbit anti ARD1 amino acids 216-235), 1/1000 antibody in 1% skim milk powder (polyclonal rabbit anti HIF-2α amino acids 632-646), 1/500 antibody (polyclonal rabbit anti PHD1), 1/5000 in 5% skim milk powder (rabbit polyclonal anti PHD2), 1/1000 antibody (polyclonal rabbit anti PHD3). The membrane was washed three times for 10 minutes in PBT prior to incubation in PBT, 2.5% skim milk powder and 1/2000 (DAKO) or 1/20000 (Pierce) horse radish peroxidase conjugated goat anti rabbit immunoglobulins or 1/5000 (DAKO) or 1/20000 (Pierce) horse radish peroxidase conjugated rabbit anti mouse immunoglobulins for 1 hour at RT. The membrane was washed three times for 10 minutes in PBT and drained of excess buffer. Equal volumes of each of the enhanced chemiluminescence reagents were mixed with each other prior to distribution over the

membrane. After 1 minute, the chemicals were drained off and the membrane was wrapped and exposed to autoradiography film.

2.3.7. TISSUE CULTURE

2.3.7.1. PC12 CELL LINE MAINTENANCE

The PC12 cell line and its derivatives were grown in PC12 medium and incubated at 37°C and 5% CO_2. A stock was grown in T175 flasks. When cells reached confluency, they were passaged. The medium was removed and the cells washed with 5 mL of 1 X PBS. 1.5 mL of trypsin solution was added. Once the cells were dislodged, 8.5 mL of PC12 medium was added to inactivate the trypsin. The cell suspension was transferred to a tube and centrifuged at 1200 RPM for 2 minutes. The supernatant was removed and the pellet dispersed in 10 mL of PC12 medium. A 2 mL or 1.25 mL aliquot of this suspension was then added to a T175 flask containing 20 mL of PC12 medium (that is, they were passaged 1:5 or 1:8 allowing for 3 and 4 days of growth, respectively, prior to confluency again being attained). An appropriate volume of this suspension was also added to receptacles employed for subsequent experiments (T75 flasks, 10 cm dishes, 6 cm dishes, 3 cm dishes or 24 well trays) as specified in the appropriate section.

2.3.7.2. TRANSFECTION OF PC12 CELLS

Cells were grown to approximately 50% confluency prior to transfection. DNA was introduced using Lipofectamine 2000 and according to the manufacturer's instructions.

2.3.7.3. HYPOXIC TREATMENT OF PC12 CELLS

Hypoxic microenvironments were attained using Anaero*Gen* sachets (Oxoid). Cultures were placed with the sachet in a 2.5 L airtight container. Within 30 minutes of the container's closure, the partial pressure of O_2 within was reduced to less than 1%.

In some experiments, PC12 medium was supplemented with 100 μM desferrioxamine (DFO), 100 μM 2, 2'-dipyridyl (DP) or 1mM dimethyloxalylglycine (DMOG). The latter two were stored as 1000 X stocks in DMSO. When these were used in experiments, a 1000 fold dilution of DMSO was added to all untreated samples.

2.3.7.4. LUCIFERASE ASSAY OF PC12 CELLS

Luciferase assays were performed on cells grown in 24 well trays. After the designated treatment, the medium was removed and replaced with 100 µL of passive lysis buffer. Trays were rocked for 15-30 minutes at RT. 10 µL of the supernatant of lysate was mixed with 25 µL Luciferase Assay Reagent II and firefly luciferase activity measured with a luminometer. When complete, 25 µL of Stop and Glow was added to measure for renilla luciferase activity in the same luminometer. The relative luciferase activity of the lysate (firefly versus renilla) was derived from the ratio of both measurements.

2.3.7.5. WHOLE CELL EXTRACTION

Whole cell extracts were obtained from cultured cells specifically for immunoblotting of proteins. Cultures were grown in 6 cm dishes. Prior to extraction, these were equilibrated to 4°C. The medium was removed and the cells suspended in 1 mL of TEN and transferred into a tube. This was centrifuged at 1200 RPM and 4°C for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1 mL of PBS and centrifuged at 1200 RPM and 4°C for 10 minutes, after which the PBS was extracted. The pellet was dispersed in four volumes of ice cold whole cell extract buffer and incubated for 30 minutes at 4°C with shaking. Following a centrifugation at 14000 RPM and 4°C, 1 µL of the supernatant was used to determine its protein concentration with Bradford protein assay reagent as suggested by the manufacturer. The remainder was stored at -80°C.

2.4. ESTABLISHMENT OF MONOCLONAL SELECTIVELY INDUCIBLE HIF-1α AND HIF-2α PC12 CELL LINES

2.4.1. CONSTRUCTION OF EXPRESSION PLASMIDS

2.4.1.1. PREPARATION OF PLASMIDS

All plasmids described below were transformed into, grown in and prepared via the ammonium acetate protocol for plasmid preparation from *E. coli* DH5α cells.

2.4.1.2. CONSTRUCTION OF pEF/TetON/IRES/NEO

pEF/TetON/IRES/PURO was digested with *Nhe*I and *Not*I and the 1 kb fragment purified and inserted into *Nhe*I and *Not*I digested pEF/IRES/NEO. The resultant construct was termed pEF/TetON/IRES/NEO

2.4.1.3. CONSTRUCTION OF pTR/HIF-2α/DC/EYFP

pHLF/SL301 was digested with *Hpa*I and *Eco*RV and the 3 kb fragment purified and inserted into *Pme*I digested pTR/DC/EYFP. The resultant construct was termed pTR/HIF-2α/DC/EYFP.

2.4.1.4. CONSTRUCTION OF pTR/HIF-1α/DC/EYFP

pET-32a/HIF-1α was digested with *Bgl*II and *Bam*HI and the 2.5 kb fragment purified and inserted into *Bgl*II digested pTR/DC/EYFP. The resultant construct was termed pTR/HIF-1α C terminus/DC/EYFP. To introduce the N terminus of HIF-1α, pET-32a/HIF-1α was digested with *Bgl*II and the 0.1 kb fragment purified and inserted into *Bgl*II digested pTR/HIF-1α C terminus/DC/EYFP. The resultant construct was termed pTR/HIF-1α/DC/EYFP.

2.4.1.5. CONSTRUCTION OF pTR/HIF-1αN803A/DC/EYFP

pEFBOS/HIF-1αN803A was digested with *Afl*II and *Hpa*I and the 1.3 kb fragment purified and inserted into pTR/HIF-1α/DC/EYFP (from which a 1.3 kb fragment was also released). The subsequent construct was termed pTR/HIF-1αN803A/DC/EYFP.

2.4.2. CHARACTERISATION OF PC12 CELLS

2.4.2.1. HIF-1α AND HIF-2α PROTEIN EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These cells were exposed to normoxia or hypoxia for 16 hours, after which time whole cell extracts were prepared. 25 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α. Furthermore, PC12 cells were plated into 24 well tray wells containing 500 μL of PC12 medium and in the conditions described previously. Each was

transfected with 100 ng of pRLTK in addition to either 100 ng $pHRE_4GL3$ or 100 ng of pGL3. 6 hours post transfection, cells were exposed to normoxia or hypoxia or treated with 100 μM DFO or 100 μM DP. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate.

2.4.2.2. HIF-2α OVEREXPRESSION

PC12 cells were plated into 24 well tray wells containing 500 μL of PC12 medium. Each was transfected with 200 ng of pEFBOS and 50 ng of pRLTK in addition to either 50 ng $pHRE_4GL3$ or 50 ng of pGL3. This was repeated but with pEFBOS/HIF-2α. 6 hours post transfection, cells were exposed to normoxia or hypoxia. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate. Also, extracts derived from cells exposed to 20% O_2 or <1% O_2 and transfected with both pGL3 and pEFBOS/HIF-2α were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of HIF-2α.

2.4.3. ESTABLISHMENT OF THE PC12/TetON/NEO CELL LINE

2.4.3.1. PREPARATION OF EXPRESSION VECTORS

All plasmids described below were transformed into, grown in and prepared via the ammonium acetate protocol for plasmid preparation from *E. coli* DH5α cells.

2.4.3.2. TRANSFECTION OF PC12 CELLS WITH THE REVERSE TETRACYCLINE TRANSACTIVATOR

PC12 cells were grown in a 6 cm dish containing 3 mL of PC12 medium. These were transfected with 10 μg of pEF/TetON/IRES/NEO. 24 hours post transfection, they were passaged 1:5 into three 10 cm dishes already containing 8 mL of complete medium. 48 hours later, 40 μL of 50 mg/mL of G418 was added. These dishes were incubated for approximately 2 weeks, after which time colonies of G418 resistant PC12 cells (presumably stably transformed with pEF/TetON/IRES/NEO and derived from a single cell) were picked and proliferated for further analysis. This entailed removal of the medium from the 10 cm dishes, addition of 10 μL of trypsin solution to the selected colony and removal of the colony via aspiration. The colony was then used it to inoculate

500 µL of PC12 medium in a well of a 24 well tray and supplemented with 200 µg/mL of G418 24 hours later. A total of 48 colonies putatively transfected with pEF/TetON/IRES/NEO were picked. 7 days later, the medium was removed from wells where growth had been successful and 100 µL of trypsin solution was added. Cells were dislodged after 2 to 3 minutes at which time 1 mL of PC12 medium was added to inactivate the trypsin. The subsequent cell suspension was passaged 1:4 into two corresponding wells of a 24 well tray containing 500 µL of PC12 medium and 1:2 into a 3 cm dish containing 2 mL of PC12 medium, with the latter serving as a master plate. 18 colonies putatively transfected with pEF/TetON/IRES/NEO were passaged in this manner. 24 hours later, all wells were transfected with 300 µg of pTR/DC/EYFP. 6 hours post transfection, cells were left untreated or treated with 2 µg/mL of doxycycline. 40 hours later, cells were checked for fluorescence. 10 of the lines demonstrated doxycycline inducible EYFP. The best was maintained and referred to as the PC12/TetON/NEO line.

2.4.3.3. ANALYSIS OF PC12/TetON/NEO CELLS

To test for doxycycline inducible TRE reporter activity, cells were plated into 24 well tray wells containing 500 µL of PC12 medium. Each well was transfected with 100 ng of pRLTK and 200 ng pBI/GL. 6 hours post transfection, cells were left untreated or exposed to 2 µg/mL of doxycycline. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate.

To test for doxycycline inducible HIF-2α expression, cells were plated into 24 well tray wells containing 500 µL of PC12 medium. Each was transfected with 200 ng of pTR/HIF-2α/DC/EYFP and 100 ng of pRLTK in addition to either 100 ng $pHRE_4GL3$ or 100 ng of pGL3. This was repeated but with pTR/DC/EYFP. 6 hours post transfection, cells were left untreated or exposed to 2 µg/mL of doxycycline. 16 hours later, cells were harvested for luciferase assays. Also, cells were plated into 6 cm dishes containing 3 mL of PC12 medium and in the conditions described previously. These were transfected with 5 µg of pTR/DC/EYFP or pTR/HIF-2α/DC/EYFP. 6 hours post transfection, cells were left untreated or exposed to 2 µg/mL of doxycycline. 16 hours later, whole cell extracts were prepared. 20 µg of each electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of HIF-1α or HIF-2α.

2.4.4. ESTABLISHMENT OF THE INDUCIBLE HIF-α PC12 CELL LINES

2.4.4.1. PREPARATION OF EXPRESSION VECTORS

All plasmids described below were transformed into, grown in and prepared via the ammonium acetate protocol for plasmid preparation from *E. coli* DH5α cells.

2.4.4.2. TRANSFECTION OF PC12/TetON/NEO CELLS WITH INDUCIBLE HIF-α

All derivatives of the PC12/TetON/NEO line were essentially generated using the same method. PC12/TetON/NEO cells were grown in a 6 cm dish containing 3 mL of PC12 medium. These were transfected with 5 μg of pTR/DC/EYFP, pTR/HIF-2α/DC/EYFP, pTR/HIF-1α/DC/EYFP or pTR/HIF-1αN803A/DC/EYFP. 24 hours post transfection, they were passaged 1:4 into four 10 cm dishes already containing 8 mL of PC12 medium. 48 hours later, 40 μL of 50 mg/mL of G418 was added. Approximately 1 week later, cells were passaged 1:2 into a T175 flask already containing 20 mL of PC12 medium. 24 hours later, 2 μg/mL doxycycline was added. 16 hours later, cells were harvested into DMEM with 3.3% HS, 1.7% FCS and 2 μg/mL doxycycline. FACS was performed (all FACS performed by Sandy MacIntyre and Alan Bishop at the Hanson Institute, Adelaide, Australia) and all fluorescing cells were collected in PC12 DMEM. These were then grown in a 6 cm dish and expanded into receptacles of increasing volume until a subconfluent T175 flask of cells was acquired. To this was added 2 μg/mL of doxycycline. 16 hours later, FACS was performed, although this time to select for the highest fluorescing cells (about 5%). All fluorescing cells were collected in PC12 DMEM. These were then grown in a 6 cm dish for 3 to 6 days prior to dilution of approximately 1 cell/well into wells of a 24 well tray. These wells were incubated for 3 to 4 weeks, after which time there were sufficient cells for monoclonal lines to be expanded and maintained as per normal PC12 cells.

2.4.4.3. ANALYSIS OF STABLE TRANSFECTANTS

To screen the PC12/TetON/NEO cell line derivatives, cells were plated into 24 well tray wells containing 500 μL of PC12 medium. Each well was transfected with 100 ng of pRLTK and 100 ng $pHRE_4GL3$. 6 hours post transfection, cells were left untreated or exposed to 2 μg/mL of doxycycline. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate.

To further characterise selected lines, cells were plated into 24 well tray wells containing 500 µL of PC12 medium. Each well was transfected with 100 ng of pRLTK in addition to either 100 ng pHRE$_4$GL3 or 100 ng of pGL3. 6 hours post transfection, cells were exposed to normoxia or hypoxia with or without additional treatment with 2 µg/mL of doxycycline. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate. Also, cells were plated into 6 cm dishes containing 3 mL of PC12 medium and in the conditions described previously. These cells were exposed to normoxia or hypoxia with or without treatment with 2 µg/mL of doxycycline. 16 hours later, whole cell extracts were prepared. 20 µg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and then transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α.

2.4.5. NORTHERN ANALYSIS OF HIF-1α TARGET GENE EXPRESSION IN THE INDUCIBLE HIF-α PC12 CELL LINES

PC12/TetON/EYFP, PC12/TetON/HIF-2α/EYFP and PC12/TetON/HIF-1αN803A/EYFP cells were plated into T175 flasks containing 20 mL of PC12 medium. These cells were exposed to 2 µg/mL doxycycline or left untreated for 16 hours, after which time total RNA was prepared. A total of four flasks of cells were required for each treatment.

From 2 µg of RNA purified from doxycycline treated and untreated PC12/TetON/HIF-2α/EYFP cells was synthesised cDNA. 1 µL of this or of H_2O was used as a template in a Taq polymerase mediated PCR reaction employing as primer combinations the following oligonucleotides: hENO1 upper and lower, rLDHA upper and lower, hTH upper and lower or β actin upper and lower. 1 µL of cDNA from the mouse endothelial cell line tEnd1 was used as a template in a similar PCR reaction employing as primer combinations aldo a sense and antisense or hGT1 upper and lower. Finally, pαFGH/VEGF164 was used as a template in a PCR reaction employing hVEGF upper and lower primers. The products were analysed via electrophoresis through a 2% agarose/1 X TBE/ethidium bromide gel and visualised with a UV transilluminator. All reaction products were of the estimated size. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified as for restriction digestion fragments and cloned directly into prelinearised pGEMTEASY and sequenced.

10 μg of total RNA from all lines and treatments was also electrophoresed through a 1 X MOPS/1% agarose/formaldehyde gel and transferred to nylon for Northern analysis of the expression of enolase 1, aldolase A, lactate dehydrogenase A, glucose transporter 1 and VEGF. The relevant pGEMTEASY derivative was digested with *Eco*RI and the appropriately sized fragments were purified and labelled with ^{32}P-dATP. The membranes were probed with the relevant labelled cDNA, stripped and then reprobed with β actin as a control.

2.4.6. COMPARISON OF GENE EXPRESSION BETWEEN PC12/TetON/HIF-2α/EYFP AND PC12/TetON/HIF-1αN803A/EYFP CELLS

2.4.6.1. MICROARRAY ANALYSIS

PC12/TetON/HIF-2α/EYFP and PC12/TetON/HIF-1αN803A/EYFP cells were plated into T175 flasks containing 20 mL of PC12 medium. These were exposed to 2 μg/mL doxycycline for 16 hours, after which time total RNA was prepared. A total of four flasks of cells were required for each treatment.

This total RNA was provided to Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia and cDNA synthesis and labelling reactions performed using a mixture of random hexamers, labelled poly dT oligonucleotides and Cy3 and Cy5 dyes as described in their protocol. The 5 K rat oligonucleotide microarray slides used were manufactured by the Clive and Vera Ramaciotti Centre for Gene Function Analysis, Australia. Hybridisation of the labelled RNA to these slides was performed according to the manufacturer's instructions. Statistical analysis was completed by Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia.

2.4.6.2. NORTHERN ANALYSIS OF SELECTED PUTATIVE HIF-2α TARGETS

From 2 μg of total RNA purified from doxycycline treated and untreated PC12/TetON/HIF-2α/EYFP cells was synthesised cDNA. 1 μL of this or of H_2O was then used as a template in a Taq polymerase mediated PCR reaction employing as primer combinations the following oligonucleotides: BARD1 upper and lower, C/ACP upper and lower, CPT1 upper and lower, NETa/b upper and lower, PGHα upper and lower, PTTG upper and lower, TAGE4 upper and lower, GIIG11 upper and lower or β actin upper and

lower. The products were analysed via electrophoresis through a 2% agarose/1 X TBE/ethidium bromide gel and visualised with a UV transilluminator. All reaction products were of the estimated. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified as for restriction digestion fragments and cloned directly into prelinearised pGEMTEASY and sequenced.

PC12/TetON/EYFP, PC12/TetON/HIF-2α/EYFP and PC12/TetON/HIF-1αN803A/EYFP cells were plated into T175 flasks containing 20 mL of PC12 medium. These cells were exposed to 2 μg/mL doxycycline or left untreated for 16 hours, after which time poly A RNA was prepared. A total of four flasks of cells were required for each treatment. 3 μg of this poly A RNA was then electrophoresed through a 1 X MOPS/1% agarose/formaldehyde gel and transferred to nylon for Northern analysis of the BARD1, CPT1, C/ACP, NETa, PGHα, PTTG, TAGE4 and GIIG11. The relevant pGEMTEASY derivative was digested with *Eco*RI and the appropriately sized fragments were purified and labelled with ^{32}P-dATP. The membranes were probed with the relevant labelled cDNA, stripped and then reprobed with β actin as a control.

2.4.7. COMPARISON OF GENE EXPRESSION BETWEEN DOXYCYCLINE TREATED AND UNTREATED PC12/TetON/HIF-2α/EYFP CELLS

2.4.7.1. MICROARRAY ANALYSIS

PC12/TetON/HIF-2α/EYFP and PC12/TetON/EYFP cells were plated into T175 flasks containing 20 mL of PC12 medium and in the conditions described previously. These were exposed to 2 μg/mL doxycycline or left untreated for 16 hours, after which time total RNA was prepared. A total of four flasks of cells were required for each treatment.

This total RNA was provided to Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia and cDNA synthesis and labelling reactions performed using a mixture of random hexamers, labelled poly dT oligonucleotides and Cy3 and Cy5 dyes as described in their protocol. The 10 K rat oligonucleotide microarray slides used were manufactured by the Clive and Vera Ramaciotti Centre for Gene Function Analysis, Australia. Hybridisation of the labelled RNA to these slides was performed according to the manufacturer's instructions.

Statistical analysis was completed by Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia.

2.4.7.2. RTPCR ANALYSIS OF SELECTED PUTATIVE HIF-2α TARGETS

PC12 cells were plated into T175 flasks containing 20 mL of PC12 medium. These were exposed to normoxia or hypoxia for 16 hours, after which time poly A RNA was prepared. Also, PC12/TetON/EYFP, PC12/TetON/HIF-2α/EYFP and PC12/TetON/HIF-1αN803A/EYFP cells were plated into T175 flasks containing 20 mL of PC12 medium and in the conditions described previously. These were exposed to 2 μg/mL doxycycline or left untreated for 16 hours, after which time total RNA was prepared. A total of four flasks of cells were required for each treatment. From 200 ng of this poly A RNA or 2 μg of total RNA was synthesised cDNA. 1 μL of this or of H_2O was used as a template in a Taq polymerase mediated PCR reaction employing as primer combinations the following oligonucleotides: VN upper and lower, Ca^+ upper and lower, INrf2 upper and lower, Na^+ upper and lower, Rgs4 upper and lower or β actin upper and lower. The products were analysed via electrophoresis through a 2% agarose/1 X TBE/ethidium bromide and visualised with a UV transilluminator.

2.5. ANALYSIS OF HIF-1α AND HIF-2α EXPRESSION IN PC12 CELLS

2.5.1. CONSTRUCTION OF EXPRESSION PLASMIDS

2.5.1.1. PREPARATION OF PLASMIDS

All plasmids described below were transformed into, grown in and prepared via the ammonium acetate protocol for plasmid preparation from *E. coli* DH5α cells.

2.5.1.2. CONSTRUCTION OF pEF/HisMycHIF-1α/P402-564A/IRES/PURO

A P402A mutation in HIF-1α P564A was performed via Quickchange mutagenesis employing pHIF-1αP564A/Bluescript as the template and HIF P402A up and HIF P402A lo primers. These primers also introduced a silent mutation allowing for the generation of an *Nhe*I site to allow for screening of mutants. The section to be further subcloned was sequenced to ensure the presence of both the P402A and P564A mutations and the

absence of aberrant mutations. The resultant successfully mutated construct was termed pHIF-1αP402-564A/Bluescript. pHIF-1αP402-564A/Bluescript was digested with *Spe*I and the 2 kb fragment purified and inserted into *Spe*I digested pEFBOS/HIF-1α (from which a 2 kb fragment was also released). The resultant construct was termed pEFBOS/HIF-1αP402-564A. pEFBOS/HIF-1αP402-564A was digested with *Nco*I and *Spe*I and the 2.5 kb fragment purified and inserted into *Nco*I and *Xba*I digested pEF/IRES/PURO5. The resultant construct was termed pEF/HIF-1αP402-564A/IRES/PURO. pEF/HisMycHIF-1α/IRES/PURO was digested with *Nco*I and the approximately 0.3 kb fragment (corresponding to the 6 consecutive histidines and 6 copies of the epitope to the anti Myc monoclonal antibody 9E10) purified and inserted into *Nco*I digested pEF/HIF-1αP402-564A/IRES/PURO. The resultant construct was termed pEF/HisMycHIF-1αP402-564A/IRES/PURO.

2.5.1.3. CONSTRUCTION OF pEF/ARD1/IRES/PURO

The entire coding sequence of rat ARD1 (nucleotides 1-708 of rat ARD1 (GenBank accession number XM_343842)) was amplified via RTPCR employing cDNA derived from reverse transcription of poly A RNA purified from PC12 cells as the template and rARD1 upper and rARD1 lower as the upper and lower primers, respectively, with the approximately 0.7 kb product cloned directly into prelinearised pGEMTEASY and sequenced. The resultant construct was termed pGEM/ARD1. pGEM/ARD1 was digested with *Eco*RI and the approximately 0.7 kb fragment purified and inserted into *Eco*RI digested pEF/IRES/PURO5. The resultant construct was termed pEF/ARD1/IRES/PURO.

2.5.1.4. CONSTRUCTION OF pSUPER/SCR

The oligonucleotides scrambled upper and scrambled lower were phosphorylated, annealed to each other and directly cloned into *Bgl*II and *Hind*III digested pSUPER. The insert that was subcloned was sequenced to ensure the absence of aberrant mutations. The resultant construct was termed pSUPER/SCR.

2.5.1.5. CONSTRUCTION OF pSUPER/PHD2 112, pSUPER/PHD2 126, pSUPER/PHD2 542 AND pSUPER/PHD2 554

The oligonucleotides PHD2 112 upper and PHD2 112 lower were phosphorylated, annealed to each other and directly cloned into *Bgl*II and *Hind*III digested pSUPER. The insert that was subcloned was sequenced to ensure the absence of aberrant mutations. The resultant construct was termed pSUPER/PHD2 112. This procedure was repeated with PHD2 126 upper and PHD2 126 lower, PHD2 542 upper and PHD2 542 lower and PHD2 554 upper and PHD2 554 lower.

2.5.1.6. CONSTRUCTION OF pSUPER/ARD1 144, pSUPER/ARD1 150, pSUPER/ARD1 552 AND pSUPER/ARD1 627

The oligonucleotides ARD1 144 upper and ARD1 144 lower were phosphorylated, annealed to each other and directly cloned into *Bgl*II and *Hind*III digested pSUPER. The insert that was subcloned was sequenced to ensure the absence of aberrant mutations. The resultant construct was termed pSUPER/ARD1 144. This procedure was repeated with ARD1 150 upper and ARD1 150 lower, ARD1 552 upper and ARD1 552 lower and ARD1 627 upper and ARD1 627 lower.

2.5.2. HIF-1α AND HIF-2α EXPRESSION AS A FUNCTION OF DURATION OF HYPOXIA

PC12 cells were plated into a 6 cm dish containing 3 mL of PC12 medium. These were exposed to hypoxia for 0, 2, 4, 6, 8, 16 or 24 hours, after which time whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and then transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α.

2.5.3. EFFECT OF IRON CHELATION ON HIF-1α AND HIF-2α EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were exposed to normoxia, hypoxia, 100 μM DFO or 100 μM DP for 16 hours, after which time whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α.

2.5.4. EFFECT OF 2-OXOGLUTARATE COMPETITION ON HIF-1α AND HIF-2α EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These cells were exposed to normoxia, hypoxia or 1 mM DMOG for 16 hours, after which time whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α. Furthermore, PC12 cells were plated into 24 well tray wells containing 500 μL of PC12 medium and in the conditions described previously. Each well was transfected with 100 ng of pRLTK in addition to either 100 ng $pHRE_4GL3$ or 100 ng of pGL3. 6 hours post transfection, cells were exposed to normoxia, hypoxia or 1 mM DMOG. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate.

2.5.5. EFFECT OF 2-OXOGLUTARATE COMPETITION ON HIF-1α CAD ACTIVITY

PC12 cells were plated into 24 well tray wells containing 500 μL of PC12 medium. Each well was transfected with 50 ng of pRLTK and 50 ng of pGRELUC in addition to 100 ng of pGal4DBD, 100ng pGal4DBD/HIF-1αCAD or 100 ng of pGal4DBD/HIF-1αCADN803A. 6 hours post transfection, cells were exposed to normoxia, hypoxia or 1 mM DMOG. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate. Furthermore, PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These cells were transfected with 2 μg of pGal4DBD, 2 μg of pGal4DBD/HIF-1αCAD or 2 μg of pGal4DBD/HIF-1αCADN803A. 6 hours post transfection, cells were exposed to normoxia, hypoxia or 1 mM DMOG. 16 hours later, whole cell extracts were prepared. 20 μg of each were electrophoresed through a 12.5% acrylamide Tris tricine PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of HIF-1α CAD.

2.5.6. RTPCR AND NORTHERN ANALYSIS OF HIF-α PROLYL-4-HYDROXYLASE EXPRESSION

PC12 cells were plated into T175 flasks containing 20 mL of PC12 medium. These cells were exposed to normoxia or hypoxia for 16 hours, after which time poly A RNA was prepared. A total of four flasks of cells were required for each treatment.

From 200 ng of this RNA was synthesised cDNA. 1 μL of this or of H_2O was then used as a template in a Taq polymerase mediated PCR reaction employing as primer combinations the following oligonucleotides: rPHD1 upper and lower, rPHD2 upper and lower, rPHD3 upper and lower or β actin upper and lower. The products were analysed via electrophoresis through a 2% agarose/1 X TBE/ethidium bromide gel and visualised with a UV transilluminator. All reaction products were of the estimated size. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified as for restriction digestion fragments and cloned directly into prelinearised pGEMTEASY (hereafter referred to as pGEM/PHD1, pGEM/PHD2, pGEM/PHD3 and pGEM/actin respectively) and sequenced.

3 μg of this poly A RNA was also electrophoresed through a 1 X MOPS/1% agarose/formaldehyde gel and transferred to nylon for Northern analysis of the expression of PHD2 and PHD3. pGEM/PHD2, pGEM/PHD3 and pGEM/actin were digested with *Eco*RI and the appropriately sized fragments were purified and labelled with ^{32}P-dATP. The membranes were then probed with the relevant PHD, stripped and then reprobed with β actin as a control.

2.5.7. IMMUNOBLOTTING FOR HIF-α PROLYL-4-HYDROXYLASE EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. Whole cell extracts were prepared from cells exposed to normoxia or hypoxia for 16 hours. 10 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of ARNT or through a 12.5% acrylamide SDS PAGE gel for immunoblotting for the presence of PHD1, PHD2 or PHD3.

2.5.8. RNA INTERFERENCE OF HIF-α PROLYL-4-HYDROXYLASE 2

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pSUPER/SCR, pSUPER/PHD2 112, pSUPER/PHD2 126, pSUPER/PHD2 542 or pSUPER/PHD2 554. 48 hours later, whole cell extracts were prepared. 10 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of ARNT or through a 12.5% acrylamide SDS PAGE gel for immunoblotting for the presence of PHD2.

Once the best PHD2 targeted constructs were determined, PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pSUPER/SCR, pSUPER/PHD2 542 or pSUPER/PHD2 554. 32 hours post transfection cells were exposed to normoxia or hypoxia. 16 hours later, whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of ARNT, HIF-1α or HIF-2α or through a 12.5% acrylamide SDS PAGE gel for immunoblotting for PHD2.

2.5.9. EFFECT OF EXOGENOUS HIF-α PROLYL-4-HYDROXYLASE ACTIVITY ON OVEREXPRESSED HIF-1α EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pEF/IRES/PURO or 2.5 μg of pEF/HisMycHIF-1α/IRES/PURO or pEF/HisMycHIF-1αP402-564A/PURO. With regards to the latter two, these were cotransfected with 2.5 μg of pcDNA3.1/HPH1, pcDNA3.1/HPH2 or pcDNA3.1/HPH3 24 hours post transfection, whole cell extracts were prepared. 10 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of the Myc epitope or ARNT.

To test the effect of iron chelation and 2-oxoglutarate competition, PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pEF/IRES/PURO or 2.5 μg of pEF/HisMycHIF-1α/IRES/PURO. With regards to the latter two, these were cotransfected with 2.5 μg of pcDNA3.1/HPH1, pcDNA3.1/HPH2 or pcDNA3.1/HPH3 6 hours post transfection, cells were left untreated or treated with 100 μM DFO or 100 μM DP. 16 hours later, whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of the Myc epitope or ARNT.

2.5.10. EFFECT OF TYROSINE KINASE INHIBITION ON HIF-1α AND HIF-2α EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These cells were exposed to normoxia or hypoxia with or without further treatment with 100 μM genistein for 16 hours. A 1000 fold dilution of DMSO was added to untreated dishes. Whole cell extracts were then prepared. 20 μg of each were electrophoresed through a

7.5% acrylamide SDS PAGE gel and then transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α. Also, PC12 cells were plated into 24 well tray wells containing 500 μL of PC12 medium. Each well was transfected with 100 μg of pRLTK in addition to either 100 μg $pHRE_4GL3$ or 100 μg of pGL3. 6 hours post transfection, cells were exposed to normoxia or hypoxia with or without further treatment with 100 μM genistein. A 1000 fold dilution of DMSO was added to untreated wells. 16 hours later, cells were harvested for luciferase assays. Each combination of transfection and treatment was performed in triplicate.

2.5.11. EFFECT OF DEACETYLASE INHIBITION ON HIF-1α AND HIF-2α EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were exposed to normoxia or hypoxia with or without further treatment with 30 ng/mL trichostatin A or 10 mM sodium butyrate for 16 hours. Whole cell extracts were then prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of either HIF-1α or HIF-2α.

2.5.12. RTPCR AND NORTHERN ANALYSIS OF ARD1 EXPRESSION

PC12 cells were plated into T175 flasks containing 20 mL of PC12 medium. These were exposed to normoxia or hypoxia for 16 hours, after which time poly A RNA was prepared. A total of four flasks of cells were required for each treatment.

From 200 ng of this RNA was synthesised cDNA. 1 μL of this or of H_2O was used as a template in a Taq polymerase mediated PCR reaction employing as primer combinations rARD1 upper and lower or β actin upper and lower. The products were analysed via electrophoresis through a 2% agarose/1 X TBE/ethidium bromide gel and visualised with a UV transilluminator. All reaction products were of the estimated size. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified as for restriction digestion fragments and cloned directly into prelinearised pGEMTEASY (hereafter referred to as pGEM/ARD1 and pGEM/actin respectively) and sequenced.

3 μg of this poly A RNA was also electrophoresed through a 1 X MOPS/1% agarose/formaldehyde gel and transferred to nylon for Northern analysis of the expression of ARD1. pGEM/ARD1 and pGEM/actin were digested with *Eco*RI and the appropriately sized fragments were purified and labelled with ^{32}P-dATP. The membranes were probed for the ARD1, stripped and reprobed for β actin as a control.

2.5.13. IMMUNOBLOTTING FOR ARD1 EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. Whole cell extracts were prepared from cells exposed to normoxia or hypoxia for 16 hours. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of ARNT or through a 15% acrylamide SDS PAGE gel for immunoblotting for the presence of ARD1.

2.5.14. RNA INTERFERENCE OF ARD1 EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pSUPER/SCR, pSUPER/ARD1 144, pSUPER/ARD1 150, pSUPER/ARD1 552 or pSUPER/PHD2 627. 48 hours later, whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and then transferred to nitrocellulose for immunoblotting for the presence of ARNT or through a 15% acrylamide SDS PAGE gel for immunoblotting for the presence of ARD1.

Once the best ARD1 targeted constructs were determined, PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pSUPER/SCR, pSUPER/ARD1 144 or pSUPER/ARD1 150. 32 hours post transfection cells were exposed to normoxia or hypoxia. 16 hours later, whole cell extracts were prepared. 10 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of ARNT, HIF-1α or HIF-2α or through a 15% acrylamide SDS PAGE gel for immunoblotting for the presence ARD1.

2.5.15. EFFECT OF EXOGENOUS ARD1 ACTIVITY ON HIF-1α AND HIF-2α EXPRESSION

PC12 cells were plated into 6 cm dishes containing 3 mL of PC12 medium. These were transfected with 5 μg of pEF/IRES/PURO or pEF/ARD1/IRES/PURO. 6 hours post

transfection, cells were exposed to normoxia or hypoxia. 16 hours later, whole cell extracts were prepared. 20 μg of each were electrophoresed through a 7.5% acrylamide SDS PAGE gel and transferred to nitrocellulose for immunoblotting for the presence of ARNT or through a 15% acrylamide SDS PAGE gel for ARD1.

CHAPTER 3

SCREENING FOR HIF-2α SPECIFIC TARGET GENES IN PC12 CELLS

CHAPTER 3

SCREENING FOR HIF-2α SPECIFIC TARGET GENES IN PC12 CELLS

3.1. INTRODUCTION

Hypoxia inducible factor 1α (HIF-1α) and HIF-2α are capable of regulating hypoxic response element (HRE) mediated reporter gene expression (Semenza *et al.*, 1992; Tian *et al.*, 1997; Ema *et al.*, 1997; Hogenesch *et al.*, 1997). Although the transcription of a number of endogenous genes has been shown conclusively to be directly induced by HIF-1α, specific HIF-2α targets are yet to be identified. Therefore, the primary aim of the research described in this chapter was to discover specific target genes of HIF-2α.

In selecting a cell system to screen for HIF-2α target genes, three important criteria were considered. Firstly, the cells should be physiologically relevant to representing the hypoxic response; secondly, they should endogenously express hypoxically inducible HIF-1α and HIF-2α; and finally they should be amenable to experimental manipulation of HIF-2α. The oxygen sensing rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976) has been utilised for some time as a model for type I carotid body cells (Seta *et al.*, 2002), particularly given that immortalised carotid body lines are unavailable. One reason for this is that they share a hypoxia inducible capability to synthesise and release catecholamines (Fishman *et al.*, 1985; Taylor and Peers, 1998; Kumar *et al.*, 1998). This is significant given the embryonic lethal phenotype of the first reported HIF-2α null mouse and its association with a significantly reduced level of catecholamines (Tian *et al.*, 1998). It therefore seemed logical to use a catecholaminergic cell line to determine HIF-2α specific modes of control and target genes. PC12 cells express both HIF-1α and HIF-2α and their respective protein products are stabilised in hypoxia (Conrad *et al.*, 1999; Agani *et al.*, 2000). Finally, it has been observed in many studies and cell types that HIF-α stability and activity can be artificially induced in normoxia by the forced overexpression of either gene, most likely due to the saturation of the HIF-α prolyl-4- and asparaginyl hydroxylases that otherwise regulate the function of the endogenous protein. With this in mind, PC12 cells were chosen and engineered to generate inducible lines capable of the selective upregulation of either HIF-α in normoxia from which

comparative gene expression profiles might be used to identify HIF-2α specific target genes.

3.2. CHARACTERISATION OF PC12 CELLS

Before this PC12 cells could be used in these studies, the biochemical properties of this line had to be further characterised. In other words, it had to be confirmed that both HIF-1α and HIF-2α could be upregulated in hypoxia.

PC12 cells were exposed to 20% O_2 (normoxia) or <1% O_2 (hypoxia) for 16 hours and whole cell extracts were analysed by immunoblotting with anti HIF-1α or anti HIF-2α rabbit polyclonal antibodies (figure 3.1.A). Bands corresponding to HIF-1α and HIF-2α were detected in hypoxic but not normoxic PC12 cell extracts. Furthermore, PC12 cells were transfected with a firefly luciferase reporter gene containing a minimal simian virus 40 (SV40) promoter and 4 copies of the HRE derived from the erythropoietin (EPO) gene ($pHRE_4GL3$, hereafter referred to as pHRE), or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to normoxia, hypoxia or to a 100 μM concentration of one of the hypoxia mimicking iron chelators desferrioxamine (DFO) or 2, 2'-dipyridyl (DP). After a further 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal control plasmid containing the herpes simplex virus thymidine kinase (TK) promoter mediating the expression of renilla luciferase (pRLTK) (figure 3.1.B). HRE reporter activity was enhanced at least 10 fold in these cells when grown in hypoxia or with DFO or DP, but that of the control reporter failed to be significantly altered by any treatment.

Collectively, these results confirm the presence of endogenous hypoxia inducible HIF-1α and HIF-2α protein in PC12 cells (Conrad *et al.*, 1999; Agani *et al.*, 2000) and HRE controlled gene activity. This supports their use in the characterisation of HIF-2α specific target genes.

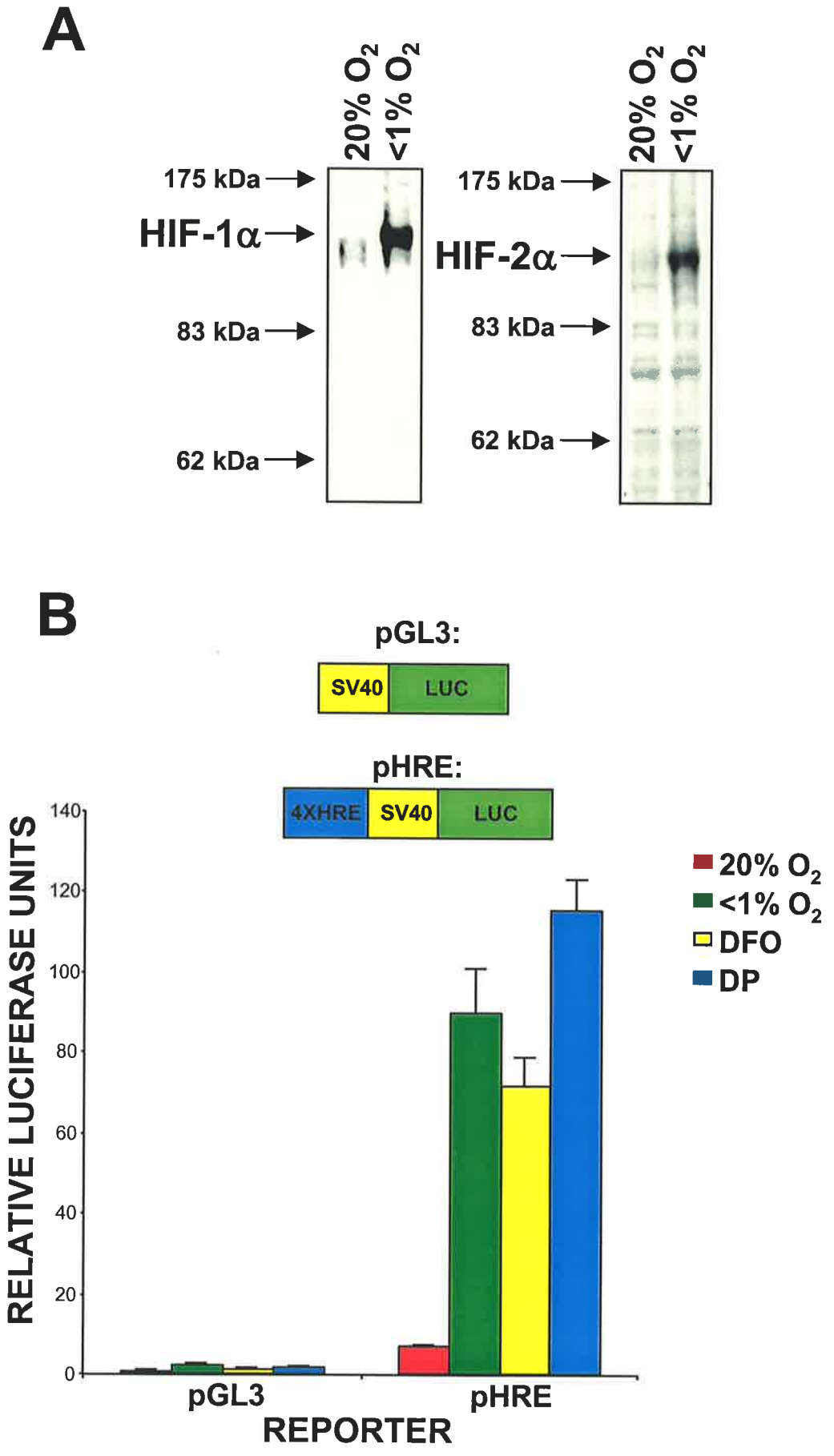
3.3. SELECTIVE EXPRESSION OF HIF-2α IN PC12 CELLS

Exposing PC12 cells to hypoxia would result in the upregulation of all hypoxically inducible genes. These would include direct and indirect targets of HIF-1α and HIF-2α and possibly genes whose transcripts are upregulated in hypoxia but not as a consequence

Figure 3.1. PC12 cells express detectable levels of both HIF-1α and HIF-2α and hypoxia and its mimetics induce HRE reporter activity in PC12 cells.

A. Whole cell extracts were prepared from PC12 cells exposed to 20% and <1% O_2 for 16 hours. 25 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (left panel) or HIF-2α (right panel).

B. PC12 cells were transiently transfected with a firefly luciferase reporter gene (LUC) containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2, 1% O_2 or to a 100 μM concentration of one of the hypoxia mimicking iron chelators desferrioxamine (DFO) or 2, 2'-dipyridyl (DP). After a further 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of three independent experiments.


A
20% O2
<1% O2
175 kDa
HIF-1α
83 kDa
62 kDa
175 kDa
HIF-2α
83 kDa
62 kDa
B
pGL3:
SV40
LUC
pHRE:
4XHRE
SV40
LUC
RELATIVE LUCIFERASE UNITS
140
120
100
80
60
40
20
0
pGL3
pHRE
REPORTER
20% O2
<1% O2
DFO
DP

of the activity of either HIF-α. As the purpose of these studies was to determine HIF-2α specific targets in PC12 cells, it had to be possible to selectively induce the protein and activity of this transcription factor only. To this end, induced protein stability and transcriptional activity of HIF-2α in normoxia via overexpression was investigated in these PC12 cells.

PC12 cells were transfected with a mammalian expression construct for HIF-2α (pEFBOS/HIF-2α) or its parent vector (pEFBOS), in addition to pHRE or pGL3 reporters. 6 hours post transfection, cells were exposed to normoxia or hypoxia. After 16 hours, cells were assayed for firefly luciferase activity against that of cotransfected pRLTK (figure 3.2.A). The forced expression of HIF-2α enhanced HRE mediated reporter activity in normoxia (compare pHRE + HIF-2α versus pHRE at 20% O_2) but not that of the reporter lacking the HRE (compare pGL3 + HIF-2α versus pGL3 at 20% O_2). Furthermore, immunoblotting of extracts from the same cells with anti HIF-2α rabbit polyclonal antibodies demonstrated that stabilised HIF-2α was present (figure 3.2.B). Therefore, it was possible to artificially induce HIF-α function in normoxia via overexpression in PC12 cells. It was thus considered feasible to generate stable derivatives of PC12 cells in which either HIF-α could be selectively induced.

3.4. ESTABLISHMENT OF MONOCLONAL TETRACYCLINE INDUCIBLE PC12 CELL LINES

To avoid problems associated with transiently introducing pEFBOS/HIF-2α into PC12 cells, such as reproducibility of transfection efficiency, poor control of expression and, not least of all, inconvenience, it was decided to engineer PC12 cells in which the HIF-2α cDNA is stably integrated into the genome.

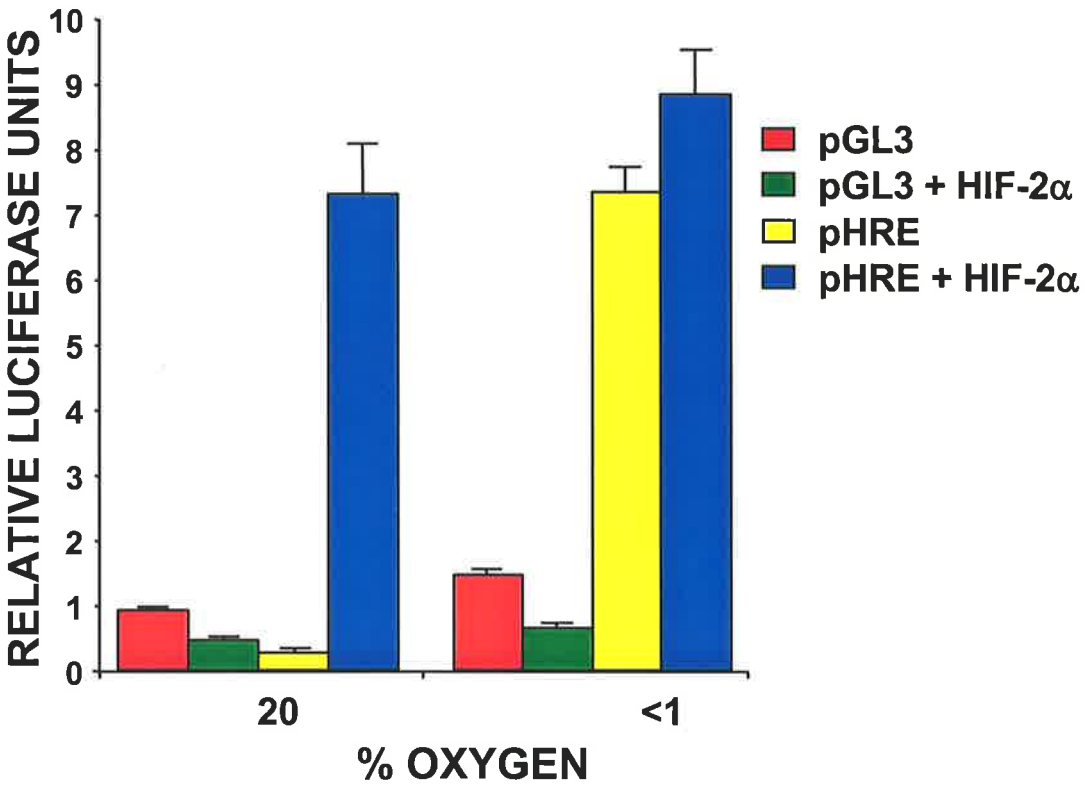
However, complications regarding the constitutive expression of the introduced HIF-2α were foreseen. For instance, direct targets of HIF-2α are more likely to be induced immediately after HIF-2α expression rather than as a consequence of extended, constitutive expression. Also, there may be deleterious effects on the cells if HIF-2α was constantly stable and active. Finally, the degradation machinery may itself be a target of HIF-α, meaning that a feedback mechanism may ultimately promote its downregulation, as appears to be the case for PHD2 (del Peso *et al.*, 2003). Therefore, it was decided to

Figure 3.2. Forced expression of HIF-2α in normoxia causes HIF-2α protein accumulation and induces HRE reporter activity.

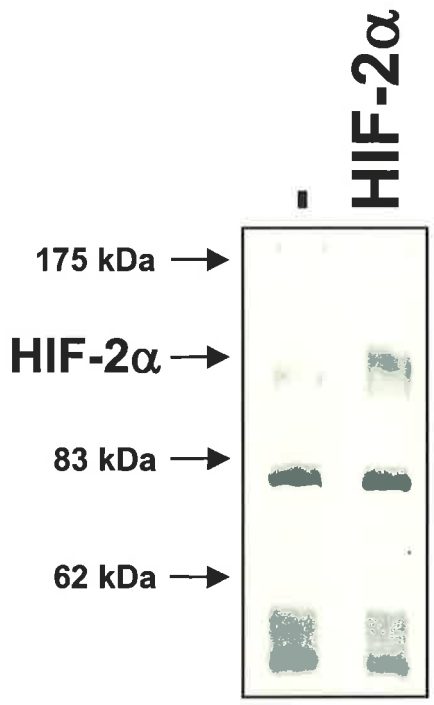
A. Cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3) and either a mammalian expression construct containing the cDNA for HIF-2α or the empty parent vector. 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2. After a further 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation.

B. Extracts from the above luciferase reporter analysis derived from cells exposed to 20% O_2 or <1% O_2 and transfected with both pGL3 and the mammalian expression construct containing the cDNA for HIF-2α were analysed by immunoblotting with antibodies to HIF-2α.

A


RELATIVE LUCIFERASE UNITS
10
9
8
7
6
5
4
3
2
1
0
20
<1
% OXYGEN
pGL3
pGL3 + HIF-2α
pHRE
pHRE + HIF-2α

B

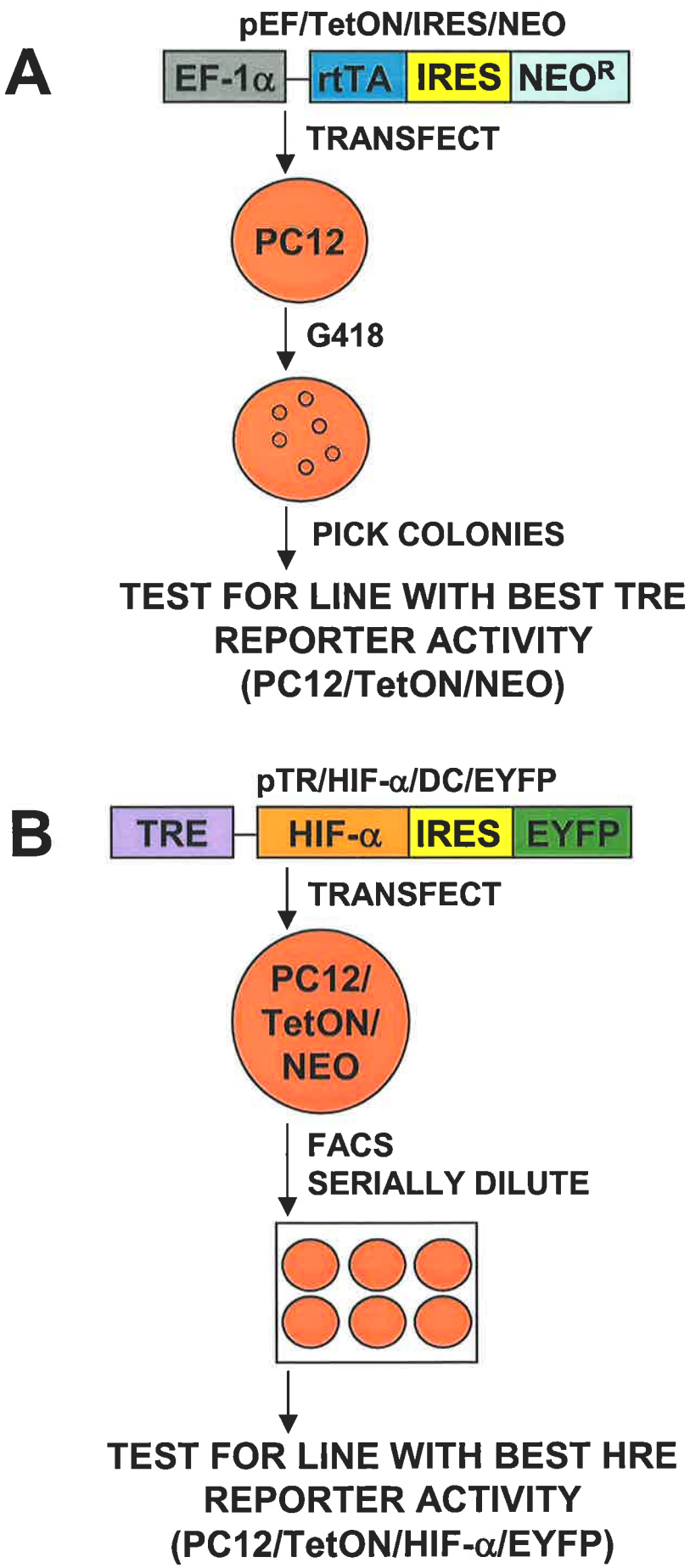

-
HIF-2α
175 kDa
HIF-2α
83 kDa
62 kDa

establish tetracycline inducible HIF-α monoclonal PC12 cell lines. Tetracycline inducible gene expression is based on the constitutive expression of an introduced gene into mammalian cell encoding the tetracycline transactivator protein (tTA), a fusion composed of the tetracycline repressor of *Escherichia coli* (*E. coli*) and the transactivation domain of the VP16 protein of the herpes simplex virus (Shockett and Schatz, 1996). When there is no tetracycline present, tTA binds with high affinity to sequences from the tetracycline resistance operator (*tetO*) of Tn10 and promotes transcription, although this is relieved upon addition of the antibiotic (Hinrichs *et al.*, 1994). The sequences to which the tTA binds are specific to *E. coli*, so there should be no aberrant association with or regulation of mammalian genes. To alleviate any potential problems associated with prolonged exposure of the stable PC12 cell lines to tetracycline or its derivative doxycycline, and given that a rapid induction of HIF-α was a desirable quality which might have been limited by the rate of disappearance of the antibiotic if the tTA was used, it was decided to utilise the reverse tTA (rtTA) protein instead. Here, the tTA has been modified such that it binds to the sequences from the *tetO* upon supplementation of growth medium with tetracycline (Gossen *et al.*, 1995). An approach that employs the rtTA protein and thus where tetracycline induces transcription of the target gene is known as the TetON system.

The generation of tetracycline inducible HIF-α monoclonal PC12 cell lines ultimately involved successive incorporation of two mammalian expression constructs into the PC12 genome (figure 3.3). First, the rtTA had to be stably transfected into the PC12 cells to generate a parent line that could constitutively express the rtTA protein. This entailed transfecting into the PC12 cells a plasmid where an internal ribosome entry sequence (IRES) allows for an antibiotic resistance gene to be translated from the same mRNA as the rtTA. In principle, the ability of the transfectants to be cultivated in the presence of the antibiotic requires the incorporation of the entire sequence as the promoter is separated from the antibiotic resistance gene by the cDNA for rtTA. The stably transfected cells could then form the basis for the establishment of subsequent lines where the stable transfection of the HIF-α genes mediated by the *tetO* could be used to induce either of the HIF-α proteins by the addition to the growth medium of doxycycline. For the introduction of inducible HIF-α, a second vector was utilised with the HIF-α cDNA separated from that of enhanced yellow fluorescent protein (EYFP) by an IRES. Transcription in this case is mediated by a *tetO* driven promoter, which is transactivated upon activation of the already integrated rtTA protein by doxycycline.

Figure 3.3. Strategy for the establishment of monoclonal PC12 cell lines capable of selective HIF-1α or HIF-2α activation.

PC12 cell lines capable of doxycycline inducible HIF-α expression were developed in two steps. First, a monoclonal line constitutively expressing the reverse tetracycline transactivator protein (rtTA) was established. This was then used as a parent line to develop derivatives that were capable of doxycycline inducible active HIF-1α or HIF-2α expression and a control line that expresses enhanced yellow fluorescent protein (EYFP) only.


A
pEF/TetON/IRES/NEO
EF-1α
rtTA
IRES
NEOR
TRANSFECT
PC12
G418
PICK COLONIES
TEST FOR LINE WITH BEST TRE REPORTER ACTIVITY (PC12/TetON/NEO)
B
pTR/HIF-α/DC/EYFP
TRE
HIF-α
IRES
EYFP
TRANSFECT
PC12/ TetON/ NEO
FACS
SERIALLY DILUTE
TEST FOR LINE WITH BEST HRE REPORTER ACTIVITY (PC12/TetON/HIF-α/EYFP)

3.4.1. ESTABLISHMENT OF THE PC12/TetON/NEO CELL LINE

Our research group already possessed a construct termed pEF/TetON/IRES/PURO that contained the gene for the rtTA driven by the strong elongation factor 1α (EF-1α) promoter. To facilitate screening for cells into which rtTA has been stably integrated, an IRES allows for the puromycin resistance gene to be translated from the same transcript as the rtTA.

As a consequence of initial problems with regards to establishing puromycin resistant PC12 cells (namely, a gross change in morphology and a marked reduction in endogenous HIF-2α expression, data not shown), PC12 cells were tested for neomycin sensitivity and it was decided that neomycin resistance would be used a selection marker for rtTA incorporation. Consequently, the cDNA for the neomycin resistance gene was substituted for that of puromycin resistance, resulting in the construction of the mammalian expression plasmid pEF/TetON/IRES/NEO.

To generate a monoclonal line capable of stably expressing the rtTA, PC12 cells were transfected with the pEF/TetON/IRES/NEO construct and expanded. After 72 hours, the medium was supplemented with 200 μg/mL of G418 for 12 days. Most cells senesced. However, a number of neomycin resistant colonies had grown to a sufficient size such that 48 colonies were isolated and maintained in separate wells. After a further 7 days, a total of 18 were still growing. To check for functional rtTA activity, these were expanded and transiently transfected with the pTR/DC/EYFP plasmid (capable of EYFP expression following doxycycline inducible activation of rtTA, more details below). 6 hours post transfection, cells were left untreated or the medium was supplemented with 2 μg/mL of doxycycline. 40 hours later, cells were checked for fluorescence. 10 of the lines demonstrated doxycycline inducible EYFP (data not shown). The best (B4) was maintained and will hereafter be referred to as the PC12/TetON/NEO line.

PC12/TetON/NEO cells were transfected with pBI/GL, a construct of the firefly luciferase reporter gene containing the minimal cytomegalovirus (CMV) promoter under the control of the tetracycline response element (TRE) which consists of seven copies of the *tetO* sequence. 6 hours post transfection, cells were exposed to 2 μg/mL doxycycline or left untreated. After 16 hours, cells were assayed for firefly luciferase activity against

that of cotransfected pRLTK (figure 3.4.A). Doxycycline treatment produced an approximately 29 fold induction in TRE mediated reporter activity.

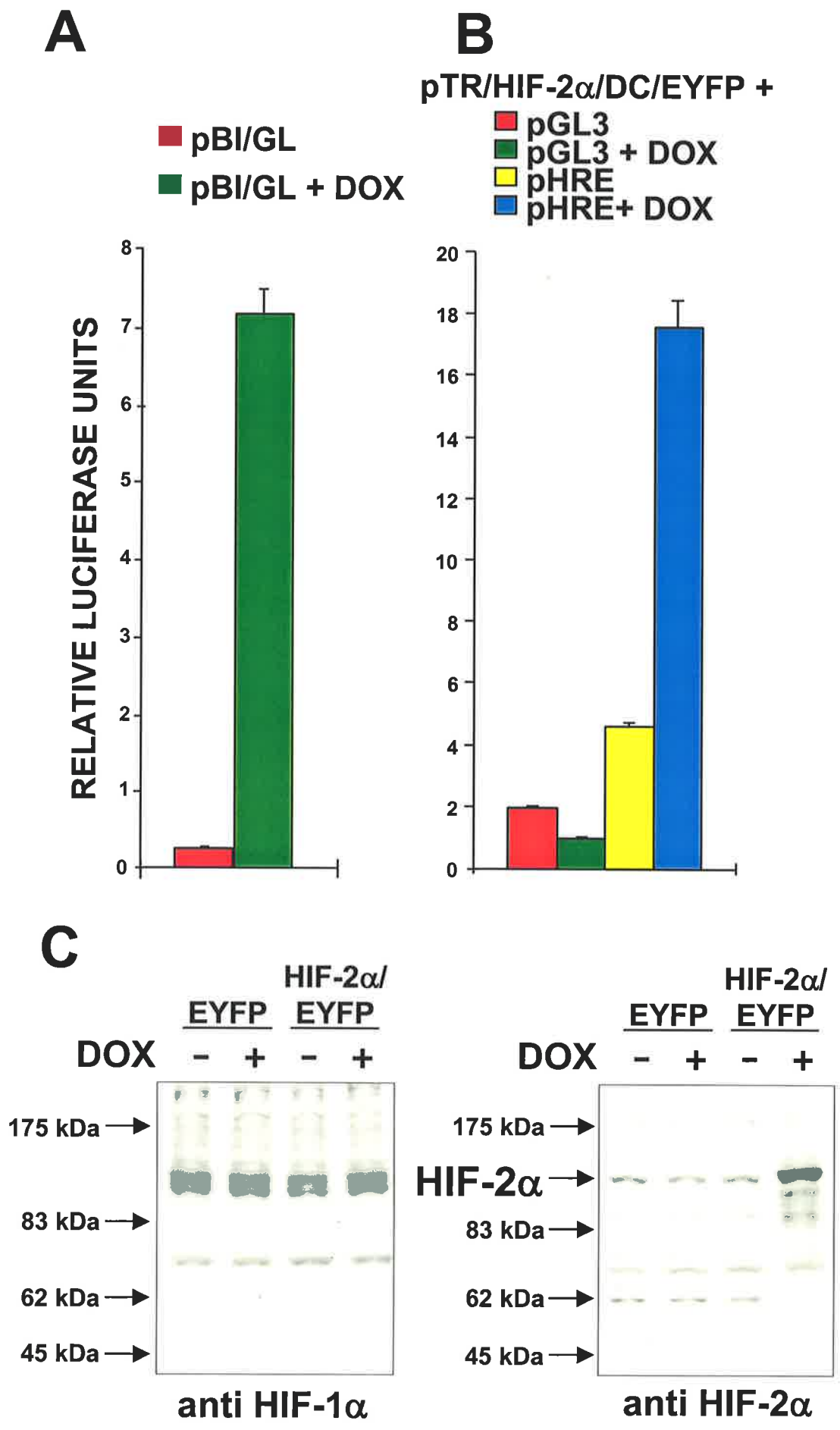
To characterise the ability of doxycycline to induce TRE mediated HIF-α function in the PC12/TetON/NEO line, cells were transfected with a mammalian expression construct containing the cDNA for HIF-2α under the control of a CMV promoter mediated by the TRE (pTR/HIF-2α/DC/EYFP, more details to below) in addition to pHRE or pGL3 reporters. 6 hours post transfection, cells were exposed to 2 μg/mL doxycycline or left untreated. After a further 16 hours, cells were assayed for firefly luciferase activity against that of cotransfected pRLTK (figure 3.4.B). The addition of doxycycline enhanced HRE mediated reporter activity (compare pHRE + DOX versus pHRE) but not that of the reporter lacking the enhancer (pGL3 + DOX versus pGL3). This suggested that rtTA activated by doxycycline is capable of inducing the HRE reporter activity of a TRE mediated HIF-α despite an absence of hypoxia. To confirm that this was due to the stabilisation of introduced HIF-2α, PC12/TetON/NEO cells were transfected with pTR/HIF-2α/DC/EYFP or its parent vector pTR/DC/EYFP. 6 hours post transfection, cells were exposed to 2 μg/mL doxycycline or left untreated. After a further 16 hours, whole cell extracts were analysed by immunoblotting with anti HIF-1α or anti HIF-2α rabbit polyclonal antibodies (figure 3.4.C). HIF-2α is detected in extracts only from PC12/TetON/NEO cells transfected with pTR/HIF-2α/DC/EYFP and treated with doxycycline (compare HIF-2α/EYFP + DOX versus HIF-2α/EYFP – DOX when immunoblotted with anti HIF-2α), thus demonstrating that a doxycycline activated rtTA can express a TRE mediated HIF-α protein in normoxia. Doxycycline itself does not cause the stabilisation of either HIF-α (compare EYFP + DOX versus EYFP – DOX), demonstrating its inability to act as a hypoxia mimetic. Furthermore, stabilisation of HIF-2α, presumably due to the saturation of the HIF-α degradation mechanisms, did not cause costabilisation of HIF-1α, so any corresponding HRE reporter activity can be attributed to the selective accumulation of HIF-2α protein. The reporter assays and immunoblotting analysis described above thus augured well for the establishment of monoclonal lines derived from the PC12/TetON/NEO line in which the function of either HIF-α could be promoted by treatment with doxycycline. Also, the immunoblotting demonstrated that the stabilisation of overexpressed HIF-2α presumably via saturation of the degradation machinery had no detectable effect on HIF-1α protein levels. This is important in the context of attempting to screen for HIF-2α specific target genes as the possibility of HIF-

Figure 3.4. Treatment of the PC12/TetON/NEO monoclonal cell line with doxycycline induces TRE mediated reporter activity. Furthermore, treatment of doxycycline causes the stabilisation and activation of a cotransfected TRE mediated HIF-2α gene.

A. Cells were transiently transfected with a firefly luciferase reporter gene containing a TRE (pBI/GL). 6 hours post transfection, cells were treated with 2 μg/mL doxycycline (DOX) or left untreated. After a further 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation.

B. Cells were transiently transfected with a mammalian expression construct containing the cDNA for HIF-2α under the control of the TRE (pTR/HIF-2α/DC/EYFP) in addition to a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were treated with 2 μg/mL doxycycline (DOX) or left untreated. After a further 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of two independent experiments.

C. Cells were transiently transfected with a mammalian expression construct containing the cDNA for HIF-2α under the control of the TRE (HIF-2α/EYFP) or the empty vector (EYFP). 6 hours post transfection, they were treated with 2 μg/mL doxycycline (DOX) or left untreated. After a further 16 hours, whole cell extracts were prepared and 20 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (left panel) or HIF-2α (right panel). Results are representative of two independent experiments.


A
pBI/GL
pBI/GL + DOX
RELATIVE LUCIFERASE UNITS
0
1
2
3
4
5
6
7
8
B
pTR/HIF-2α/DC/EYFP +
pGL3
pGL3 + DOX
pHRE
pHRE+ DOX
0
2
4
6
8
10
12
14
16
18
20
C
HIF-2α/
EYFP
EYFP
DOX
- + - +
175 kDa
83 kDa
62 kDa
45 kDa
anti HIF-1α
HIF-2α/
EYFP
EYFP
DOX
- + - +
175 kDa
HIF-2α
83 kDa
62 kDa
45 kDa
anti HIF-2α

1α involvement can be discounted. However, it is also interesting insofar as it suggests that the respective HIF-α proteins may be regulated by independent mechanisms of degradation.

3.4.2. ESTABLISHMENT OF THE PC12/TetON/EYFP CELL LINE

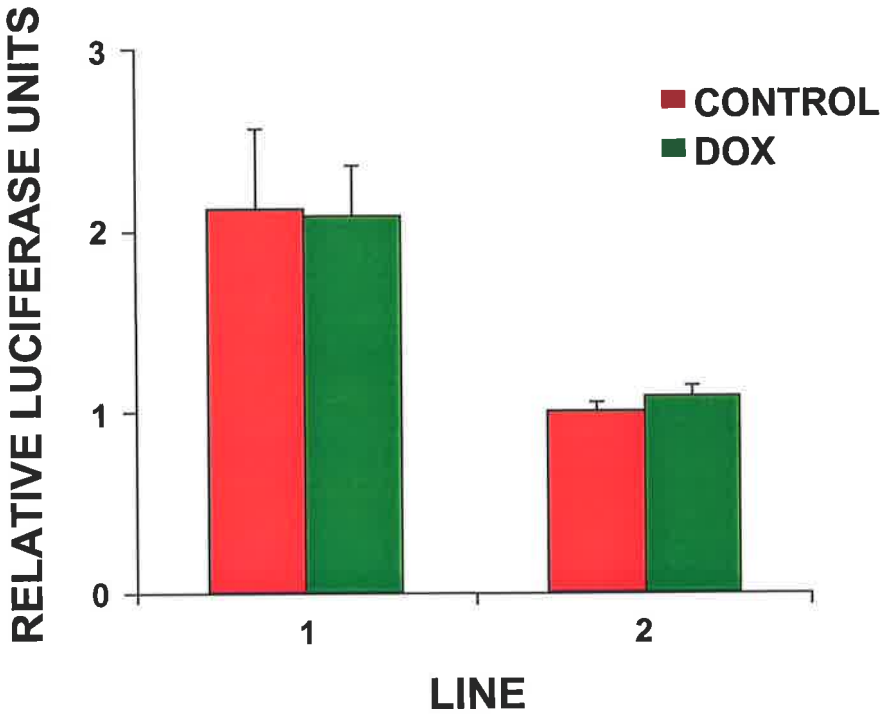
Although the PC12 derived line of most significance to this study is that in which HIF-2α can be selectively induced by treatment with doxycycline, it was also imperative that a control line be established in order to account for any genes whose transcripts are upregulated by doxycycline or the selective marker.

The construct which formed the basis of all subsequent doxycycline inducible cell lines derived from PC12/TetON/NEO was termed pTR/DC/EYFP (briefly introduced above), into which the HIF-α cDNA could be cloned. Transcription is mediated by the TRE which is activated upon the activation of the previously incorporated rtTA. Also, an IRES separates the introduced upstream cDNA from that of EYFP within the same transcript. The presence of doxycycline induced EYFP thus infers expression of the protein coded by the inserted cDNA.

To generate the control line, PC12/TetON/NEO cells were transfected with pTR/DC/EYFP. After cultivation for 7 days, followed by two rounds of FACS (all FACS was performed by Sandy MacIntyre and Alan Bishop at the Hanson Institute, Adelaide, Australia) after a 16 hour treatment with 2 μg/mL doxycycline (the first to select for all fluorescing cells, the second to select for the highest fluorescing cells (about 5%)), the selected cells were grown for a further 5 days prior to dilution of approximately 1 cell/well into wells of a 24 well tray. There ultimately remained 2 monoclonal lines that displayed EYFP expression after a 24 hour treatment with 2 μg/mL doxycycline. To examine these further, both were transfected with the pHRE reporter plasmid. 6 hours post transfection, cells were left untreated or were exposed to 2 μg/mL doxycycline. After a further 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.5). As expected, neither demonstrated doxycycline inducible HRE mediated reporter activity. Given that one of these lines (2) upon prolonged maintenance demonstrated a gross change in morphology, it was decided to continue with the other (1), which will hereafter be referred to as the PC12/TetON/EYFP cell line.

Figure 3.5. Screening for the control PC12/TetON/EYFP monoclonal cell line.

Selected cell lines were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE). 6 hours post transfection, cells were either treated with 2 μg/mL doxycycline (DOX) or left untreated (CONTROL). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation.


RELATIVE LUCIFERASE UNITS
3
2
1
0
CONTROL
DOX
1
2
LINE

To further characterise this line with respect to HIF-α expression, PC12/TetON/EYFP cells were transfected with pHRE or pGL3 reporters. 6 hours post transfection, cells were treated with 2 μg/mL doxycycline or left untreated and exposed to normoxia or hypoxia. After a further 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.6.A). Doxycycline was unable to induce HRE reporter activity (compare pHRE + DOX versus pHRE at 20% O_2), which was only enhanced by hypoxic treatment (pHRE and pHRE + DOX at <1%O_2). Furthermore, immunoblotting of whole cell extracts from similarly treated cells (figure 3.6.B) showed that HIF-1α and HIF-2α could only be stabilised upon a reduction in oxygen levels, regardless of doxycycline treatment. In short, HIF-α protein accumulation and transcriptional activity in the PC12/TetON/EYFP line is unaltered by doxycycline treatment. Therefore, it serves as an ideal control line from which genes regulated by doxycycline treatment or EYFP can be determined and eliminated from the gene expression profile of the other PC12/TetON/NEO derivatives.

3.4.3. ESTABLISHMENT OF THE PC12/TetON/HIF-2α/EYFP CELL LINE

The cDNA for HIF-2α was subcloned into pTR/DC/EYFP, resulting in pTR/HIF-2α/DC/EYFP. This construct was then transfected into PC12/TetON/NEO cells to generate stable cell lines. After doxycycline treatment, FACS and limiting dilution as for the control line, 7 monoclonal lines were generated, two of which (1 and 2) displayed EYFP expression after treatment with 2 μg/mL doxycycline for 24 hours. To examine these further, both lines were transfected with the pHRE reporter plasmid. 6 hours post transfection, cells were left untreated or were exposed to 2 μg/mL doxycycline. After a further 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.7). As expected, all displayed some degree of doxycycline inducible HRE mediated reporter activity (compare DOX versus CONTROL), although the best two (1 and 2) were those which expressed most EYFP after 24 hours doxycycline treatment. The overall best clone (2 with an approximately 21 fold increase in HRE reporter after exposure to doxycycline) was maintained and will hereafter be referred to as the PC12/TetON/HIF-2α/EYFP cell line.

To analyse this line in further detail, PC12/TetON/HIF-2α/EYFP cells were transfected with pHRE or pGL3 reporters. 6 hours post transfection, cells were treated with 2 μg/mL

Figure 3.6. In PC12/TetON/EYFP cells, HRE reporter activity is increased and HIF-1α and HIF-2α are stabilised in hypoxia but are not influenced by the presence of doxycycline.

A. Cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of three independent experiments.

B. Whole cell extracts were prepared from cells exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX) for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to HIF-1α (left panel) or HIF-2α (right panel). Results are representative of three independent experiments.

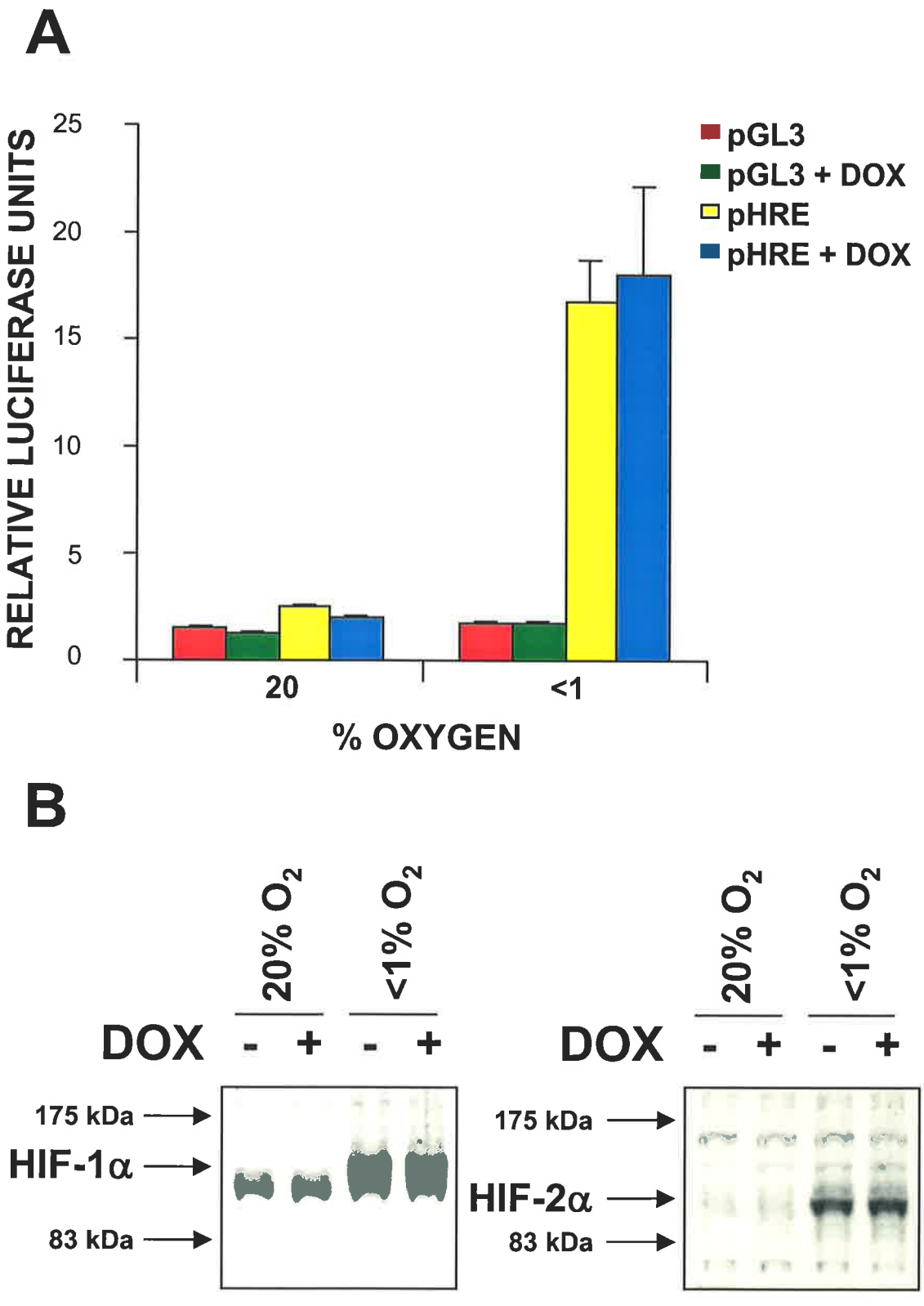
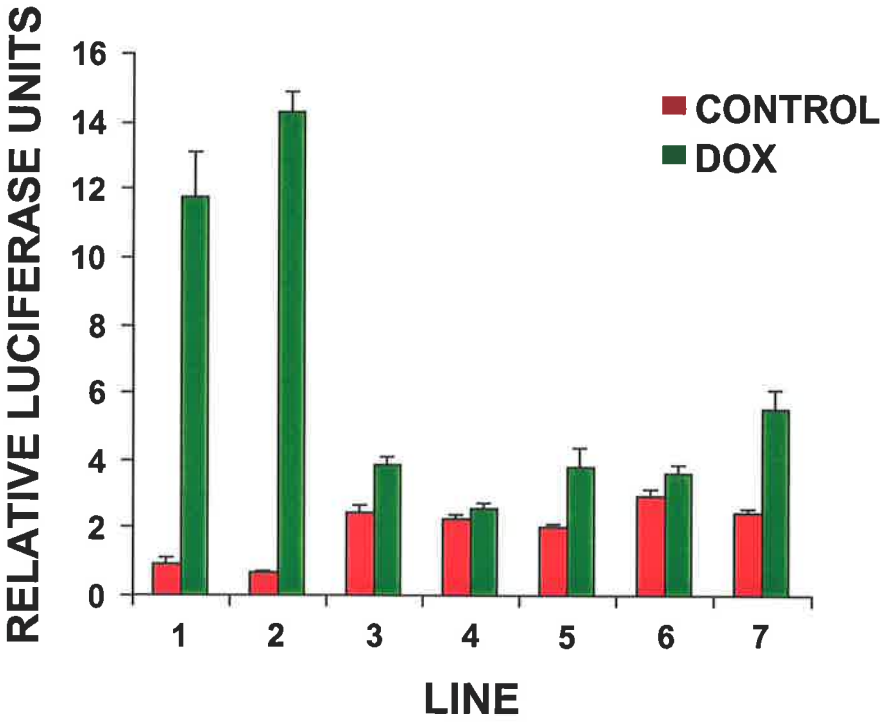

A
RELATIVE LUCIFERASE UNITS
25
20
15
10
5
0
20
<1
% OXYGEN
pGL3
pGL3 + DOX
pHRE
pHRE + DOX
B
20% O2
<1% O2
DOX
- + - +
175 kDa
HIF-1α
83 kDa
20% O2
<1% O2
DOX
- + - +
175 kDa
HIF-2α
83 kDa

Figure 3.7. Screening for the PC12/TetON/HIF-2α/EYFP monoclonal cell line. Selected cell lines were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE). 6 hours post transfection, cells were either treated with 2 μg/mL doxycycline (DOX) or left untreated (CONTROL). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation.


RELATIVE LUCIFERASE UNITS
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
LINE
CONTROL
DOX

doxycycline or left untreated and exposed to normoxia or hypoxia. After a further 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.8.A). Doxycycline enhanced HRE reporter activity in normoxia (compare pHRE + DOX versus pHRE). To attribute this phenomenon to doxycycline inducible HIF-2α, whole cell extracts from similarly treated cells were immunoblotted with antibodies to HIF-1α or HIF-2α (figure 3.8.B). HIF-2α but not HIF-1α protein was induced as a consequence of exposure to doxycycline (compare + DOX versus – DOX at 20% O_2), whereas both forms of HIF-α were present at hypoxia. Therefore, any doxycycline induced HRE mediated activity in the PC12/TetON/HIF-2α/EYFP line in normoxia can be credited to the selective accumulation and transactivation of HIF-2α. Moreover, any direct HIF-α target gene induced upon exposure of this line to doxycycline would indeed be a HIF-2α target.

3.4.4. ESTABLISHMENT OF THE PC12/TetON/HIF-1α/EYFP CELL LINE

The primary aim of this research was to determine HIF-2α specific target genes. Therefore, a means had to be developed by which genes also upregulated by HIF-1α could be discounted from the complement of putative HIF-2α targets. To that end, HIF-1α cDNA was subcloned into pTR/DC/EYFP, resulting in the construction of pTR/HIF-1α/DC/EYFP. This construct was then transfected into PC12/TetON/NEO cells to generate stable cell lines. After doxycycline treatment, FACS and limiting dilution as for the control line, there remained 32 monoclonal lines. Of these, 4 displayed EYFP expression which was induced by treatment with 2 μg/mL doxycycline for 18 hours. To examine these further, all four lines were transfected with the pHRE reporter plasmid. 6 hours post transfection, cells were left untreated or were exposed to 2 μg/mL doxycycline. After 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.9). All displayed poor doxycycline inducible HRE reporter activity (compare DOX versus CONTROL). E6 (hereafter referred to as PC12/TetON/HIF-1α/EYFP) displayed the best with still only about a 2 fold increase in luciferase activity after 16 hours of doxycycline treatment.

Doxycycline inducible HIF-1α stability was not an issue with these cells, but the transcriptional activity of this protein as determined by a transiently transfected HRE mediated reporter was low (see chapter 4.3.3). This lack of inducible HIF-1α activity discounted the use of this cell line for the identification of HIF-1α target genes and

Figure 3.8. In PC12/TetON/HIF-2α/EYFP cells, HRE reporter activity is via doxycycline inducible HIF-2α.

A. Cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of three independent experiments.

B. Whole cell extracts were prepared from cells exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX) for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to HIF-1α (left panel) or HIF-2α (right panel). Results are representative of three independent experiments.

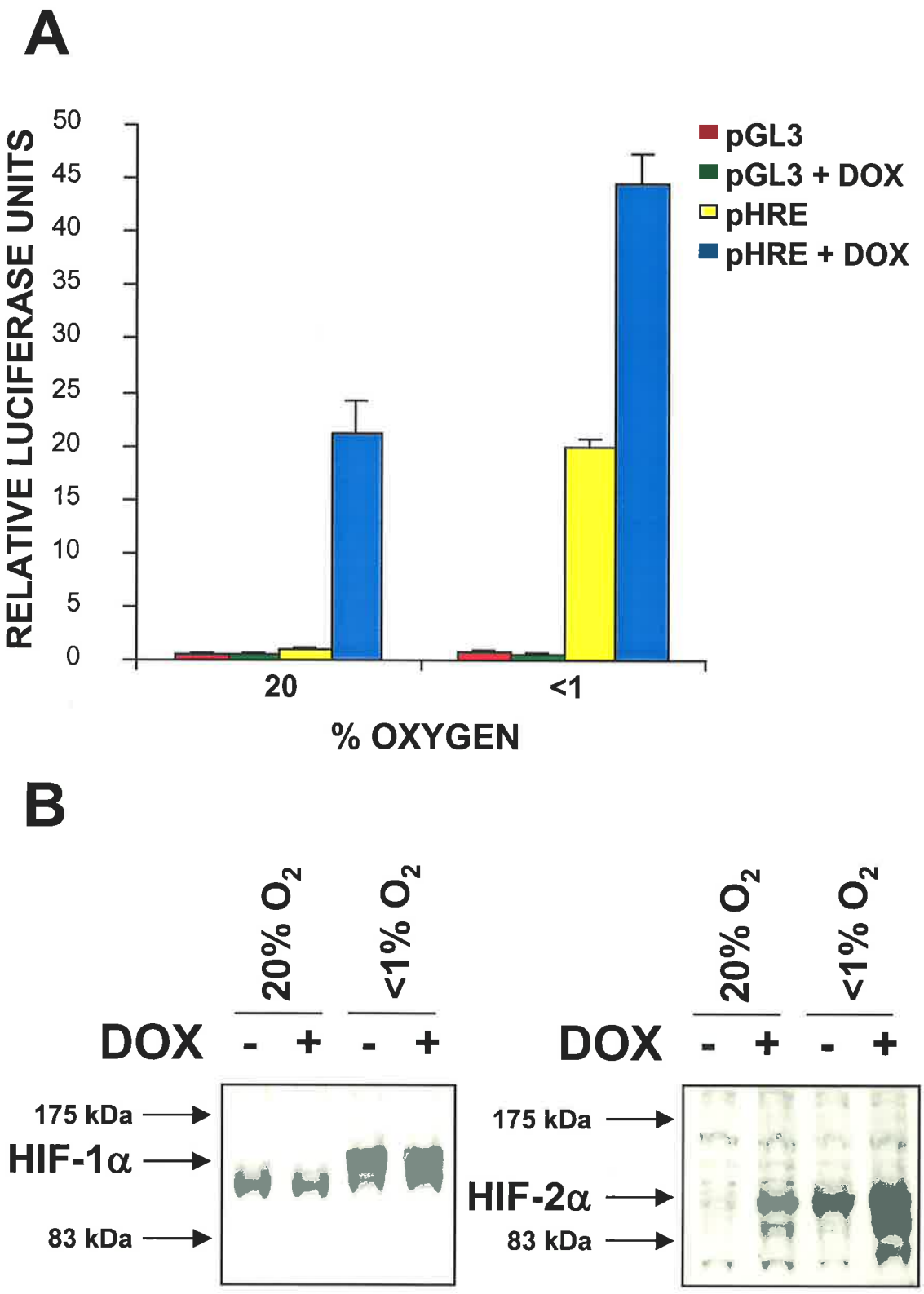
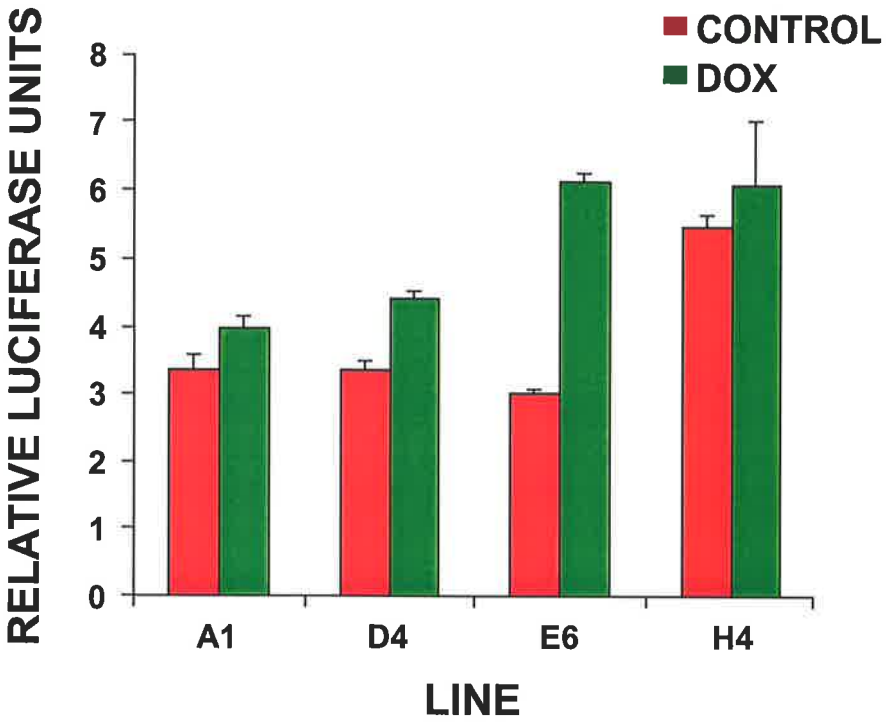

A
RELATIVE LUCIFERASE UNITS
50
45
40
35
30
25
20
15
10
5
0
20
<1
% OXYGEN
pGL3
pGL3 + DOX
pHRE
pHRE + DOX
B
20% O2
<1% O2
DOX
- + - +
175 kDa
HIF-1α
83 kDa
20% O2
<1% O2
DOX
- + - +
175 kDa
HIF-2α
83 kDa

Figure 3.9. Screening for the PC12/TetON/HIF-1α/EYFP monoclonal cell line. Selected cell lines were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE). 6 hours post transfection, cells were either treated with 2 μg/mL doxycycline (DOX) or left untreated (CONTROL). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation.


CONTROL
DOX
RELATIVE LUCIFERASE UNITS
8
7
6
5
4
3
2
1
0
A1
D4
E6
H4
LINE

therefore a second modified HIF-1α cell line had to be generated. The previously discussed phenomenon that HIF-1α protein which has been overexpressed and stabilised in normoxia still displays a high hypoxia inducibility of its transcriptional activity (O'Rourke *et al.*, 1999) may explain the poor doxycycline inducibility of HRE reporter activity in PC12/TetON/HIF-1α/EYFP cells.

3.4.5. ESTABLISHMENT OF THE PC12/TetON/HIF-1αN803A/EYFP CELL LINE

The fact that the PC12/TetON/HIF-1α/EYFP cell line expressed stable HIF-1α upon doxycycline treatment demonstrated that there was clearly no need to mutate the targeted residues (P402 and P564) of the HIF-α prolyl-4-hydroxylases to induce stability (chapter 4.3.3). Rather, it was postulated that the activity of the introduced HIF-1α in normoxia could be enhanced by the mutation of the target residue of the HIF-α asparaginyl hydroxylase so as to prevent normoxic repression of its transcriptional potency. Consequently, HIF-1α N803A was generated and subcloned into pTR/DC/EYFP resulting in the construction of pTR/HIF-1αN803A/DC/EYFP.

PC12/TetON/NEO cells were transfected with pTR/HIF-1αN803A/DC/EYFP. After doxycycline treatment, FACS and limiting dilution as for the control line, 23 monoclonal lines were obtained, 8 of which displayed EYFP expression after exposure to 2 μg/mL doxycycline for 23 hours. These were transfected with the pHRE reporter plasmid. 6 hours post transfection, cells were left untreated or were exposed to 2 μg/mL doxycycline. After a further 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.10). All displayed doxycycline inducible HRE mediated reporter activity (compare DOX versus CONTROL). The most inducible clone (E1 with an approximately 26 fold increase in HRE reporter after exposure to doxycycline) was maintained and will hereafter be referred to as the PC12/TetON/HIF-1αN803A/EYFP cell line.

The PC12/TetON/HIF-1αN803A/EYFP line was then evaluated in a fashion similar to that of the previous lines. Cells were transfected with the pHRE or pGL3 reporters. 6 hours post transfection, cells were treated with 2 μg/mL doxycycline or left untreated and exposed to normoxia or hypoxia. After a further 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 3.11.A). As with the PC12/TetON/HIF-2α/EYFP line, doxycycline induced HRE reporter activity in normoxia

Figure 3.10. Screening for the PC12/TetON/HIF-1αN803A/EYFP monoclonal cell line.

Selected cell lines were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE). 6 hours post transfection, cells were either treated with 2 μg/mL doxycycline (DOX) or left untreated (CONTROL). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation.

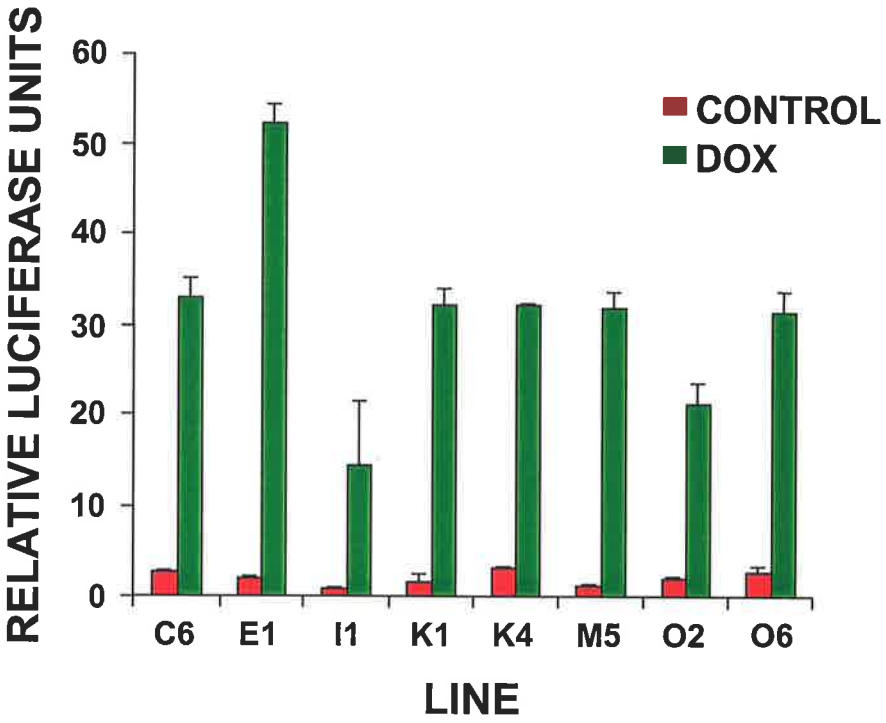
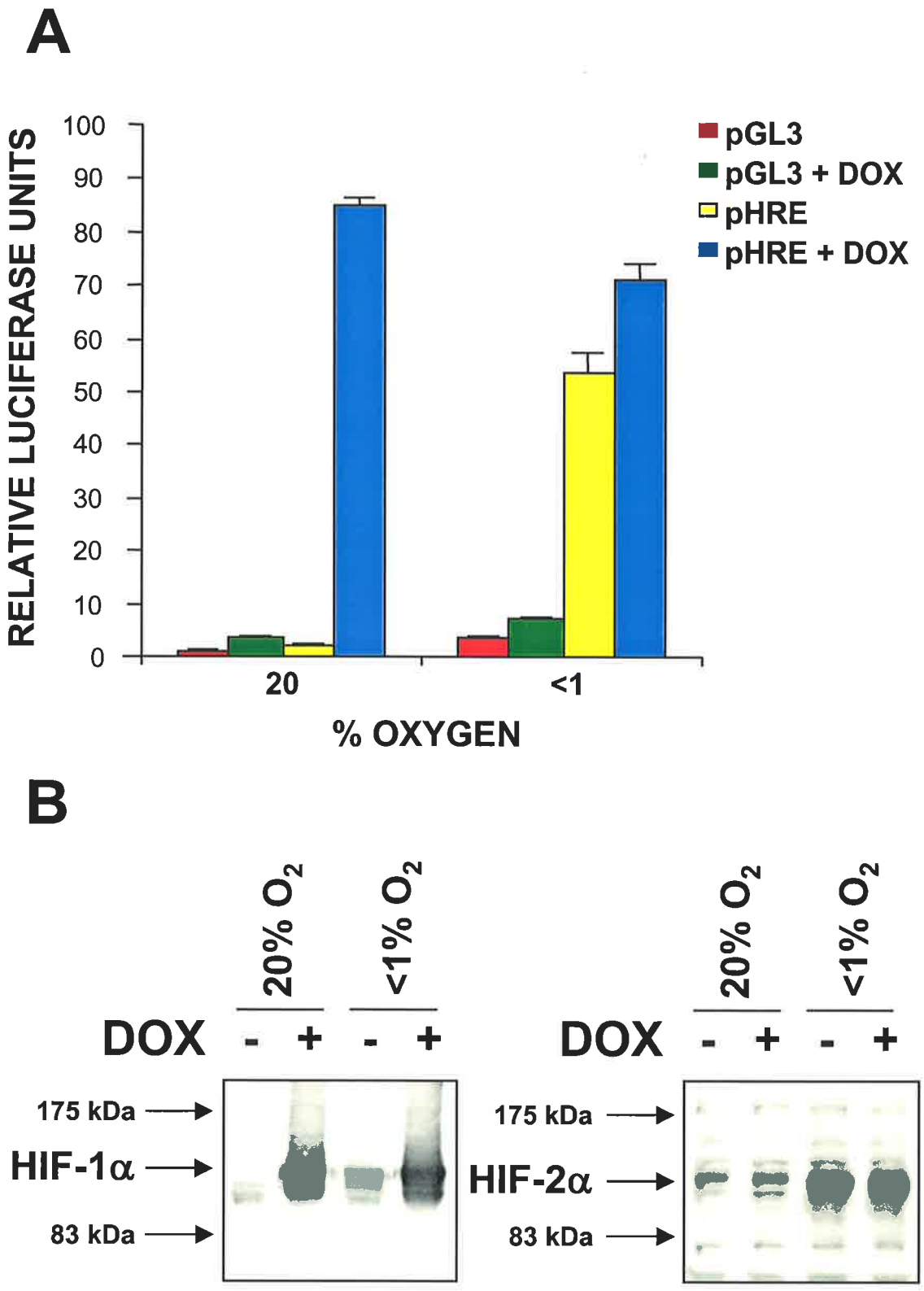

RELATIVE LUCIFERASE UNITS
60
50
40
30
20
10
0
CONTROL
DOX
C6
E1
I1
K1
K4
M5
O2
O6
LINE

Figure 3.11. In PC12/TetON/HIF-1αN803A/EYFP cells, HRE reporter activity is via doxycycline inducible HIF-1αN803A.

A. Cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of two independent experiments.

B. Whole cell extracts were prepared from cells exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX) for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to HIF-1α (left panel) or HIF-2α (right panel). Results are representative of two independent experiments.


A
RELATIVE LUCIFERASE UNITS
100
90
80
70
60
50
40
30
20
10
0
20
<1
% OXYGEN
pGL3
pGL3 + DOX
pHRE
pHRE + DOX
B
20% O2
<1% O2
DOX
- + - +
175 kDa
HIF-1α
83 kDa
20% O2
<1% O2
DOX
- + - +
175 kDa
HIF-2α
83 kDa

(compare pHRE + DOX versus pHRE). To associate this activity with doxycycline inducible HIF-1αN803A expression, whole cell extracts from similarly treated cells were immunoblotted with antibodies to HIF-1α or HIF-2α (figure 3.11.B). HIF-1α but not HIF-2α protein was detected to have accumulated as a result of doxycycline treatment at normoxia (compare + DOX versus – DOX at 20% O_2). However, HIF-1α and HIF-2α were present at hypoxia. In this line, therefore, HRE mediated activity caused by doxycycline treatment can be ascribed to HIF-1α N803A. As the N803A mutation in HIF-1α alleviated any problems caused by a lack of activity displayed by this protein in normoxia, a line in which HIF-1α target genes could be upregulated by doxycycline treatment had been established. It was thus possible to distinguish HIF-1α targets from HIF-2α regulated transcripts in the PC12/TetON/HIF-2α/EYFP line.

It must be mentioned that there were multiple attempts to generate a corresponding PC12/TetON/HIF-2αN851A/EYFP line but with no success (data not shown). Since the PC12/TetON/HIF-2α/EYFP line demonstrated maximum induction of active HIF-2α protein, it was decided to use this line for all subsequent studies.

3.5. ANALYSIS OF PUBLISHED HIF-1α TARGET GENE EXPRESSION

3.5.1. HIF-1α TARGET GENES

There are a number of hypoxia inducible genes that are direct targets of HIF-1α (table 1.1). However, there is not a single gene that has been unequivocally characterised as being regulated by HIF-2α, let alone one which is considered as unique to HIF-2α. Upon their establishment, it was of immediate interest to determine the expression of transcripts known to be directly upregulated by HIF-1α in the inducible HIF-α cell lines upon their treatment with doxycycline. The reasons for this were two fold. Firstly, induction of direct HIF-1α target genes in the PC12/TetON/HIF-1αN803A/EYFP cell line as a consequence of exposure to doxycycline would confirm the ability of HIF-α to enhance the transcription of a target despite the absence of hypoxia. In addition, it would provide novel evidence with regards to the ability of HIF-2α in PC12 cells to regulate classical HIF-1α target genes.

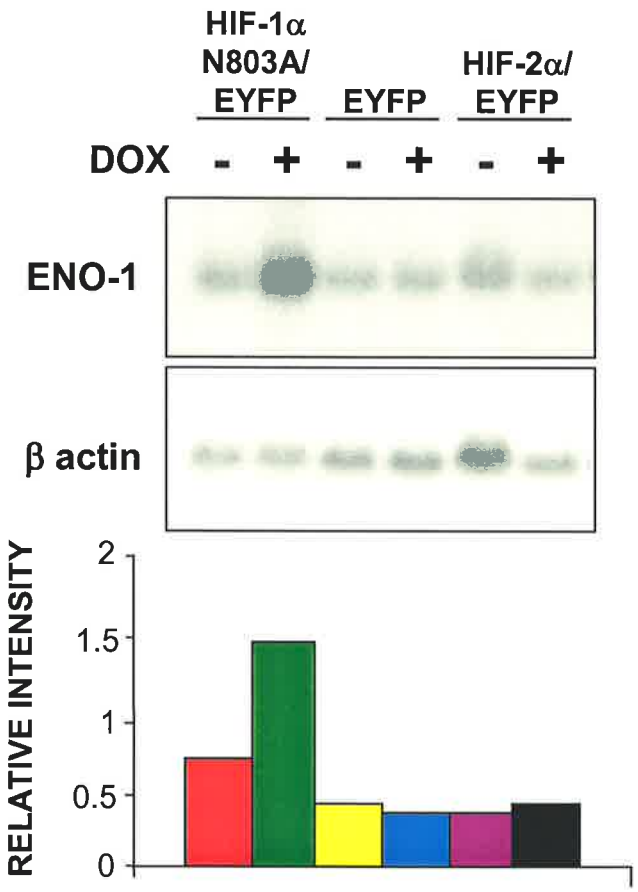
3.5.2. NORTHERN ANALYSIS OF PUBLISHED HIF-1α TARGET GENE EXPRESSION IN PC12/TetON/EYFP, PC12/TetON/HIF-1αN803A/EYFP AND PC12/TetON/HIF-2α/EYFP CELLS

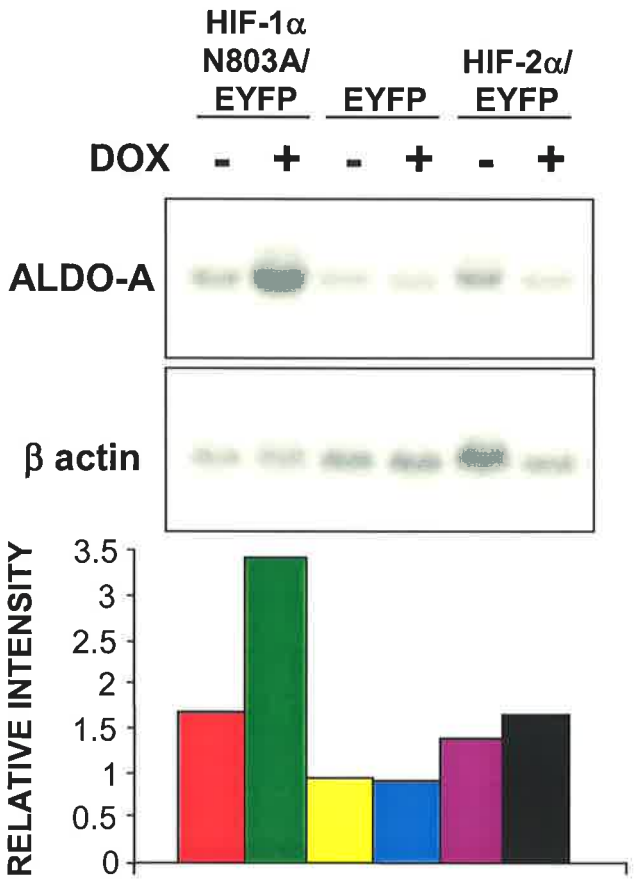
The probes to the chosen HIF-1α targets were acquired as follows. Primers were designed to amplify nucleotides 37-513 of VEGF (GenBank accession number AJ010438), 1236-1678 of aldolase a (GenBank accession number NM_000034), 335-724 of enolase 1 (GenBank accession number NM_001428), 173-552 of lactate dehydrogenase A (GenBank accession number NM_017025), 353-852 of glucose transporter 1 (GenBank accession number K03195), 589-1436 of tyrosine hydroxylase (GenBank accession number NM_199292) and 327-564 of β actin (GenBank accession number BC013835). These were employed to perform RTPCR on mRNA derived from PC12 cells. In short, cDNA was derived from reverse transcription of total RNA purified from 2 μg/mL doxycycline treated and untreated PC12/TetON/HIF-2α/EYFP cells. The cDNA (or water as a negative control) provided the template for PCR reactions employing the above primers. The exceptions were VEGF (where the probe was obtained via PCR employing pαFGH/VEGF164 as the template) and aldolase A and glucose transporter 1 (where the probe was obtained via RTPCR of total RNA from the mouse endothelial cell line tEnd1). All products were of the estimated size when compared to the DNA markers. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified, cloned into pGEMTEASY and sequenced.

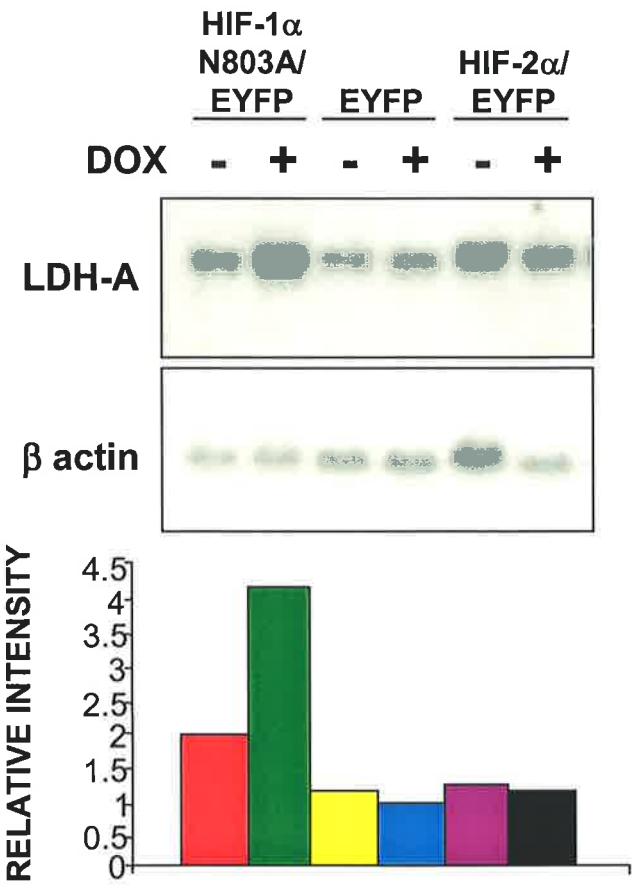
Total RNA was prepared from control PC12/TetON/EYFP, PC12/TetON/HIF-1αN803A/EYFP and PC12/TetON/HIF-2α/EYFP cells grown with or without 2 μg/mL doxycycline for 16 hours. 10 μg of each RNA were analysed by Northern blotting with various ^{32}P labelled cDNA probes corresponding to known HIF-1α target genes. The probes used were chosen to cover a range of classical physiological responses to hypoxia, namely neovasularisation (VEGF, Goldberg *et al.*, 1994), expression of glycolytic and glucose transporting enzymes (enolase 1, aldolase A, lactate dehydrogenase A and glucose transporter 1 (Semenza *et al.*, 1994; Ebert *et al.*, 1995)) and enhanced ventilation and heart rate (tyrosine hydroxylase (Schnell *et al.*, 2003)). These were generated from the insert released from the *Eco*RI digestion of the relevant pGEMTEASY derivative. Hybridisation was normalised against that of β actin (figure 3.12). Treatment of the PC12/TetON/HIF-1αN803A monoclonal line with doxycycline caused the upregulation

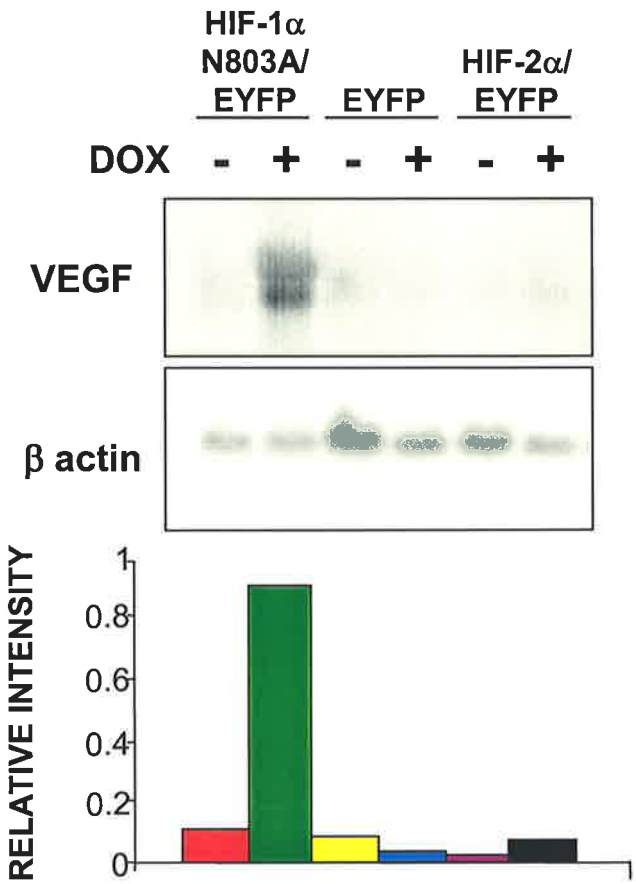
Figure 3.12. Known HIF-1α inducible genes are upregulated by HIF-1α but not HIF-2α in PC12 cells.

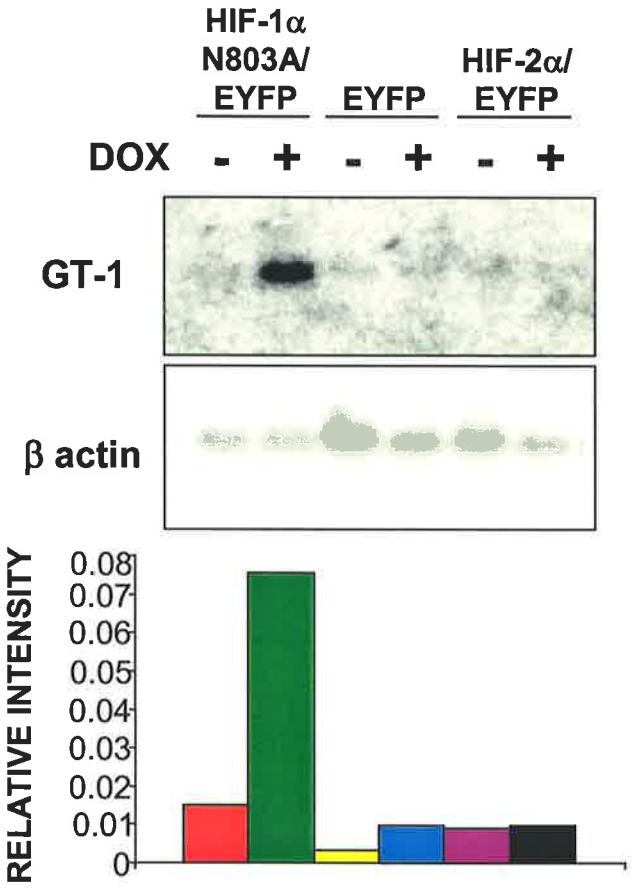
Total RNA was prepared from PC12/TetON/HIF-1αN803A/EYFP (HIF-1α N803A/EYFP), PC12/TetON/EYFP (EYFP) and PC12/TetON/HIF-2α/EYFP (HIF-2α/EYFP) cells grown in the presence or absence of 2 μg/mL doxycycline (DOX) for 16 hours. 10 μg of each were analysed by blotting with the following ^{32}P labelled cDNA probes: enolase 1 (ENO-1), aldolase A (ALDO-A), lactate dehydrogenase A (LDH-A), vascular endothelial growth factor (VEGF), glucose transporter 1 (GT-1) and tyrosine hydroxylase (TH). Hybridisation was normalised against that of a cDNA probe for β actin and represented as relative intensity. Results are representative of two independent experiments. Please note that with respect to the data presented, the ENO-1, ALDO-A and LDH-A Northern analyses were performed on the same blot (with stripping between each probing) and thus compared to the same β actin hybridisation control, as was the case with the VEGF and GT-1 Northern analyses.

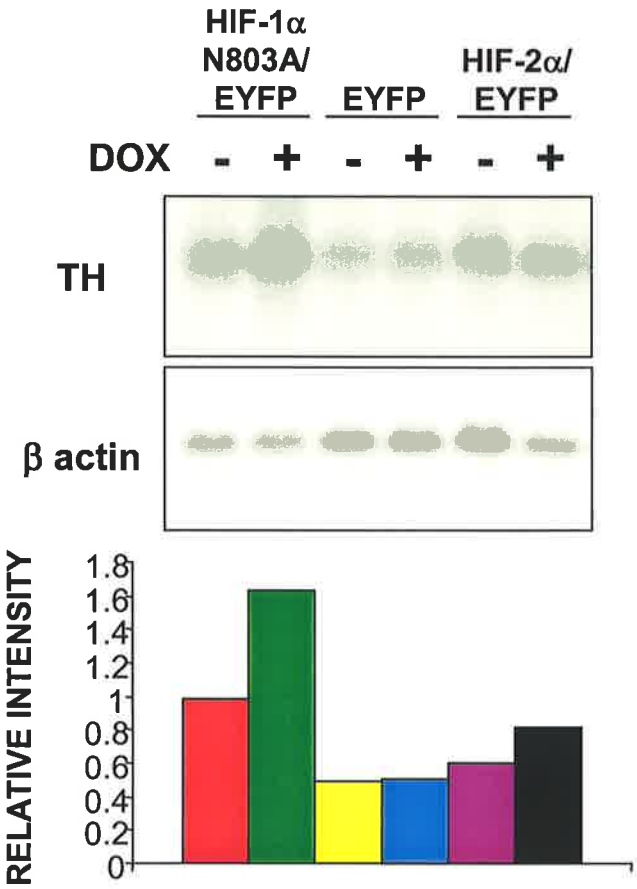

HIF-1α N803A/ EYFP
EYFP
HIF-2α/ EYFP
DOX - + - + - +
ENO-1
β actin
RELATIVE INTENSITY
2
1.5
1
0.5
0


HIF-1α N803A/ EYFP
EYFP
HIF-2α/ EYFP
DOX - + - + - +
ALDO-A
β actin
RELATIVE INTENSITY
3.5
3
2.5
2
1.5
1
0.5
0


HIF-1α N803A/ EYFP
EYFP
HIF-2α/ EYFP
DOX - + - + - +
LDH-A
β actin
RELATIVE INTENSITY
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0


HIF-1α N803A/ EYFP
EYFP
HIF-2α/ EYFP
DOX - + - + - +
VEGF
β actin
RELATIVE INTENSITY
1
0.8
0.6
0.4
0.2
0


HIF-1α N803A/ EYFP
EYFP
HIF-2α/ EYFP
DOX - + - + - +
GT-1
β actin
RELATIVE INTENSITY
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0


HIF-1α N803A/ EYFP
EYFP
HIF-2α/ EYFP
DOX - + - + - +
TH
β actin
RELATIVE INTENSITY
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

of all probed transcripts relative to β actin levels (compare HIF-1α N803A/EYFP + DOX versus HIF-1α N803A/EYFP – DOX). As expected, doxycycline treatment did not alter the expression of the HIF-1α targets in the control PC12/TetON/EYFP cells (compare EYFP + DOX versus EYFP – DOX). Moreover, induction of HIF-2α in the PC12/TetON/HIF-2α/EYFP cell line did not result in any obvious change in the RNA levels of the analysed transcripts (compare HIF-2α/EYFP + DOX versus HIF-2α/EYFP – DOX). Therefore, it appears that the targets of HIF-1α are not also those of HIF-2α in the context of the PC12 cell line. This data also confirms the validity of the system as known HIF-1α targets are consistently upregulated by doxycycline treatment of the HIF-1α inducible line.

In short, by employing a system whereby only HIF-1α or HIF-2α function can be initiated, it is apparent that many of the known specific targets of HIF-1α are not shared with HIF-2α in the PC12 cell line. Unfortunately, therefore, no leads from these experiments were provided with respect to a gene that is convincingly regulated by HIF-2α. However, the upregulation of RNA known to be induced by HIF-1α upon the stabilisation and activation of HIF-α despite the absence of reduced oxygen levels validates this system for use in the screening for novel target genes. As HIF-2α accumulated in normoxia can function as a transcription factor to activate an HRE mediated luciferase reporter, the next logical step was thus to employ cDNA microarray technology to screen for the elusive HIF-2α target gene.

3.6. SCREENING FOR HIF-2α SPECIFIC TARGET GENES

3.6.1. COMPARISON BETWEEN DOXYCYCLINE TREATED PC12/TetON/HIF-2α/EYFP CELLS AND PC12/TetON/HIF-1αN803A/EYFP

The ultimate aim of this research was to discover genes that are directly transcriptionally regulated by HIF-2α but not HIF-1α. Initially, it was therefore decided to compare gene expression in the doxycycline inducible HIF-2α line versus the doxycycline inducible HIF-1α line using microarrays. It was proposed that HIF-1α specific target genes would be upregulated in the latter, whereas there would be no relative change in the levels of transcripts of those upregulated by both transcription factors, or, for that matter, any that are mediated for whatever reason by doxycycline. However, it was predicted that the mRNA of HIF-2α specific target genes would be increased in the doxycycline inducible

HIF-2α line when compared with the expression profile of the doxycycline inducible HIF-1α line.

To that end, total RNA was prepared from PC12/TetON/HIF-2α/EYFP and PC12/TetON/HIF-1αN803A/EYFP cells treated with 2 μg/mL of doxycycline for 16 hours. 16 hours was chosen as it was determined that this duration of time was required for optimal doxycycline inducible HIF-2α protein stability and HRE reporter activity in the PC12/TetON/HIF-2α/EYFP line (data not shown). The RNA from PC12/TetON/HIF-2α/EYFP cells was labelled with Cy3 and PC12/TetON/HIF-1αN803A/EYFP cells with Cy5 and these were hybridised to two 5K rat oligonucleotide slides (that is, half of each sample per slide). This was repeated, but for a substitution in the dyes used to label each sample. A selection of some of the most differentially regulated genes is shown in table 3.1. Note that the results with respect to the fold changes in RNA levels compare expression in the doxycycline treated inducible HIF-2α PC12 line versus that in similarly treated inducible HIF-1α N803A PC12 cells. When RNA levels are considered downregulated in the inducible HIF-2α line (and thus relatively upregulated in the inducible HIF-1α N803A line), the value given is equivalent to the inverse of the fold change. The RNA labelling, microarray hybridisations and data analysis were performed by Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia.

Of prime importance was the relative upregulation of the respective HIF-α transcripts in each cell line. When comparing mRNA from 2μg/mL doxycycline treated PC12/TetON/HIF-2α/EYFP cells with that from similarly treated PC12/TetON/HIF-1αN803A/EYFP cells, HIF-2α (GenBank accession number AJ277828) was one of the most upregulated transcripts, whereas HIF-1α (GenBank accession number AF057308) was one of the most relatively downregulated. Furthermore, a number of the same genes or similar from the Northern analysis of known HIF-1α specific target genes (figure 3.12) were relatively downregulated, as expected given that they appeared to be transactivated by HIF-1α and not HIF-2α. These included VEGF (GenBank accession number AF215726), aldolase C (GenBank accession number AB017483), lactate dehydrogenase A (GenBank accession number M54926) and tyrosine hydroxylase (GenBank accession number L22651).

Table 3.1. A list of genes differentially regulated between doxycycline treated PC12/TetON/HIF-2α/EYFP cells and doxycycline treated PC12/TetON/HIF-1αN803A/EYFP cells.

Total RNA was prepared from PC12/TetON/HIF-2α/EYFP cells and PC12/TetON/HIF-1αN803A/EYFP cells treated with 2 μg/mL doxycycline for 16 hours. The RNA from PC12/TetON/HIF-2α/EYFP cells was labelled with Cy3 and PC12/TetON/HIF-1αN803A/EYFP cells with Cy5 and these were hybridised to two 5K rat oligonucleotide slides (that is, half of each sample per slide). This was repeated, except for a substitution in the dyes used to label each sample. Fold changes in RNA levels are a measure of the chosen gene's expression in PC12/TetON/HIF-2α/EYFP cells when compared to PC12/TetON/HIF-1αN803A/EYFP cells. When RNA levels are considered downregulated in the inducible HIF-2α line (and thus relatively upregulated in the inducible HIF-1α line), the value given is equivalent to the inverse of the fold change. The *B* value is used to describe the probability that a gene is differentially regulated. The RNA labelling, microarray hybridisations and data analysis were performed by Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia.

GenBank number	Gene	Fold change	*B*
AJ277828	hypoxia inducible factor 2α (HIF-2α)	4.82 (up)	2.06
D00575	pituitary glycoprotein hormone α subunit precursor	4.09 (up)	3.46
U44845	vitronectin	2.29 (up)	2.33
L07736	carnitine palmitoyltransferase 1	2.21 (up)	0.82
AB021971	norepinephrine transporter b	2.17 (up)	0.21
AB021970	norepinephrine transporter a	1.94 (up)	-1.10
U73030	pituitary tumour transforming gene (PTTG)	1.81 (up)	0.07
AF182946	BRCA1-associated RING domain protein 1 (BARD1)	1.80 (up)	2.20
X97831	carnitine/acylcarnitine carrier protein	1.74 (up)	0.29
AF125562	tumour associated glycoprotein E4 gene, complete cds	1.73 (up)	-1.58
M54926	lactate dehydrogenase A 3' end	1.85 (down)	2.30
L22651	tyrosine hydroxylase (TH) 5' flank and complete cds	1.90 (down)	2.71
AF324255	global ischaemia inducible protein 11 (GIIG11)	2.40 (down)	5.32
AB017483	aldolase C	2.79 (down)	5.28
AF243515	Nip3	2.91 (down)	7.69
U06713	SM-20	3.18 (down)	7.17
AF057308	hypoxia inducible factor 1α (HIF-1α)	4.23 (down)	1.27
AF215726	vascular endothelial growth factor A120 alternatively spliced	4.34 (down)	8.83

The list of genes upregulated in the doxycycline PC12/TetON/HIF-2α/EYFP line was by no means exhaustive but there were a sufficient number of potential HIF-2α targets to warrant some prioritising with respect to further analysis. Among those chosen were genes correlated with tumour development (given the suggestion of a tumour promoting role for HIF-2α and not HIF-1α, at least in the context of 786-O cells (Maranchie *et al.*, 2002; Kondo *et al.*, 2003; Kondo *et al.*, 2003)), such as BRCA-1 associated RING domain protein (BARD1) pituitary tumour transforming gene (PTTG), pituitary glycoprotein hormone α subunit precursor (PGHα) and tumour associated glycoprotein E4 (TAGE4); genes associated with the oxidation of long chain fatty acids for energy production, namely carnitine palmitoyltransferase 1 (CPT1) and carnitine/acylcarnitine carrier protein (C/ACP); and norepinephrine transporter (NET) a and NETb (accounting for an apparent role for HIF-2α in catecholamine synthesis and release(Tian *et al.*, 1998)). In addition, a randomly chosen putative HIF-1α target known as global ischaemia inducible protein 11 (GIIG 11) was assessed.

3.6.2. NORTHERN ANALYSIS OF GENES UPREGULATED BY DOXYCYCLINE TREATMENT OF PC12/TetON/HIF-2α/EYFP CELLS

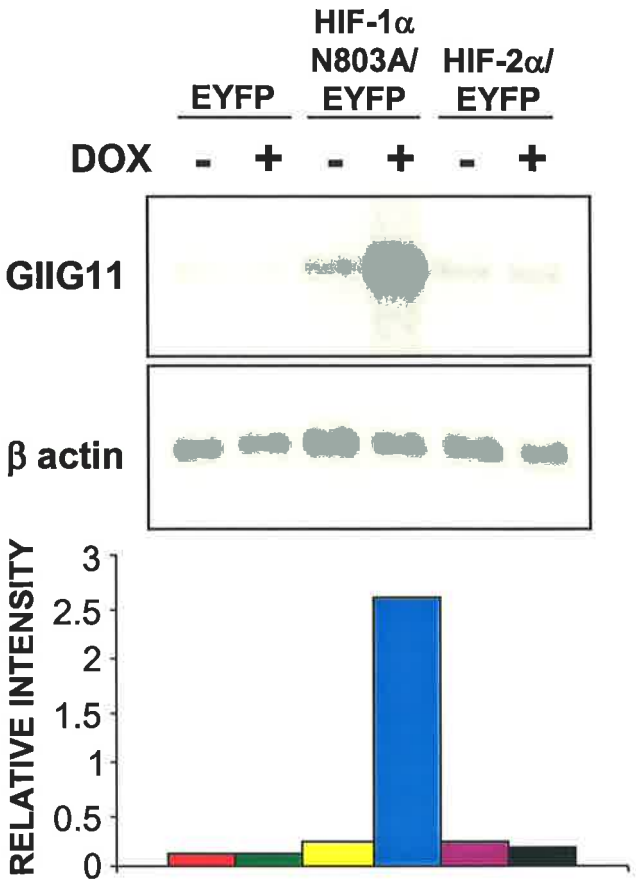
The probes to the chosen putative HIF-2α targets were acquired as follows. Primers were designed to amplify nucleotides 1019-1220 of BARD1 (GenBank accession number AF182946), 65-551 of TAGE4 (GenBank accession number AF125562), 661-1080 of CPTI (GenBank accession number L07736), 220-840 of C/ACP (GenBank accession number X97831), 1584-1932 of NETa (GenBank accession number AB021970), 515-715 of PTTG (GenBank accession number U73030), 80-406 of PGHα (GenBank accession number D00575), 202-430 of GIIG11 (GenBank accession number AF324255) and β actin (see chapter 3.5.2). These were employed to perform RTPCR on mRNA derived from PC12 cells. In short, cDNA was derived from reverse transcription of total RNA purified from 2 μg/mL doxycycline treated and untreated PC12/TetON/HIF-2α/EYFP cells. The cDNA (or water as a negative control) provided the template for PCR reactions employing the above primers. All products were of the estimated size when compared to the DNA markers. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified, cloned into pGEMTEASY and sequenced.

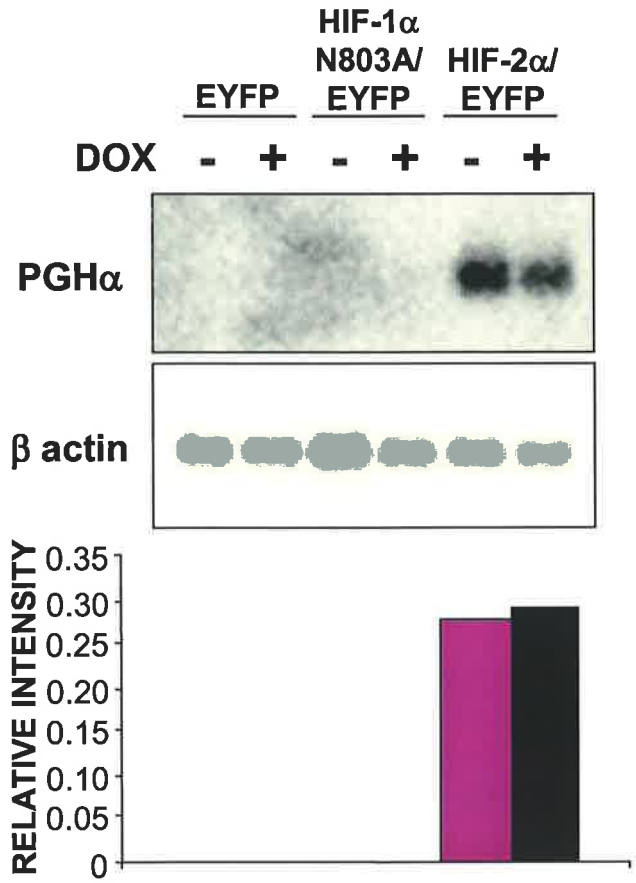
Poly A RNA was prepared from PC12/TetON/HIF-1αN803A/EYFP, PC12/TetON/EYFP and PC12/TetON/HIF-2α/EYFP cells grown with or without 2 μg/mL doxycycline for 16 hours. 3 μg of each were analysed by blotting with various ^{32}P labelled cDNA probes corresponding to genes selected from the list in table 3.1, specifically BARD1, CPT1, C/ACP, NETa, PGHα, PTTG, TAGE4 and GIIG11. These were generated from the insert released from the *Eco*RI digestion of the relevant pGEMTEASY derivative. Hybridisation was normalised against that of β actin (figure 3.13). In short, the putative HIF-1α specific target (designated as such by virtue of the fact that it was relatively downregulated in the doxycycline treated PC12/TetON/HIF-2α/EYFP line when compared with the expression profile of the PC12/TetON/HIF-1αN803A/EYFP line) was indeed upregulated by HIF-1α as only treatment of the PC12/TetON/HIF-1αN803A monoclonal line with doxycycline caused the induction of GIIG11 transcript relative to β actin levels (compare HIF-1α N803A/EYFP + DOX versus all other lanes on the GIIG11 Northern blot). However, the putative HIF-2α specific targets (classified as such because they were induced in the doxycycline treated PC12/TetON/HIF-2α/EYFP line when contrasted with PC12/TetON/HIF-1αN803A/EYFP line) were upregulated in the PC12/TetON/HIF-2α/EYFP line regardless of doxycycline treatment (compare HIF-2α/EYFP + DOX or HIF-2α/EYFP – DOX versus all other lanes on the PGHα, C/ACP and CPT1 Northern blots). The same phenomenon was also observed with the Northern analysis of BARD1 and PTTG (data not shown). There was no signal generated as a consequence of probing for NETa and TAGE4 (data not shown). In other words, the putative HIF-2α specific targets were instead constitutively upregulated in the doxycycline inducible HIF-2α PC12 derivative, thus explaining why they were upregulated in this line versus the PC12/TetON/HIF-1αN803A/EYFP cells.

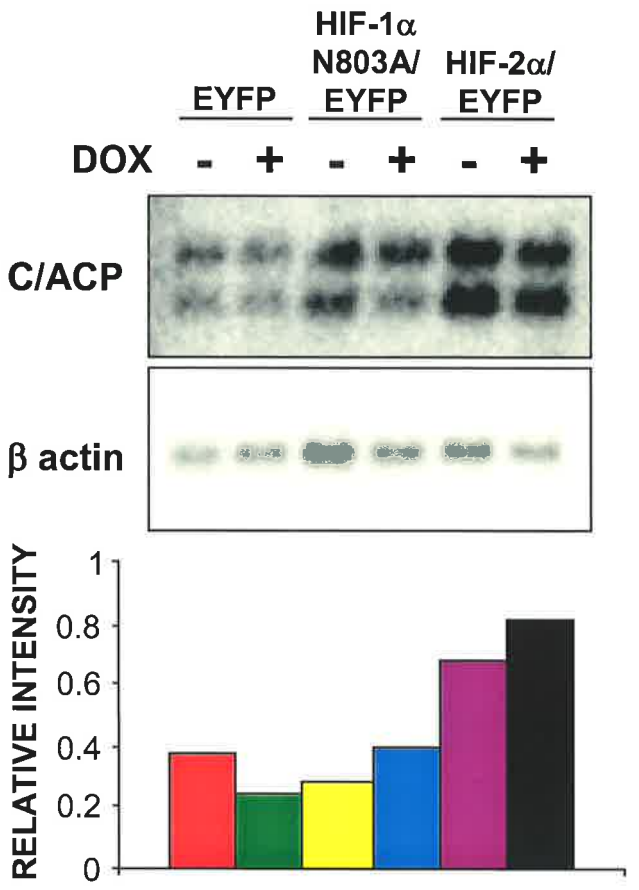
A possible cause of altered gene regulation in stable cell lines stems from the site of incorporation of the transfected plasmid. This may disrupt the expression of the gene which has been interrupted itself and/or other genes which may be positively or negatively mediated by the disturbed gene. It must be recalled that all three doxycycline inducible PC12 monoclonal lines (PC12/TetON/EYFP, PC12/TetON/HIF-2α/EYFP and PC12/TetON/HIF-1αN803A/EYFP) were derived from the same parent transfectant (PC12/TetON/NEO). Therefore, any changes to overall PC12 gene expression caused by the inclusion of the pEF/TetON/IRES/NEO construct into the genome would be common to all three lines. Differential modifications must have been a result of the stable

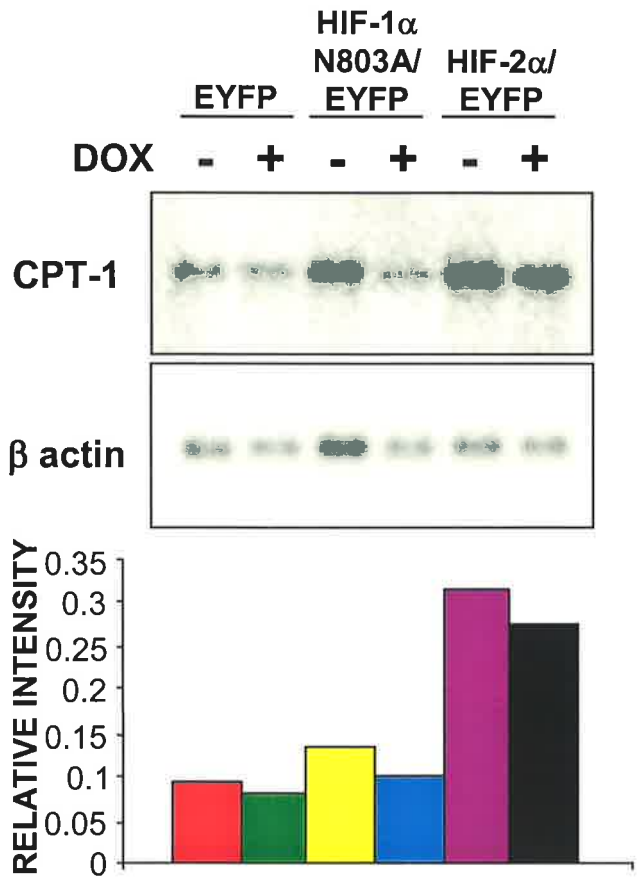
Figure 3.13. Genes that are relatively upregulated by HIF-2α are in fact constitutively upregulated in the doxycycline inducible HIF-2α PC12 cell line.

Poly A RNA was prepared from PC12/TetON/HIF-1αN803A/EYFP (HIF-1α N803A/EYFP), PC12/TetON/EYFP (EYFP) and PC12/TetON/HIF-2α/EYFP (HIF-2α/EYFP) cells grown in the presence or absence of 2 μg/mL doxycycline (DOX) for 16 hours. 3 μg of each were analysed by Northern blotting with the following ^{32}P labelled cDNA probes: global ischaemia inducible gene product 11 (GIIG11, a potential novel HIF-1α specific target), pituitary glycoprotein hormone alpha subunit precursor (PGHα), carnitine/acetylcarnitine carrier protein (C/ACP) and carnitine palmitoyltransferase 1 (CPT-1). Hybridisation was normalised against that of a cDNA probe for β actin and represented as relative intensity.


HIF-1α N803A/ EYFP
HIF-2α/ EYFP
EYFP
DOX - + - + - +
GIIG11
β actin
RELATIVE INTENSITY
3
2.5
2
1.5
1
0.5
0


HIF-1α N803A/ EYFP
HIF-2α/ EYFP
EYFP
DOX - + - + - +
PGHα
β actin
RELATIVE INTENSITY
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0


HIF-1α N803A/ EYFP
HIF-2α/ EYFP
EYFP
DOX - + - + - +
C/ACP
β actin
RELATIVE INTENSITY
1
0.8
0.6
0.4
0.2
0


HIF-1α N803A/ EYFP
HIF-2α/ EYFP
EYFP
DOX - + - + - +
CPT-1
β actin
RELATIVE INTENSITY
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0

transfection of the pTR/DC/EYFP, pTR/HIF-2α/DC/EYFP or pTR/HIF-1αN803A/DC/EYFP plasmids into the PC12/TetON/NEO line as this would most likely have occurred in different sites of the genome with respect to each cell line.

In short, the array and subsequent Northern analysis of the selected targets was a technical success and supported the use of the TetON system for the selective upregulation of either HIF-α. Firstly, only the HIF-1α or the HIF-2α transcript was markedly upregulated upon exposure of the relevant inducible HIF-α PC12 cell line to doxycycline (table 3.1). This explains the increase in HRE reporter observed in these cells during their initial characterisation (figure 3.8 and figure 3.11). A number of the same or similar genes from the Northern analysis of known HIF-1α specific target genes (figure 3.12) were again shown to be preferentially transactivated by HIF-1α and not HIF-2α in the PC12 cells. Also, Northern analysis confirmed that a putative HIF-1α target (GIIG11) was upregulated only in doxycycline treated PC12/TetON/HIF-1αN803A/EYFP cells (figure 3.13), thus suggesting that it is indeed transactivated by HIF-1α and not HIF-2α. However, as the Northern analysis provided for a more extensive scrutiny of the expression of the genes selected for further investigation as potential HIF-2α targets (that is, by including RNA extracted from cells not treated with doxycycline), it was evident that these potential HIF-2α targets were constitutively upregulated in the PC12/TetON/HIF-2α/EYFP cell line regardless of doxycycline treatment. An alternative comparison in the microarray screen had to be employed.

3.6.3. COMPARISON BETWEEN DOXYCYCLINE TREATED AND UNTREATED PC12/TetON/HIF-2α/EYFP CELLS

It was thus decided to compare gene expression in the absence and presence of doxycycline in the inducible HIF-2α line using microarrays. To ensure that any putative targets are not aberrantly upregulated by doxycycline, it was decided to perform a similar comparison with the control PC12/TetON/EYFP line. General doxycycline inducible genes could thus be eliminated from any subsequent analytical techniques. Furthermore, as gene expression within each line was compared (rather than between lines), any aberrant changes in gene expression unique to any given line (for instance, as a consequence of the stable integration of exogenous DNA) would be accounted for.

To that end, total RNA was prepared from PC12/TetON/HIF-2α/EYFP cells treated with 2 μg/mL of doxycycline or left untreated for 16 hours. The RNA from doxycycline treated PC12/TetON/HIF-2α/EYFP cells was labelled with Cy3 and that of untreated PC12/TetON/HIF-2α/EYFP cells with Cy5 and these were hybridised to one 10K rat oligonucleotide slide. This was repeated, except for a substitution in the dyes used to label each sample. This entire experiment was then repeated, meaning that each comparison was performed in duplicate. PC12/TetON/EYFP cells were experimented upon in the same way, although doxycycline treatment of the PC12/TetON/EYFP cells caused no significant change in the regulation of any of the genes analysed (data not shown). The most upregulated genes upon doxycycline treatment of PC12/TetON/HIF-2α/EYFP cells are shown in table 3.2. The RNA labelling, microarray hybridisations and data analysis were performed by Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia.

Of prime importance was the fact that HIF-2α was itself the most doxycycline inducible transcript in the PC12/TetON/HIF-2α/EYFP cell line (GenBank accession number AJ277828), whereas it remained unchanged in the PC12/TetON/EYFP control cell line upon doxycycline treatment (data not shown). The relative levels of HIF-1α remained unchanged in all lines and treatments (data not shown). Any change in HIF-α mediated gene expression can therefore be attributed to HIF-2α and not HIF-1α. Of particular significance were vitronectin, cytosolic inhibitor of Nrf2 (INrf2), various ion channels including voltage gated sodium channel β3 subunit (Na^+ β3) and voltage gated calcium channel $\alpha_2\delta$-1 subunit (Ca^+ $\alpha_2\delta$-1) and regulator of G protein signalling 4 (Rgs4). Of interest also were ion channel from the heart (GenBank accession number AB022331), Na^+-Ca^+ exchanger isoform NACA7 (GenBank accession number U04933) and Nip3 (GenBank accession number AF243515). Interestingly, there was no change in any known genes involved in catecholamine synthesis (data not shown).

3.6.4. RTPCR OF GENES UPREGULATED BY DOXYCYCLINE TREATMENT OF PC12/TetON/HIF-2α/EYFP CELLS

The expression of the chosen putative HIF-2α targets in the PC12 derivatives was analysed as follows. Primers were designed to amplify nucleotides 70-708 of vitronectin (GenBank accession number U44845), 78-580 of INrf2 (GenBank accession number AF304364), 363-1010 of Na^+ β3 (GenBank accession number AJ243395), 561-1221 of

Table 3.2. A list of genes upregulated upon the doxycycline treatment of PC12/TetON/HIF-2α/EYFP cells.

Total RNA was prepared from PC12/TetON/HIF-2α/EYFP treated with 2 μg/mL doxycycline or left untreated for 16 hours. The RNA from doxycycline treated PC12/TetON/HIF-2α/EYFP cells was labelled with Cy3 and that of doxycycline untreated PC12/TetON/HIF-2α/EYFP cells with Cy5 and these were hybridised to one 10K rat oligonucleotide slide. This was repeated, except for a substitution in the dyes used to label each sample. Experiments were performed in duplicate. Fold changes in RNA levels are a measure of the chosen gene's expression in doxycycline treated PC12/TetON/HIF-2α/EYFP cells when compared to untreated cells. The *B* value is used to describe the probability that a gene is differentially regulated. PC12/TetON/EYFP cells were experimented upon in the same way although doxycycline treatment of the PC12/TetON/EYFP cells caused no significant change in the regulation of any of the genes analysed (data not shown). The RNA labelling, microarray hybridisations and data analysis were performed by Ashley Connolly and Mark van der Hoek at the Adelaide Microarray Facility, University of Adelaide, Australia.

GenBank number	Gene	Fold change	*B*
AJ277828	hypoxia inducible factor 2α (HIF-2α)	15.52	6.13
AJ277828	*Rattus norvegicus* mRNA for HIF-2α	8.04	6.74
AF304364	cytosolic inhibitor of Nrf2 (INrf2)	4.30	8.42
L03556	(clone RAHB2 8/10) hox1.3 protein (hox1.3) 3' end	4.04	3.72
U44845	vitronectin (S-protein, epibolin, serum spreading factor)	3.23	6.64
AB022331	ion channel from heart	2.48	1.90
NM_017214	*Rattus norvegicus* mRNA for regulator of G-protein signalling 4 (Rgs4)	2.12	5.36
AF215726	vascular endothelial growth factor A120 alternatively spliced	1.97	2.77
U27767	RGP4	1.89	2.93
M36410	Rat sepiapterin reductase mRNA, partial cds	1.80	4.61
U70778	Doc2B (highly enriched in neurons, similar to Doc2B)	1.79	4.19
AJ243395	voltage gated sodium channel β3 subunit.	1.72	0.69
AF243515	Nip3	1.69	3.64
AJ243395	*Rattus norvegicus* mRNA for voltage-gated sodium channel β3 subunit	1.62	1.18
NM_012716	*Rattus norvegicus* mRNA for solute carrier 16 (monocarboxylic acid transporter), member 1	1.61	2.42
U04933	*Rattus norvegicus* mRNA for Sprague-Dawley (CD-1) Na^+-Ca^+ exchanger isoform NACA7	1.59	2.21
AF286488	voltage gated calcium channel $\alpha_2\delta$-1 subunit	1.55	1.15
U78123	zinc finger protein 12 (AZF12) partial cds.	1.52	1.11
AB010119	Tctex-1	1.52	1.79
M31178	Rat mRNA for calbindin D28	1.51	3.04

Ca^{2+} $\alpha_2\delta$-1 (GenBank accession number AF286488), 110-727 of Rgs4 (GenBank accession number NM_017214) and β actin (see chapter 3.5.2). These were employed to perform RTPCR on mRNA derived from PC12 cells. In short, cDNA was derived from reverse transcription of poly A RNA extracted from PC12 cells exposed to normoxia or hypoxia for 16 hours (figure 3.14.A) or from total RNA purified from PC12/TetON/EYFP, PC12/TetON/HIF-1αN803A/EYFP and PC12/TetON/HIF-2α/EYFP cells grown with or without 2 μg/mL doxycycline for 16 hours (figure 3.14.B). The cDNA (or water as a negative control) provided the template for PCR reactions employing the above primers.

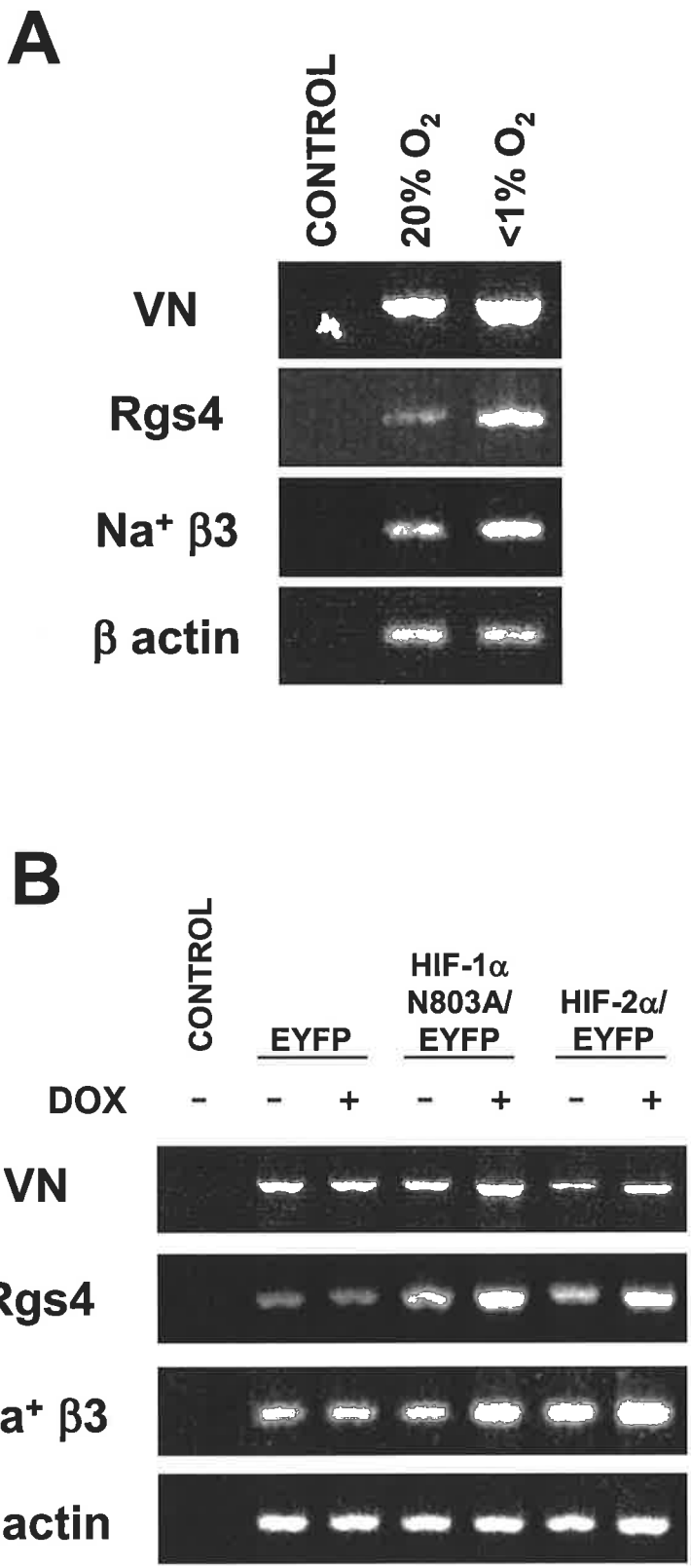
Unfortunately, PCR reactions employing primers to INrf2 and Ca^{2+} $\alpha_2\delta$-1 yielded no products. However, vitronectin, Rgs4 and Na^+ β3 all showed a slight hypoxic induction in their mRNA levels by RTPCR when compared to β actin levels (compare <1% O_2 versus 20% O_2). However, treatment of the PC12/TetON/HIF-2α/EYFP monoclonal line with doxycycline caused the upregulation of vitronectin and Rgs4 but not Na^+ β3 relative to β actin levels (compare HIF-2α/EYFP + DOX versus HIF-2α/EYFP – DOX). Interestingly, induction of HIF-1α in the PC12/TetON/HIF-1αN803A/EYFP cell line also resulted in an increase in the RNA levels of vitronectin and Rgs4 but not Na^+ β3 (compare HIF-1αN803A/EYFP + DOX versus HIF-1αN803A/EYFP – DOX). As expected, doxycycline treatment did not alter the expression of any genes in the control PC12/TetON/EYFP cells (compare EYFP + DOX versus EYFP – DOX).

Therefore, it appears that the putative HIF-2α targets in the context of the PC12 cell are all hypoxically inducible, although it appears that Na^+ β3 is a target of neither HIF-α. It was expected that Rgs4 may be upregulated by the initiation of either HIF-1α or HIF-2α function because, despite being induced upon doxycycline treatment of the HIF-2α line compared with untreated cells (table 3.2), there was no change in expression when comparing the profile of the doxycycline treated HIF-2α and HIF-1α inducible cell lines (data not shown), which thus already suggested that it was targeted by both factors. That this was also the case for vitronectin was somewhat of a surprise as it was upregulated in doxycycline treated inducible HIF-2α PC12 cells when compared with untreated cells (table 3.2) or with similarly treated inducible HIF-1α PC12 cells (table 3.1). In other words, HIF-2α in the microarray analysis appeared to be the preferential transactivator of vitronectin, although the RTPCR results suggest that it is targeted by both HIF-1α and

Figure 3.14. Putative HIF-2α target genes are all hypoxically inducible. However, VN and Rgs4 may be targets of both HIF-1α and HIF-2α.

A. cDNA was prepared from reverse transcription of 200 ng of poly A RNA extracted from PC12 cells exposed to 20% O_2 or <1% O_2 for 16 hours. 1 μL of this cDNA (20 μL total) was used as a template in a PCR reaction employing primers designed to amplify a portion of the sequences of vitronectin (VN), regulator of G protein signalling 4 (Rgs4), voltage gated sodium channel β3 subunit (Na^+ β3) or β actin. Reactions were electrophoresed through a 2% agarose/TBE/ethidum bromide gel.

B. cDNA was prepared from reverse transcription of 2 μg of total RNA extracted from PC12/TetOn/EYFP (EYFP), PC12/TetON/HIF-1αN803A/EYFP (HIF-1α N803A/EYFP) and PC12/TetON/HIF-2α/EYFP (HIF-2α/EYFP) cells grown in the presence or absence of 2 μg/mL doxycycline (DOX) for 16 hours. 1 μL of this cDNA (20 μL total) was used as a template in a PCR reaction employing VN, Rgs4, Na^+ β3 or β actin. Reactions were electrophoresed through a 2% agarose/TBE/ethidum bromide gel.


A
CONTROL
20% O2
<1% O2
VN
Rgs4
Na+ β3
β actin
B
CONTROL
EYFP
HIF-1α N803A/ EYFP
HIF-2α/ EYFP
DOX
- - + - + - +
VN
Rgs4
Na+ β3
β actin

HIF-2α. However, it must be noted that this is preliminary data and in the first instance requires some confirmation, not only in the form of an additional RTPCR employing independently acquired RNA, but also via a more sensitive and quantifiable technique such as Northern analysis.

3.7. GENERAL DISCUSSION

3.7.1. NOVEL HIF-2α TARGET GENES

The possibility that HIF-1α and HIF-2α possess mutually exclusive target genes despite the ability to both bind the HRE is an interesting concept and not without precedent. The *Drosophila melanogastor* bHLH/PAS proteins trachealess (trh), and single-minded (sim) both heterodimerise with the *Drosophila* equivalent of ARNT and bind the same DNA sequence, although they are responsible for mediating the expression of a distinct complement of target genes (the former controls tracheal formation, while the latter performs a role in inducing midline cell fates) (Zelzer *et al.*, 1997). This raises the question of whether the mutation of the crucial asparagine targeted for hydroxylation by FIH-1 in the transfected HIF-1α but not HIF-2α protein may explain the ability of HIF-1α but not HIF-2α to upregulate the hypoxia inducible transcripts tested in this thesis. Structural analysis of the HIF-1α CAD when associated with CBP/p300 demonstrated that N803 is deeply buried within the complex (Dames *et al.*, 2002; Freedman *et al.*, 2002). It is therefore highly unlikely that the asparagine is required for the direct recruitment of any other factors, given its engulfing by CBP/p300. In other words, the status of the asparagine only determines the ability to complex with CBP/p300. As HRE mediated reporter activity infers association with CBP/p300, it appears that stabilised wildtype HIF-2α is able to avoid asparaginyl hydroxylation more effectively than wildtype HIF-1α in the PC12 cell line. The *in vivo* and *in vitro* data that reflects and explains this will be discussed in the succeeding chapter. In short, the N803A mutation in HIF-1α merely permits it to display transcriptional activity which is favourably comparable with that of wildtype HIF-2α when both are induced by doxycycline in normoxia.

To date, there are no genes whose transcription can be conclusively accredited to HIF-2α transcriptional activity, let alone any that can be classed as particular to HIF-2α. As mentioned previously, the best evidence that HIF-1α and HIF-2α possess genes whose transactivation is unique to the function of one and not the other stems from studies into

HIF-1α$^{-/-}$ or HIF-2α$^{-/-}$ mice where it is evident that they are not functionally redundant (chapter 1.7.2). Indeed, a reason for the choice of the PC12 cell line from which to derive a system to discover direct HIF-2α target genes was its catecholaminergic characteristics, which are considerable when one accounts for the embryonic lethal phenotype of the first HIF-2α null mouse and its correlation with a significantly reduced level of catecholamines (Tian *et al.*, 1998). Therefore, one way to assign importance to a particular potential HIF-2α target gene is by determining whether there is a logical correlation between its upregulation upon the induction HIF-2α activity and the phenotype of any of the HIF-2α null mice which is what has been done in choosing the following to discuss. It must be noted that none of these have been proposed in the published literature as genes whose transcription is regulated by HIF-2α.

Vitronectin is a glycoprotein found in plasma and involved in the blood coagulation, fibrinolytic and complement systems although it is also required in the association of cells to their surrounding matrix and has been proposed roles in the regulation of cell proliferation, differentiation and morphogenesis (Preissner and Seiffert, 1998). It was of most interest as it was shown to be upregulated in both arrays (table 3.1. and table 3.2). In other words, it was probably the best candidate for being a HIF-2α specific target gene. Ultimately, this was not supported by the RTPCR results which suggested that vitronectin was a hypoxia inducible gene (figure 3.14.A), albeit one targeted by both HIF-1α and HIF-2α (figure 3.14.B). The phenotypes of HIF-2α null mice provide almost no clues with respect to the precise role vitronectin may play in the developing mouse as a consequence of being a HIF-2α target gene. However, the phenotype of one HIF-2α null mouse implicates this transcription factor in vascular remodelling (Peng *et al.*, 2000), a process in which vitronectin is also involved by virtue of its being anchored to the extracellular matrix and promoting cellular adhesion and migration via interaction with the integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha IIb\beta_3$ (Schvartz *et al.*, 1999).

The NF-E2 related factor (Nrf), Nrf2 and Nrf3 form a group of transcription factors that are activated by reactive oxygen species (ROS) and subsequently serve to promote the transcription of an array of antioxidant enzymes (AOEs) via binding *in cis* to an antioxidant response element (ARE) (Jaiswal *et al.*, 2004). Among these, Nrf2 is the major regulator of ARE regulated gene expression (Ishii *et al.*, 2000). The cytosolic inhibitor of Nrf2 (INrf2) inhibits the transcriptional activity of Nrf2 by maintaining it in the cytoplasm

and also by enhancing its degradation (Itoh *et al.*, 1999; McMahon *et al.*, 2003; Zhang and Hiddink, 2003). INrf2 was identified as a gene upregulated upon the doxycycline treatment of the doxycycline inducible HIF-2α line but not of the control PC12/TetON/EYFP line (table 3.2), therefore classifying INrf2 as a putative HIF-2α target gene, although confirmation of this via RTPCR proved to be unsuccessful (data not shown). HIF-2α may thus serve to downregulate Nrf2 and subsequent AOE transcription by inducing the expression of INrf2. This makes sense as hyperoxia enhances Nrf2 levels and AOE expression in the lungs of mice (Ho *et al.*, 1996; Clerch *et al.*, 2000; Cho *et al.*, 2002). Therefore, oxygen levels may serve in various ways to regulate Nrf2 activity by controlling of levels of Nrf2 and INrf2. However, a phenotype of one of the published HIF-2α null mice was a mitochondrial dysfunction resulting from oxidative stress caused by increased ROS (Scortegagna *et al.*, 2003b). This was correlated with a downregulation of primary antioxidant enzyme (AOE) transcript (Scortegagna *et al.*, 2003b). Also, HIF-2α overexpressed in 293T cells from a mammalian expression construct enhanced the activity of a luciferase reporter fused downstream of the promoters of the aforementioned AOEs, thus implicating at least an indirect role for HIF-2α in the regulation of these genes (Scortegagna *et al.*, 2003b). The HIF-2α null mice in this study thus suggest that hypoxia increases AOE expression, whereas the array data suggests that it would contribute to doing the opposite by increasing INrf2 levels and other studies suggest that indeed hyperoxia enhances AOE transcript levels. It must be noted that, despite being known as high expressors of HIF-2α (Tian *et al.*, 1997; Ema *et al.*, 1997; Ema *et al.*, 1998), neither the lung (where hyperoxia causes Nrf2 and AOE upregulation) nor the adrenal gland (the source of the PC12 cell line) were analysed in this particular null mouse, so the response to O_2 levels with respect to AOE gene regulation mediated by Nrf2 may be cell specific. Nonetheless, Scortegagna and coworkers propose that the increase in ROS due to HIF-2α elimination may be the cause of the reduction in catecholamine levels and subsequent bradycardia in the first HIF-2α null mouse (Tian *et al.*, 1998), as ROS are known to directly inhibit tyrosine hydroxylase activity and general catecholamine synthesis (Ischiropoulos *et al.*, 1995; Kuhn *et al.*, 1999), thus inferring that their observations are common to all cells and organs (Scortegagna *et al.*, 2003b). However, given the somewhat nonoverlapping characteristics of the published HIF-2α$^{-/-}$ mice (chapter 1.7.2) or for that matter the conjecture with respect to the role of ROS in the regulation of at least HIF-1α (chapter 1.5.1), it is perhaps not surprising that the role of HIF-2α in AOE expression, dependent or independent of Nrf2 regulation, if indeed there is one, is unclear at best.

As mentioned previously, the immediate response of both PC12 cells and the type I carotid body to hypoxia is a rapid depolarisation caused by the inhibition of oxygen sensitive K^+ channels (Conforti and Millhorn, 1997; López-Barneo *et al.*, 1988), followed by an increase in intracellular Ca^{2+} (Zhu *et al.*, 1996; Buckler and Vaughan-Jones, 1994). Therefore, it would not be surprising if some of the genes involved in the regulation of these ion channels are targets of HIF-1α and/or HIF-2α. Admittedly, the regulation of the ion channels that permits this depolarisation probably occurs too rapidly for their transcriptional upregulation via HIF-α to be of relevance to an acute response, but an increase in their levels is no doubt of benefit for prolonged periods of hypoxia. Ion channels that were upregulated in the doxycycline treated PC12/TetON/HIF-2α/EYFP cell line included voltage gated sodium channel β3 subunit (Na^+ β3), voltage gated calcium channel $\alpha_2\delta$-1 (Ca^{2+} $\alpha_2\delta$-1) and the Na^+-Ca^{2+} exchanger isoform NACA7 (table 3.2). Regulator of G protein signalling 4 (Rgs4) which is involved in calcium signalling among other processes (Muallem and Wilkie, 1999) was also upregulated. In other words, these are all putative targets of HIF-2α. There was no apparent change in the expression of these when comparing the doxycycline treated inducible HIF-1α versus HIF-2α line, which means that it is likely that these may be generally transcriptionally upregulated by HIF-α. Surprisingly, although that of Ca^{2+} $\alpha_2\delta$-1 proved to be unsuccessful, the RTPCR employing primers to Na^+ β3 showed that it was a hypoxia inducible gene (figure 3.14.A) but not one that is targeted by either HIF-1α or HIF-2α (figure 3.14.B). Rgs 4 was also demonstrated to have a hypoxia inducible transcript (figure 3.14.A), but in this case it also appears to be a target of both HIF-1α and HIF-2α by virtue of its being upregulated upon doxycycline treatment of the inducible HIF-1α and HIF-2α PC12 cells, but not the control line (figure 3.14.B). Finally, it is important to note that the α_{1H} gene of the T type voltage gated calcium channel has also been implicated as a target of HIF-2α in another study (Del Toro *et al.*, 2003), although this is discussed in more detail below.

Finally, the proapoptotic Nip3 protein has already been described as a gene whose transcription is induced by hypoxia or the forced expression of HIF-1α and thus may be a direct target of HIF-1α by virtue of the presence of a functional HRE upstream of its translation start codon (Bruick, 2000). Therefore, the proposed scenario is that Nip3 is involved in hypoxia induced apoptosis (Bruick, 2000). It was of interest that doxycycline caused the upregulation of Nip3 in the PC12/TetON/HIF-2α/EYFP but not the control line

(table 3.2), suggesting that it is a putative target of HIF-2α and that there is a role for HIF-2α in hypoxia mediated apoptosis in PC12 cells. However, Nip3 was also upregulated in the doxycycline exposed inducible HIF-1α PC12 cell line when compared with similarly treated inducible HIF-2α PC12 cells (table 3.1). This reconfirms Nip3 as at least an indirect target of HIF-1α as originally hypothesised (Bruick, 2000). However, the data from the two arrays suggest that it is a target of both HIF-α subunits, although HIF-1α is clearly the predominant transactivator. This reflects another study where RNA interference (RNAi) of HIF-1α completely ablated the hypoxic induction of Nip3 protein in both the breast cell carcinoma MDA 468 and human umbilical vein endothelial cells (HUVECs), whereas RNAi of HIF-2α either had no effect on NIP-3 levels (MDA 468 cells) or did not completely inhibit Nip3 accumulation in response to hypoxia, even though both lines express both HIF-1α and HIF-2α (Sowter *et al.*, 2003).

3.7.2. PREVIOUSLY PUBLISHED HIF-2α ARRAYS

During the completion of this work, a number of studies have been published the aims and/or consequences of which were all to determine direct target genes of HIF-2α.

The most detailed of these used a number of cell lines to partially classify genes into those which are unique or common to HIF-1α and HIF-2α (Hu *et al.*, 2003). In most detail, array analysis of hypoxia inducible gene expression within the following cell lines were performed: 786-O cells (a renal clear cell carcinoma (RCC) which is defective for the von Hippel-Lindau protein (pVHL) and therefore has HIF-α protein and activity which is constitutively upregulated but only in the form of HIF-2α), RCC4 cells (another RCC line also defective for pVHL but capable of expressing both HIF-1α and HIF-2α) and 293 cells (a human embryonic kidney line definitely capable of expressing HIF-1α and, according to this paper, HIF-2α, although the ability of 293 cells to accumulate HIF-2α in hypoxia is controversial (Wiesener *et al.*, 1998). Briefly, it was concluded that the glycolytic enzymes in the chosen cell lines (such as enolase 1, aldolase A and lactate dehydrogenase A) are specific target genes of HIF-1α (Hu *et al.*, 2003). This reflects what was seen in our doxycycline inducible PC12 cell lines in that their mRNA was upregulated only upon doxycycline treatment of the PC12/TetON/HIF-1α/EYFP line (figure 3.12), so it can be concluded that these are direct targets of HIF-1α and not HIF-2α. However, it is also concluded that some genes are also transactivated by both HIF-α proteins, most notably VEGF and glucose transporter 1 (Hu *et al.*, 2003). According to

the Northern analysis described in this thesis, these appeared to be targets of HIF-1α (or in the case of VEGF, substantially preferentially regulated by HIF-1α, see below). Interestingly, there was no indication of the existence of a HIF-2α specific target gene.

The remaining studies have been more limited with their analysis of the complement of HIF-1α and HIF-2α target genes. In an array comparing expression in a 786-O line derivative in which wildtype pVHL had been reintroduced with another derivative where only the parent vector had been transfected, the HIF-α prolyl-4-hydroxylase PHD3 was determined to be a differentially expressed gene and, by virtue of its exclusive expression, one mediated by HIF-2α (Aprelikova *et al.*, 2004). Northern analysis confirmed that PHD3 mRNA levels were decreased when pVHL was reintroduced, although treatment of these cells with hypoxia restored levels to those in the wildtype cells in normoxia (Aprelikova *et al.*, 2004). The pVHL deficient cells in hypoxia expressed higher still PHD3 transcript (Aprelikova *et al.*, 2004), suggesting an influence of hypoxia inducible transcription independent of HIF-2α (as its protein levels are not altered by hypoxia (Maxwell *et al.*, 1999)) or the necessity to inhibit the HIF-α asparaginyl hydroxylase FIH-1 to ensure maximum upregulation. In some respects, this reflects the general hypoxia inducibility (whether HIF-α dependent or otherwise) of PHD3 seen by other groups in various cell lines (Epstein *et al.*, 2001; Metzen *et al.*, 2003a; del Peso *et al.*, 2003; D'Angelo *et al.*, 2003). However, in one of these (del Peso *et al.*, 2003), the same 786-O cells were used, but pVHL reintroduction caused the oxygen dependent regulation of PHD2 and not PHD3 mRNA, whereas Aprelikova and coworkers saw no change in PHD2 mRNA upon pVHL expression in the 786-O cells (Aprelikova *et al.*, 2004). Although the former study was acknowledged by the latter, there was no discussion of these discrepancies. Nonetheless, in the human hepatoma cell line Hep3B which expresses both HIF-α subunits, it was shown that RNAi of either HIF-1α or HIF-2α reduced the hypoxic induction of PHD3 transcript (unlike PHD2 whose hypoxic induction was downregulated only by HIF-1α RNAi) (Aprelikova *et al.*, 2004), it is thus difficult to classify PHD3 as a HIF-2α specific target. In the PC12 cells, PHD3 (GenBank accession number U06713, also known as SM-20) was relatively enhanced upon doxycycline treatment of the doxycycline inducible HIF-1α PC12 line when compared with the levels seen in the doxycycline inducible HIF-2α cells (table 3.1). However, it is not upregulated in the latter line upon exposure to doxycycline when compared to untreated cells. Therefore, PHD3 may be a direct target of HIF-1α but probably not HIF-2α in the PC12 cell line.

In another study comparing HUVECs that overexpress HIF-2α against those transfected with an empty vector, VEGF receptor Flt-1 was upregulated. It was shown that HIF-2α and ARNT overexpression enhance Flt-1 promoter reporter activity and that the promoter contains a functional HRE, the mutation of which attenuated reporter activity (Takeda *et al.*, 2004). The other differentially regulated genes of significance to this thesis were VEGF (see chapter 3.7.3), adrenomedullin (see chapter 3.7.8) and surprisingly lactate dehydrogenase A (see chapter 3.7.4) (Takeda *et al*; 2004).

3.7.3. VEGF AND ITS RECEPTORS

Initial experiments into HIF-2α performed at or shortly after the time of its discovery suggested a predominant role over HIF-1α in vascularisation, especially with regards to the regulation of VEGF, with perhaps less of a significance in the mediation of general hypoxic responses than has HIF-1α.

For instance, levels of VEGF mRNA expression appeared to be synchronised with the transcript of HIF-2α but not HIF-1α (Ema *et al.*, 1997). When the development of a tissue of high HIF-2α expression (the lung) was analysed, it was shown that, although HIF-1α levels remained constantly low throughout, the upregulation of both HIF-2α and VEGF commenced at the same time point (embryonic day (E) 17.5) and continued until the formation of the adult lung (Ema *et al.*, 1997). Furthermore, *in situ* hybridisation showed the coexpression of HIF-2α and VEGF in the alveolar epithelial cells of postnatal day (P) 0 and adult mice (Ema *et al.*, 1997).

Also, it has been postulated that HIF-2α is the preferential transactivator of the VEGF promoter. Using a mutant Chinese hamster ovary (CHO) derived cell line which expresses neither HIF-1α nor HIF-2α at detectable levels (Wood *et al.*, 1998), it was shown that the activity of a VEGF promoter mediated luciferase reporter was unaltered by exposure to hypoxia. However, cotransfection with HIF-2α caused an approximately 10 fold increase in reporter activity in hypoxia compared to a 2 fold increase upon overexpression of exogenous HIF-1α (Wiesener *et al.*, 1998). However, hypoxic induction of reporters driven either by multiple copies of an EPO derived HRE or the lactate dehydrogenase A promoter was identical independent of which HIF-α was introduced (Wiesener *et al.*, 1998). In addition, the expression of reporter genes mediated by promoters to the

endothelial specific VEGF receptor tyrosine kinase Tie-2 (Tian *et al.*, 1997) and the endothelial VEGF receptor Flk-1 (Kappel *et al.*, 1999) have been shown to be increased upon cotransfection of HIF-2α but not HIF-1α at least in normoxia (however, the higher activity of overexpressed HIF-2α when compared to HIF-1α at normoxia cannot be excluded as a possible explanation for the differential activity). Collectively, these results, albeit based on reporter analyses, hint at a role for HIF-2α in vascular development by transactivating VEGF and some of its effectors.

However, only one of the studies into HIF-2α null mice has correlated its phenotype with a reduction in VEGF levels, and even then in an unexpected fashion. Here, HIF-2α deletion caused an inhibition in foetal lung maturation (Compernolle *et al.*, 2002). The HIF-2α$^{-/-}$ mice that did not die due to cardiac failure before E13.5 died shortly after birth due to respiratory distress syndrome. This was shown to be due to decreased VEGF expression in alveolar epithelia, resulting in insufficient lung surfactant production, and thus suggested that VEGF is a HIF-2α target gene.

This data is of interest given the relative induction of VEGF by HIF-1α and HIF-2α in the doxycycline inducible HIF-1α and HIF-2α PC12 lines. Northern analysis of the established cell lines without and with doxycycline treatment (figure 3.12) showed a large induction of VEGF RNA by HIF-1α and essentially no signal in the other lanes, including that derived from doxycycline treated HIF-2α PC12 cells. This suggests that VEGF is a target of HIF-1α and not HIF-2α in the PC12 cells. This is supported by the comparison of the expression profile of doxycycline treated HIF-1α PC12 cells with similarly treated HIF-2α PC12 cells (table 3.1). However, the comparison between the doxycycline treated and untreated doxycycline inducible HIF-2α PC12 line showed an upregulation in VEGF transcript (table 3.2). This suggests that HIF-2α can induce VEGF but HIF-1α is the preferential transactivator of VEGF and that there is at best a limited role for HIF-2α in the regulation of VEGF when compared to that of HIF-1α.

3.7.4. GLYCOLYTIC ENZYMES AND GLUCOSE TRANSPORTERS

The initial expression profile of the doxycycline inducible lines with respect to known direct HIF-1α target genes showed that transcripts of the glycolytic enzymes tested (aldolase A, enolase 1 and lactate dehydrogenase A) were upregulated only upon doxycycline treatment of the HIF-1α inducible but not HIF-2α PC12 line (figure 3.12).

This suggests that they are targets of HIF-1α but not HIF-2α in the PC12 cell line. Given the observations of other studies, a general conclusion with respect to a lack of HIF-2α involvement in regulating glycolytic enzyme message can be established. Hu and coworkers demonstrated that in 786-O cells (an RCC line where pVHL does not function and only HIF-2α is present), glycolytic mRNA levels could not be altered, regardless of oxygen tension or the reintroduction of pVHL activity (Hu *et al.*, 2003). However, in the pVHL defective RCC4 line where both HIF-α proteins are present, the glycolytic enzymes displayed hypoxia inducibility in a derivative line stably expressing pVHL, probably via HIF-1α activity (Hu *et al.*, 2003). The role of HIF-1α as the dominant hypoxic transactivator of glycolytic enzymes was further supported by similar experiments in the human embryonic kidney line 293 where either HIF-1α or HIF-2α could be specifically upregulated (Hu *et al.*, 2003).

However, there is some conjecture pertaining to the control of glucose transporter 1 by HIF-2α. The transcript to this gene was only upregulated upon the doxycycline treatment of the PC12/TetON/HIF-1αN803A/EYFP and not the PC12/TetON/HIF-2α/EYFP line (figure 3.12), thus suggesting that it is directly targeted by HIF-1α but not HIF-2α. This is supported by a study involving HIF-1$\alpha^{-/-}$ and HIF-2$\alpha^{-/-}$ mouse embryonic stem (ES) cells (Brusselmans *et al.*, 2001). However, in the 786-O line, hypoxia inducibility of glucose transporter 1 is restored upon stable transfection of these cells with functional pVHL and thus the reestablishment of the oxygen dependent regulation of the HIF-2α protein, thus suggesting that it may be a target of HIF-2α at least in this line (Iliopoulos *et al.*, 1996; Ohh *et al.*, 2000).

3.7.5. ERYTHROPOIETIN

The *EPO* gene was among the first discovered to undergo hypoxia and specifically HIF-1α induced transcription (Goldberg *et al.*, 1988; Semenza and Wang, 1992). Originally, it was supposed that EPO served a purely erythropoietic purpose and its expression was limited to the kidney and liver (Jacobsen *et al.*, 1957; Naughton *et al.*, 1977; Caro *et al.*, 1983). However, it is becoming clearer that its expression is more widespread and that it is multipurposed. For instance, it has recently been shown that EPO is expressed in the brain and serves to prevent the apoptosis of neurons during cerebral ischaemia (Digicaylioglu and Lipton, 2001).

Of interest also is the role of EPO in the protection of the retina. It has recently been shown that exposing mice to mild hypoxia (6% O_2) for 6 hours followed by 4 hours of reoxygenation almost completely ablates light induced apoptosis of photoreceptors in the mouse retina and that systemic administration of recombinant EPO serves the same purpose (Grimm *et al.*, 2002). Interestingly, EPO mRNA is expressed in the retina and is enhanced after exposure of mice to mild hypoxia, thus suggesting that the hypoxically induced EPO is responsible for the neuroprotection (Grimm *et al.*, 2002). Although HIF-1α protein is also substantially upregulated in the retina as a consequence of the same treatment, it is not known whether HIF-1α protein was directly responsible for the enhanced EPO (Grimm *et al.*, 2002).

Retinal neovascularisation in the disease known as retinopathy of prematurity (ROP) can be induced in mice via exposure to 75% O_2 (hyperoxia) followed by returning the mice to normoxia (Smith *et al.*, 1994). The hyperoxia causes the destruction of the retinal capillary network such that when the mice are returned to normal oxygen tension they are present in a relatively hypoxic environment, resulting in a hyperproliferation of vessels (Smith *et al.*, 1994). This has been correlated with an increase in retinal VEGF (Pierce *et al.*, 1995). Interestingly, neovascularisation of the retina caused by the ROP model was almost totally inhibited in mice where HIF-2α expression has not been eliminated but significantly decreased (HIF-2α knockdown mice) (Morita *et al.*, 2003). Surprisingly, there were no changes in the levels of various angiogenic factors (VEGF, Tie-2 and Flk-1) induced by the ROP in wildtype and HIF-2α knockdown retinas, although the ROP model's induction of EPO mRNA and protein in wildtype mice was attenuated in the HIF-2α knockdown (Morita *et al.*, 2003). This suggests that EPO may be a target of HIF-2α and not HIF-1α in this context, a concept which has been also concluded upon the ablation of hypoxia inducible EPO message upon RNAi of HIF-2α in human hepatoma Hep3B and neuroblastoma Kelly cells (Warnecke *et al.*, 2004).

It was not thought that EPO was expressed in PC12 cells. Consequently, its expression was not probed in the Northern analysis of the doxycycline inducible PC12 derivatives. Even if it were, the array data suggests that it is not a target of HIF-2α in PC12 cells.

3.7.6. GENE EXPRESSION IN 786-O CELLS

The 786-O line is of interest for studies into the downstream effects of the HIF-α genes as it expresses a non functioning truncated pVHL, so HIF-α protein is not regulated by hypoxia but rather is constitutively upregulated (Maxwell *et al.*, 1999). However, only HIF-2α is expressed in this cell line (Maxwell *et al.*, 1999). The hypoxia inducibility of this HIF-2α protein can be reintroduced upon transfection into these cells of pVHL (Maxwell *et al.*, 1999). The exclusive presence of HIF-2α in the 786-O cell line demands the question: should a system have been derived from this cell line to screen for HIF-2α target genes, possibly via a comparison of the expression profile between wildtype 786-O cells and a variant which stably produces pVHL (as has since been done by Hu *et al.*, 2003, see above and below)? The answer is possibly. However, the absence of HIF-1α in this line means that the scope for determining HIF-2α specific target genes (that is, the ability to differentiate between those whose transcription is directly mediated by HIF-1α and HIF-2α) is not provided for. Furthermore, 786-O cells are the only line known to express solely HIF-2α, whereas all others have both HIF-1α and HIF-2α or merely HIF-1α. It was proposed that the role of HIF-2α in the 786-O line may not be reflective of its significance in other lines or *in vivo* as it may serve to partially compensate for the absence of HIF-1α. This is unlike the PC12 cell line where both HIF-1α and HIF-2α proteins are accumulated in hypoxia and where both therefore may have distinct functions. However, it has subsequently been shown that HIF-2α may indeed not serve as a general hypoxia inducible factor in the 786-O line as, unlike glucose transporter 1, the transcripts of glycolytic enzymes such as phosphoglycerate kinase 1 and lactate dehydrogenase A are not upregulated by HIF-2α, as mentioned above (Hu *et al.*, 2003). Interestingly, the mRNA of the glycolytic enzymes could be increased by the introduction of the otherwise absent HIF-1α activity (Hu *et al.*, 2003).

The apparent incapacity of HIF-2α to mediate glycolytic enzyme expression in 786-O cells (Hu *et al.*, 2003) reflects what is seen in the doxycycline inducible HIF-1α and HIF-2α PC12 lines in this thesis, whether by the Northern analysis for the expression of known HIF-1α targets (figure 3.12) or subsequent microarray analysis. That is, only HIF-1α appears to regulate the transcription of glycolytic enzymes (as discussed more in depth above). Nonetheless, there are distinct differences between the two cell lines. Most notable are the ability of 786-O cells to control glucose transporter 1 and VEGF expression (Iliopoulos *et al.*, 1996; Ohh *et al.*, 2000; Hu *et al.*, 2003) compared with no

change in glucose transporter 1 levels (figure 3.12) and the very limited VEGF upregulation (at least when compared with that resulting from doxycycline treatment of the inducible HIF-1α PC12 line) (figure 3.12, table 3.1 and table 3.2) upon the induction of HIF-2α in the doxycycline inducible HIF-2α PC12 cell line. This serves to confirm the dependence on cellular context in determining HIF-2α function.

3.7.7. GENE EXPRESSION IN PC12 CELLS

PC12 cells have been employed for many years for studies into the physiology and biochemistry of cellular responses to hypoxia (Seta *et al.*, 2002). During the course of the research described in this thesis, they have also been used in a limited capacity to provide hints as to possible HIF-2α specific targets.

Ca^{2+} entry via voltage gated calcium channels is crucial for adapting neurosecretory cells (such as the PC12 cell line) to reduced oxygen tension (López-Barneo *et al.*, 2001), so it is perhaps no surprise that the mRNA of the α_{1H} gene of the T type voltage gated calcium channel is upregulated in hypoxia (Del Toro *et al.*, 2003). The involvement of at HIF-α activity in increasing transcript levels is suggested by the ability of the HIF-α prolyl-4- and asparaginyl hydroxylase inhibitors $CoCl_2$, DFO and dimethlyoxalylglycine (DMOG) to also enhance α_{1H} levels and the presence of a number of HRE sequences approximately 1300 bases upstream of the coding sequence (Del Toro *et al.*, 2003). Interestingly, transfection of PC12 cells with antisense oligonucleotides to HIF-2α showed a reduction in hypoxically inducible α_{1H} transcript, suggesting that it is a target of HIF-2α (Del Toro *et al.*, 2003). This is of particular interest since the voltage gated calcium channel $\alpha_2\delta$-1 subunit was identified as a potential HIF-2α target gene in the array analysing the expression of genes induced by doxycycline treatment of the PC12/TetON/HIF-2α/EYFP line (table 3.2).

A final mention must be made of tyrosine hydroxylase, perhaps the most studied gene with respect to its regulation by hypoxia in PC12 cells. HIF-1α and HIF-2α binding to and mediating transcription from an HRE present in the tyrosine hydroxylase gene has been shown, thus at least implicating HIF-α in the hypoxia inducibility of its product (Schnell *et al.*, 2003). However, with respect to the studies described in this thesis, HIF-1α appears to be the primary transactivator of tyrosine hydroxylase (figure 3.12 and table 3.1).

3.7.8. OTHER PUTATIVE HIF-2α TARGET GENES

As mentioned previously, there are a number of direct targets of HIF-1α (table 1.1), many of which are indicative of its function as a general molecular mediator of physiological responses to hypoxia. However, there is also a large contingent of genes that are directly mediated by HIF-1α whose known functions are difficult to reconcile with any perceived necessity to be induced by hypoxia. This may also be the case for HIF-2α. In other words, as an appreciation of the complement of HIF-2α target genes slowly emerges, it is possible that some of these may also play no obvious role with regards to the adaptation of an organism to hypoxia or for that matter with the processes whose interruption in any of the null mice are responsible for their phenotype. A couple of recent studies are already suggesting that this is the case.

One such gene may be adrenomedullin (AM) (Tanaka *et al.*, 2002). AM has been reported to perform a beneficial function by decreasing blood pressure and increasing cardiac index and stroke volume without any coincident alteration of heart rate and also by elevating the volume and sodium excretory capacity of urine (Ishiyama *et al.*, 1995; Majid *et al.*, 1996; Nagaya *et al.*, 2000). The expression pattern of AM mRNA is essentially identical to that of HIF-2α in that it is predominant in the heart, lung and adrenal gland and in endothelial cells (Sakata *et al.*, 1994; Ema *et al.*, 1997). The original observation was that the proinflammatory cytokine interleukin (IL) 1β could induce both HIF-2α mRNA and protein levels in neonatal rat cardiac myocytes, unlike hypoxia which only enhanced HIF-2α protein (Tanaka *et al.*, 2002). As AM was known to be regulated by both hypoxia and proinflammatory cytokines such as IL-1β (Horio *et al.*, 1998), a correlation between HIF-2α and AM expression was sought and it was shown that overexpressed HIF-2α could upregulate endogenous AM transcript in cardiac myocytes and an AM promoter driven reporter (containing a putative HRE whose mutation abolished activity) in the CV-1 cell line (Tanaka *et al.*, 2002). It is interesting to note that, although this study was conducted in cardiac myocytes, AM was cloned from a human pheochromocytoma (Kitamura *et al.*, 1993), the tumour type from which PC12 cells originate (Greene and Tischler, 1976). Also, in the first published study with respect to HIF-2α null mice, embryos died as a result of blood congestion caused by bradycardia (Tian *et al.*, 1998). AM may thus be a means by which HIF-2α can regulate the cardiovascular system in response to hypoxia, perhaps in conjunction with the synthesis and release of catecholamines which in turn regulate heart rate, a function not performed

by AM. AM, however, is not listed as a gene that is upregulated by doxycycline treatment of the PC12/TetON/HIF-2α/EYFP line.

Another proposed target of HIF-2α is plasminogen activator inhibitor (PAI) 1 (Sato *et al.*, 2004). PAI-1 is a relatively uncharacterised protease linked to the ability of tumours to vascularise and metastasise (Bajou *et al.*, 1998; Chazaud *et al.*, 2002). PAI-1 was originally shown to be a target of HIF-1α in rat hepatocytes (Kietzmann *et al.*, 1999). However, it has since been demonstrated to also be a hypoxia inducible gene in the adenocarcinoma A549 cell line, but in this case overexpressed HIF-2α was shown to increase endogenous PAI-1 protein and a PAI-1 promoter mediated luciferase reporter (Sato *et al.*, 2004). Also, a hypoxically inducible binding activity in an electromobility shift assay (EMSA) was supershifted (albeit weakly) with an anti HIF-2α antibody (Sato *et al.*, 2004). It is impossible to rule out the involvement of HIF-1α in PAI-1 induction in the A549 line. However, the function of HIF-2α in mediating the increase in PAI-1 protein in hypoxia is of interest, particularly given the suggestion of a tumour promoting role for HIF-2α and not HIF-1α, at least in the context of 786-O cells (Maranchie *et al.*, 2002; Kondo *et al.*, 2003; Kondo *et al.*, 2003). There is no reason to suggest from the array data that PAI-1 is a HIF-2α target gene. However, PAI-1 binds to and is stabilised by vitronectin (Declerck *et al.*, 1988), a putative HIF-2α target gene (see above), so HIF-2α may also perform a role in PAI-1 function by mediating vitronectin.

3.7.9. FURTHER ANALYSIS OF PUTATIVE HIF-2α TARGET GENES

Analysis of the putative HIF-2α target genes (table 3.2) would commence with a confirmation of the RTPCR results (figure 3.14) and additional Northern analysis of RNA derived from all three lines with or without doxycycline treatment. This may demonstrate that the relevant transcript is upregulated upon doxycycline treatment of the inducible HIF-2α line and not the control line such that it can be classed as at least an indirect target gene of HIF-2α. If it is additionally upregulated in the inducible HIF-α line upon its doxycycline treatment, then it would also be that of HIF-1α. If not, then it would specifically be associated with HIF-2α function. In this case, it would then be determined whether HIF-2α is a direct or indirect transactivator of the analysed gene.

The existence of a putative HRE would immediately suggest that it is a direct target of HIF-α. However, one cannot discount the requirement of other sequences for the HIF-2α

inducibility of the transcript. Therefore, luciferase reporters can be constructed with various portions of the putative target gene's promoter to determine the necessary sequences for the upregulation of the gene. Once known, the upregulation of the reporter upon doxycycline treatment of the inducible HIF-2α line but not the inducible HIF-1α line would further confirm the distinct necessity for HIF-2α in its hypoxia inducibility. That HIF-2α binds to these sequences can be initially characterised via an EMSA. Also, a chromatin immunoprecipitation of the putative binding sequence from extracts from hypoxic PC12 cells with anti HIF-2α antibodies will confirm that this HIF-α associates directly with the endogenous promoters *in vivo*.

3.7.10. ALTERNATIVE METHODS

Perhaps another method of discovering HIF-2α specific target genes would be to derive monoclonal lines whereby either HIF-1α or HIF-2α is downregulated via RNAi. Monoclonal PC12 cells expressing doxycycline inducible dsRNA directed towards HIF-1α or HIF-1α or (as a negative control to account for any non specific effects of expressing dsRNA) a scrambled sequence. All cells would be exposed to doxycycline and compared with the expression of untreated cells. Presumably, HIF-1α or HIF-2α would be downregulated in the respective inducible HIF-α RNAi line and in association with their respective target genes. The advantage of this method is that any effects seen are mediated by the endogenous protein, as opposed to the system used in this thesis, which utilised selective overexpression of a stably incorporated HIF-α protein, so any downstream effects may be due to artificially high levels of expression. However, it should be noted that the expression and activity of the introduced HIF-1α or HIF-2α in PC12 derivatives were comparable to that seen in hypoxia, so any problems arising from having an excessive amount of protein are unlikely. RNAi would be a poor substitute for the method used above if the HIF-α transcript were not efficiently reduced and this may be difficult to achieve. Besides, the development of these cell lines was well in progress by the time the criteria required for successful RNAi in mammalian cells had been published (Elbashir *et al.*, 2001).

Regardless of which method of HIF-1α or HIF-2α selective regulation were utilised (that is, induction via the TetON system or inhibition via RNAi), it would be of major interest to determine the complement of HIF-1α and HIF-2α target genes in the PC12 cell line via microarray. By using the cell lines engineered in this thesis, this would entail the analysis

of gene expression of doxycycline treated PC12/TetON/HIF-1αN803A/EYFP cells versus untreated cells. When collated with the data from the second array (table 3.2), genes can be classified as strictly HIF-1α or HIF-2α targets (that is, upregulated upon doxycycline treatment of the inducible HIF-1α or HIF-2α line, respectively) or general HIF-α targets (that is, their transcripts are enhanced after exposure of both lines to doxycycline). Although the first array (table 3.1) may have in theory been sufficient to identify putative HIF-2α specific target genes (which in itself proved to be a problem as highlighted in chapter 3.6.2), this more detailed array will permit the discovery of transcripts that are selectively mediated by either HIF-1α or HIF-2α as well as those whose control is common to both. This will no doubt provide clues as to the respective roles of HIF-1α and HIF-2α *in vivo*.

CHAPTER 4

DIFFERENTIAL REGULATION OF HIF-1α AND HIF-2α IN PC12 CELLS

CHAPTER 4

DIFFERENTIAL REGULATION OF HIF-1α AND HIF-2α IN PC12 CELLS

4.1. INTRODUCTION

Although for some time elusive, the mechanisms by which hypoxia results in the stabilisation and transactivation of the otherwise labile and nonfunctioning hypoxia inducible factor α (HIF-α) protein have recently become apparent. In short, this involves inhibition of the oxygen dependent prolyl-4-hydroxylation of two conserved proline residues within HIF-α to prevent association with the von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex and destruction by the ubiquitin proteasome pathway (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001; Epstein *et al.*, 2001; Bruick and McKnight, 2001). Furthermore, transactivation during hypoxia is accomplished by the inhibition of the oxygen dependent asparaginyl hydroxylation of a conserved asparagine to permit association with the transcriptional coactivator CBP/p300 and ultimately transcription of HIF-α target genes (Lando *et al.*, 2002a; Lando *et al.*, 2002b; Hewitson *et al.*, 2002). The HIF-α prolyl-4-hydroxylases (known as prolyl hydroxylase domain containing (PHD) 1, 2 and 3) and asparaginyl hydroxylase (also called factor inhibiting HIF-1 (FIH-1)) therefore appear to function as the oxygen sensors which detect cellular O_2 levels (Epstein *et al.*, 2001; Bruick and McKnight, 2001; Mahon *et al.*, 2001; Lando *et al.*, 2002b; Hewitson *et al.*, 2002).

It has generally been assumed that the same pathways are involved in inhibiting the accumulation and enhanced function of both HIF-1α and HIF-2α. Consequently, there is little insight into any specificity in the posttranslational modifications that may regulate these respective proteins, although it is clear that there are some. For instance, it has been shown that a cysteine in the DNA binding basic region of HIF-2α requires reduction, possibly by the redox factor Ref-1, prior to the activation of HIF-2α's DNA binding ability (Lando *et al.*, 2000). This is not so for HIF-1α, where serine is present in the equivalent position (the only difference between the amino acid sequences of their basic regions) and DNA binding is constitutive (Lando *et al.*, 2000). A controversial mechanism of HIF-2α regulation has been proposed in a recent study using mouse

embryo fibroblasts (MEFs) where HIF-1α protein was shown to be stabilised and undergo nuclear translocation during hypoxia, whereas HIF-2α was easily detectable regardless of oxygen levels and primarily cytoplasmic (Park *et al.*, 2003). However, HIF-2α can function as a transcription factor when overexpressed in MEFs, signifying that the cytoplasmic entrapment of endogenous HIF-2α inhibits its ability to act as a transcription factor and suggesting that an as yet unknown mechanism may stimulate HIF-2α to enter the nucleus and invoke target gene expression (Park *et al.*, 2003).

Based on the assumption that the major mechanisms of regulation are common to both proteins, it could be postulated that the saturation of the HIF-α hydroxylases and other factors that mediate degradation by the overexpression of either HIF-1α or HIF-2α would result in at least some stabilisation of both endogenous HIF-1α and HIF-2α. Indeed, this hypothesis is supported by previous findings. The renal cell carcinoma (RCC) 786-O line expresses a non functioning truncated pVHL and therefore unregulated HIF-2α expression (HIF-1α is absent) (Maxwell *et al.*, 1999). Using a derivative which stably expresses wildtype pVHL and therefore which has its oxygen dependent degradation of HIF-2α restored, HIF-2α could also be stabilised by the overexpression of HIF-1α 541-580 (that is, containing P564 and the pVHL binding site) (Maranchie *et al.*, 2002). In other words, it seems that HIF-1α and HIF-2α compete for the same degradation machinery and the forced expression of one permits the stabilisation of the other.

However, during the development of the doxycycline inducible HIF-1α and HIF-2α PC12 cell lines, it was evident that this was not the case. Whether HIF-2α was overexpressed from a transiently transfected plasmid (figure 3.2.B and figure 3.4.C) or the accumulation of either HIF-α from a stably incorporated transgene (figure 3.8.B and 3.11.B), only the induced HIF-α could be detected via immunoblotting of cell extracts with the relevant antibody. Therefore, it was proposed that there are differences in the processes by which HIF-1α and HIF-2α are degraded, at least in the PC12 cell line. An aim of this research, therefore, became to characterise any differences between the respective mechanisms of HIF-1α and HIF-2α degradation in PC12 cells.

4.2. HIF-α EXPRESSION IN PC12 CELLS AS A FUNCTION OF DURATION OF HYPOXIA

The accumulation of HIF-1α protein upon exposure of cells to hypoxia occurs rapidly. In HeLaS3 cells for instance, nuclear HIF-1α is evident within 2 minutes, with protein levels reaching their maximum after only 60 minutes of exposure to 0.5% O_2 (Jewell *et al.*, 2001). Given that the PHD enzymes function as a major sensor and effector of reduced oxygen and since dioxygen is a necessary substrate of their hydroxylase activity, it is perhaps expected that the exposure of cells to hypoxia elicits such a rapid increase in HIF-1α levels. Similar experiments were thus designed to compare the induction of HIF-1α and HIF-2α protein in PC12 cells.

Whole cell extracts were prepared from PC12 cells exposed to <1 % O_2 (hypoxia) for 0 to 24 hours and were analysed by immunoblotting with antibodies to either HIF-1α or HIF-2α (figure 4.1). It was predicted that if both HIF-1α and HIF-2α protein were regulated by prolyl-4-hydroxylation, the kinetics of their accumulation and/or degradation should be similar. HIF-1α levels appear to have been maximised within 2 hours of hypoxic treatment, which is consistent with a mechanism of decreased PHD function. However, HIF-2α protein is only detectable after 4 hours and reaches a maximum after 16 hours, by which time HIF-1α levels have peaked and decreased. This result demonstrates a difference in the hypoxic regulation of endogenous HIF-1α and HIF-2α in PC12 cells. Given the similarity in the respective O_2 binding capabilities of the three PHDs (Hirsilä *et al.*, 2003), it is thus possible that prolyl-4-hydroxylation is not involved in the targeting of HIF-2α for normoxic destruction in PC12 cells.

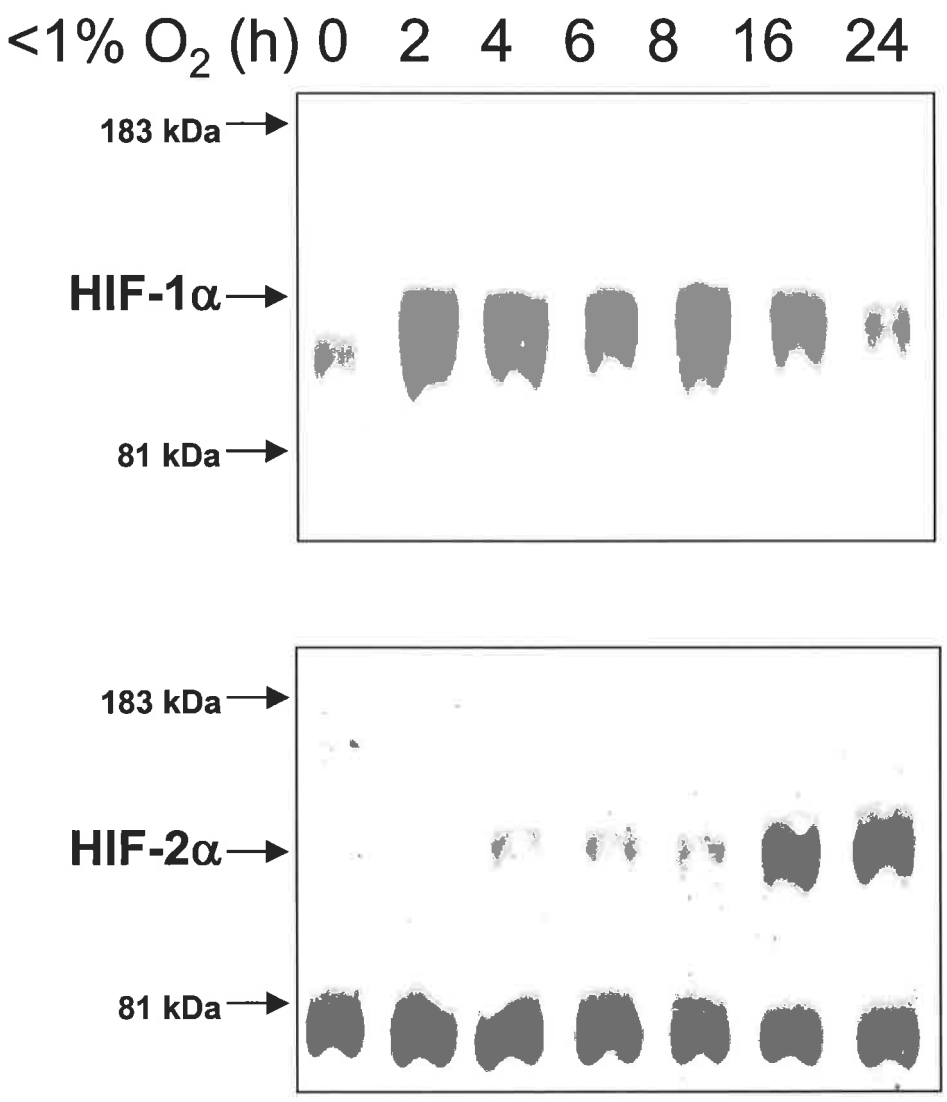
4.3. EFFECT OF PROLYL-4-HYDROXYLASE INHIBITORS ON HIF-α EXPRESSION IN PC12 CELLS

4.3.1. IRON CHELATION STABILISES HIF-1α BUT NOT HIF-2α PROTEIN

In addition to low oxygen levels, hypoxic responses such as the increase in erythropoietin gene expression can be induced by the iron chelators desferrioxamine (DFO, Wang and Semenza, 1993b) and 2, 2'-dipyridyl (DP, Kallio *et al.*, 1998). These function by inducing both HIF-α protein levels and transcriptional potency (Jiang *et al.*, 1997, Pugh *et al.*, 1997). It is now clear that DFO and DP can stabilise and transactivate HIF-α by sequestering the Fe^{2+} required for prolyl-4- and asparaginyl hydroxylase activity (Epstein

Figure 4.1. Hypoxia causes rapid increase in HIF-1α protein but a delayed accumulation of HIF-2α protein in PC12 cells.

Whole cell extracts were prepared from PC12 cells exposed to <1% O_2 for 0, 2, 4, 6, 8, 16 or 24 hours. 20 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (upper panel) or HIF-2α (lower panel). Results are representative of three separate experiments.


<1% O_2 (h) 0 2 4 6 8 16 24
183 kDa
HIF-1α
81 kDa
183 kDa
HIF-2α
81 kDa

et al., 2001; Bruick and McKnight, 2001; Lando *et al.*, 2002b; Hewitson *et al.*, 2002). Therefore, to further decipher the differential mechanisms of HIF-1α and HIF-2α protein regulation in the PC12 cell line, experiments were performed utilising these hypoxia mimetics.

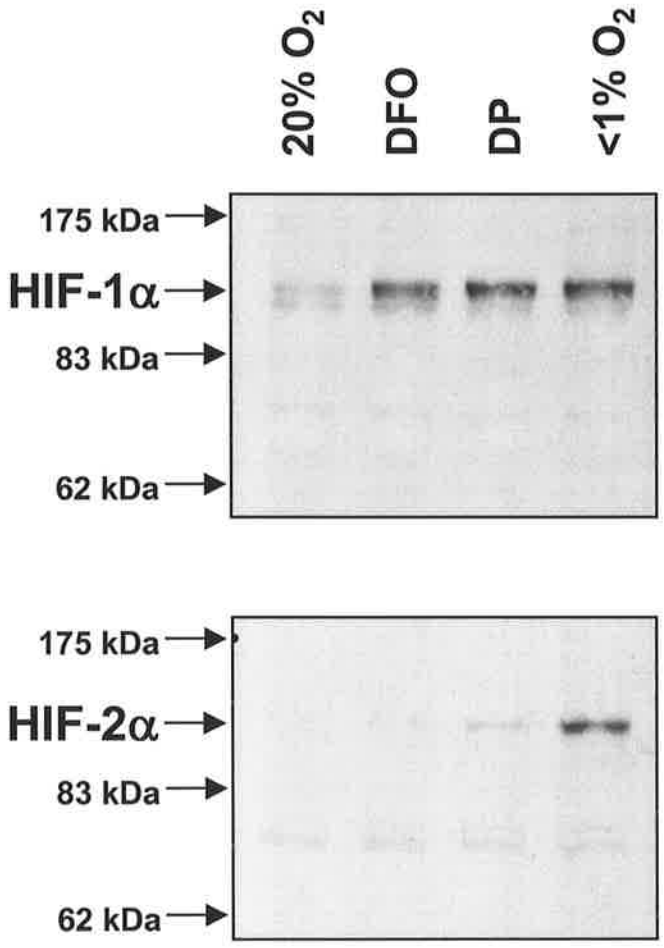
Whole cell extracts were derived from PC12 cells exposed to 20% O_2 (normoxia), hypoxia or to 100 μM DFO or DP for 16 hours and were analysed by immunoblotting with antibodies to either HIF-1α or HIF-2α (figure 4.2). It was hypothesised that iron chelation would cause the stabilisation of HIF-1α and/or HIF-2α if their levels are regulated by PHD activity. The levels of HIF-1α generated after exposure to hypoxia, DFO or DP were equivalent (upper panel), whereas the induction of HIF-2α by DFO or DP was minimal when compared to that stabilised in hypoxia (lower panel). It thus seems that in PC12 cells HIF-1α but not HIF-2α degradation is initiated by prolyl-4-hydroxylation as chelating the iron required for this reaction causes the stabilisation of HIF-1α but not HIF-2α. These observations, coupled with the time course experiments in which the duration of hypoxia required for the accumulation of maximum HIF-2α was longer than expected if the inhibition of prolyl-4-hydroxylase activity were involved, suggests that HIF-1α but not HIF-2α protein is regulated by PHD function.

4.3.2. 2-OXOGLUTARATE COMPETITION STABILISES NEITHER HIF-1α NOR HIF-2α PROTEIN

The HIF-α prolyl-4- and asparaginyl hydroxylases are classified as 2-oxoglutarate dependent dioxygenases (Epstein *et al.*, 2001; Bruick and McKnight, 2001; Lando *et al.*, 2002b; Hewitson *et al.*, 2002). In the hydroxylation reaction, the splitting of O_2 allows for one oxygen atom to be incorporated within the alcohol group of the hydroxylated substrate (in this case, HIF-α) and the other to be received by succinate during the decarboxylation of 2-oxoglutarate, a process which also results in the formation of CO_2 (Schofield and Ratcliffe, 2004). *N*-oxalylglycine (NOG) is an analogue of 2-oxoglutarate. However, it cannot be utilised by the 2-oxoglutarate dependent dioxygenases and so serves as a competitive inhibitor of these enzymes (Jaakkola *et al.*, 2001). Dimethyloxalylglycine (DMOG) is a cell permeable analogue of 2-oxoglutarate that is converted to NOG upon cellular entry. DMOG can thus stabilise HIF-α in cells by virtue of its inhibiting PHD function (Jaakkola *et al.*, 2001).

Figure 4.2. The levels of HIF-1α protein generated after exposure to hypoxia or the iron chelators DFO or DP are equivalent, unlike HIF-2α induction.

Whole cell extracts were prepared from PC12 cells exposed to 20% O_2, 100 μM desferrioxamine (DFO), 100 μM 2, 2'-dypyridyl (DP) or <1% O_2 for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (upper panel) or HIF-2α (lower panel). Results are representative of three independent experiments.


20% O2
DFO
DP
<1% O2
175 kDa
HIF-1α
83 kDa
62 kDa
175 kDa
HIF-2α
83 kDa
62 kDa

Whole cell extracts were derived from PC12 cells exposed to normoxia, hypoxia or to 1 mM DMOG for 16 hours and were analysed by immunoblotting with antibodies to either HIF-1α or HIF-2α (figure 4.3.A). Given that iron chelation caused the stabilisation of HIF-1α but not HIF-2α in PC12 cells, it appears that prolyl-4-hydroxylation initiates the degradation of the former but not the latter. Therefore, it was proposed that DMOG treatment would cause HIF-1α but not HIF-2α accumulation. Surprisingly, DMOG induced neither HIF-1α (left panel) nor HIF-2α (right panel) accumulation. Furthermore, PC12 cells were transfected with a firefly luciferase reporter gene containing the simian virus 40 (SV40) promoter and 4 copies of the HRE (pHRE$_4$GL3, hereafter referred to as pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to normoxia, hypoxia or to 1mM DMOG. After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal control reporter gene consisting of the herpes simplex virus thymidine kinase (TK) promoter which mediates the expression of renilla luciferase (pRLTK) (figure 4.3.B). HRE mediated reporter activity resulting from DMOG treatment was relatively low when compared with that caused by hypoxic treatment (compare pHRE + DMOG versus pHRE at 20% O_2 and pHRE at <1% O_2).

Collectively, this data supports the novel suggestion that HIF-2α levels are not regulated by prolyl-4-hydroxylation in PC12 cells. However, it is difficult to reconcile the fact that one class of PHD inhibition (iron chelation) did result in the upregulation of HIF-1α with the observation that another (2-oxoglutarate competition) did not. The immunoblot (figure 4.3.A) does not rule out the possibility that DMOG cannot permeate into PC12 cells or, that once it enters, it is processed into a compound that cannot inhibit hydroxylase activity. However, the approximately 3 fold increase in HRE reporter activity in response to DMOG treatment (figure 4.3.B) suggests that DMOG enters and stimulates the transactivation of the little amount of HIF-α present. In other words, DMOG may not inhibit PHD activity but may still interfere with asparaginyl hydroxylation function in PC12 cells.

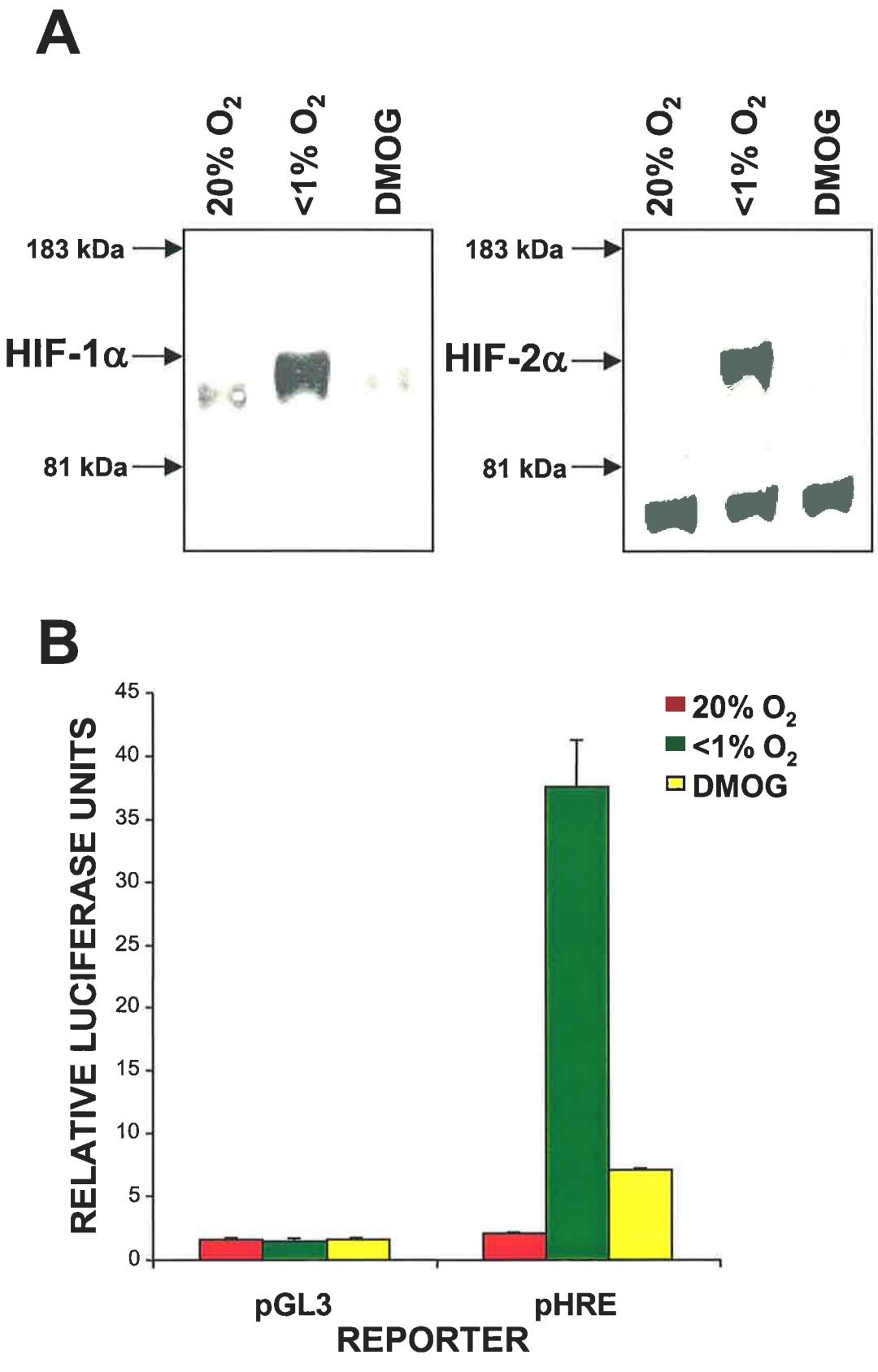
4.3.3. 2-OXOGLUTARATE COMPETITION INHIBITS ASPARAGINYL HYDROXYLASE MEDIATED TRANSCRIPTIONAL REPRESSION OF HIF-1α

As with the PHD enzymes, the HIF-α asparaginyl hydroxylase FIH-1 is also a 2-oxoglutarate dependent dioxygenase. Therefore, its ability to hydroxylate the crucial

Figure 4.3. Treatment of PC12 cells with the 2-oxoglutarate competitor DMOG causes the stabilisation of neither HIF-1α nor HIF-2α.

A. Whole cell extracts were prepared from PC12 cells exposed to 20% O_2, 1 mM dimethyloxalylglycine (DMOG) or <1% O_2 for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (left panel) or HIF-2α (right panel). Results are representative of three independent experiments.

B. PC12 cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2, <1% O_2 or to 1 mM DMOG. After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of three independent experiments.


A
20% O2
<1% O2
DMOG
183 kDa
HIF-1α
81 kDa
20% O2
<1% O2
DMOG
183 kDa
HIF-2α
81 kDa
B
RELATIVE LUCIFERASE UNITS
45
40
35
30
25
20
15
10
5
0
20% O2
<1% O2
DMOG
pGL3
pHRE
REPORTER

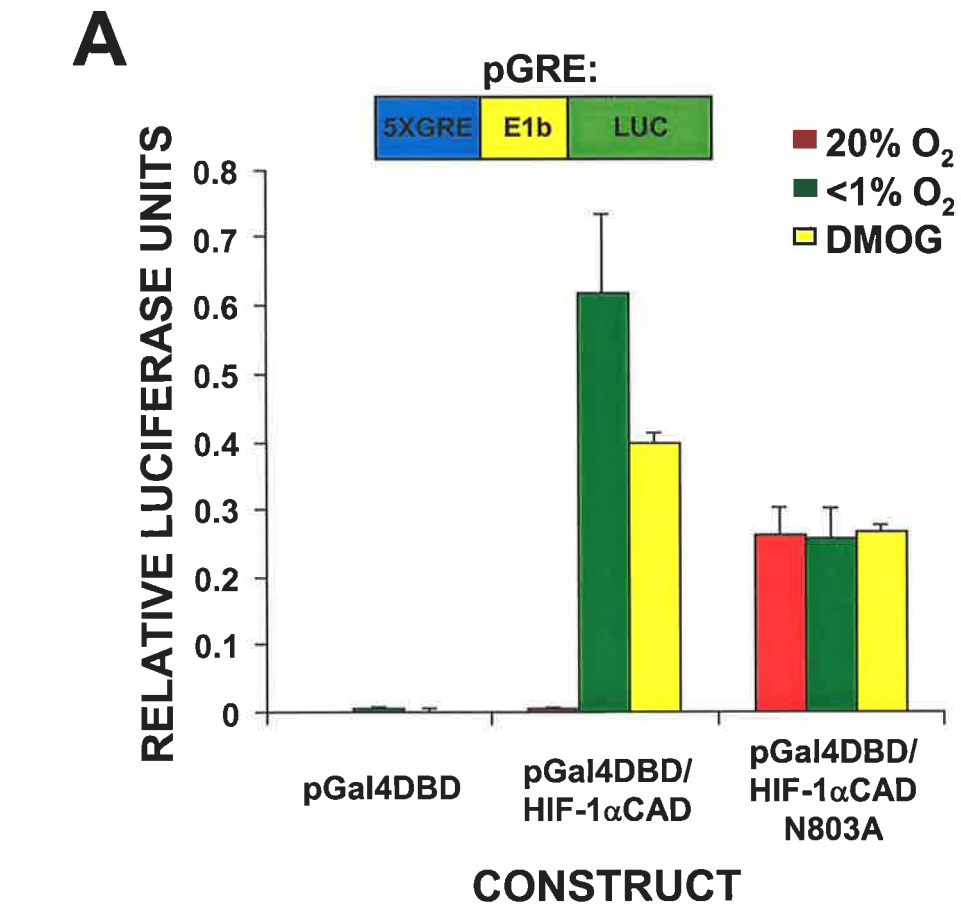
asparagine and downregulate the transcriptional potency of the carboxy terminal transactivation domain (CAD) of HIF-α can be ablated by the treatment of cells with DMOG (Lando *et al.*, 2002b; Hewitson *et al.*, 2002). This is inferred by the fact that the C terminal 100 amino acids of HIF-1α and HIF-2α when fused to the Gal4 DNA binding domain (Gal4DBD) cause a substantial increase in Gal4 response element (GRE) mediated reporter activity when 293T cells cotransfected with these constructs are treated with DMOG. This activation by DMOG is comparable with hypoxia and does not involve any change in protein levels (Lando *et al.*, 2002a).

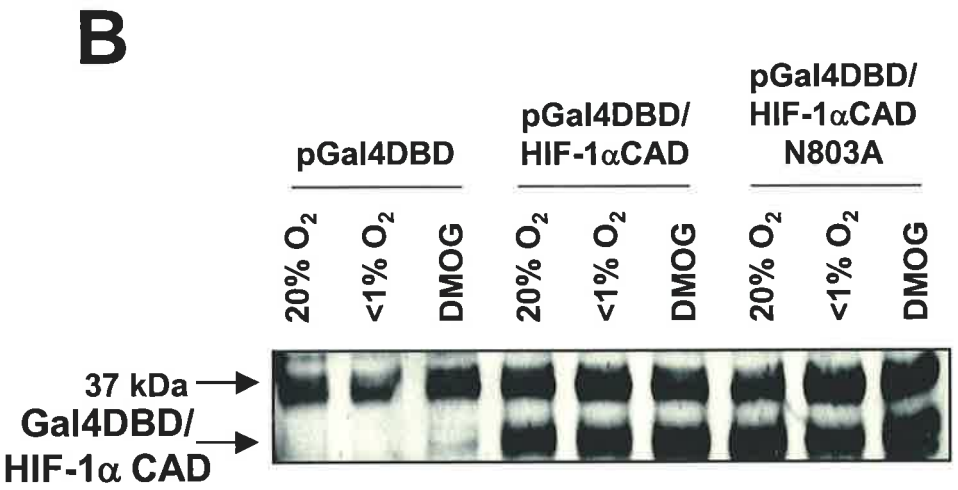
Therefore, to confirm the functionality of DMOG in PC12 cells, reporter analysis of HIF-1α CAD activity was employed. PC12 cells were transfected with a firefly luciferase reporter gene containing the E1b promoter and 5 copies of the GRE (pGRELUC) in addition to mammalian expression constructs expressing either of the following: the Gal4 DNA binding domain (pGal4DBD), the Gal4DBD fused to amino acids 737-826 of HIF-1α (pGal4DBD/HIF-1αCAD) or the Gal4DBD fused to a HIF-1α CAD with an N803A mutation (pGal4DBD/HIF-1αCADN803A). 6 hours post transfection, cells were exposed to normoxia, hypoxia or to 1 mM DMOG. After 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 4.4.A). If PC12 cells were impermeable to this compound or alter it upon entry into a chemical other than NOG, then presumably DMOG would not inhibit FIH-1 and would thus be incapable of increasing the activity of the Gal4DBD/HIF-1α CAD. However, DMOG markedly induced the transcriptional potency of Gal4DBD/HIF-1α CAD on a GRE reporter almost to the levels caused by hypoxia (compare Gal4DBD/HIF-1α CAD + DMOG versus Gal4DBD/HIF-1α CAD at 20% or <1% O_2). As expected (Lando *et al.*, 2002a), mutation of the asparagine targeted by FIH-1 for hydroxylation (N803) to an alanine resulted in a high level of GRE reporter activity regardless of oxygen tension or DMOG treatment, suggesting that this chemical's capacity to induce Gal4DBD/HIF-1α CAD transactivation is via its inhibition of FIH-1. To ensure that the differences in Gal4DBD/HIF-1α CAD activity invoked by the treatments or mutations were not due to differences in protein stability, whole cell extracts from similarly transfected and treated cells were analysed by immunoblotting with antibodies to HIF-1α (figure 4.4.B). HIF-1α CAD protein levels were not significantly altered by a reduction in oxygen tension, DMOG treatment or the mutation of N803 to alanine. It is thus concluded that HIF-1α CAD activity is elicited by

Figure 4.4. Treatment of PC12 cells with the 2-oxoglutarate competitor DMOG causes the transactivation of the HIF-1α CAD.

A. PC12 cells were transiently transfected with a firefly luciferase reporter gene (LUC) containing 5 copies of the GRE (pGRE) in addition to mammalian expression constructs containing the cDNA of either of the following: the Gal4 DNA binding domain (pGal4DBD), the Gal4DBD fused to amino acids 737-826 of HIF-1α (pGal4DBD/HIF-1αCAD) or the Gal4DBD fused to HIF-1α CAD with a mutation of N803 to A (pGal4DBD/HIF-1αCADN803A). 6 hours post transfection, cells were exposed to 20% O_2, <1% O_2 or 1 mM DMOG. After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of two independent experiments.

B. PC12 cells were transiently transfected with mammalian expression constructs pGal4DBD, pGal4DBD/HIF-1αCAD or pGal4DBD/HIF-1αCADN803A. 6 hours post transfection, cells were exposed to 20% O_2, <1% O_2 or 1 mM DMOG. After 16 hours, whole cell extracts were prepared. 20 μg of each were analysed via immunoblotting with antibodies to HIF-1α. Results are representative of two independent experiments.


A
pGRE:
5XGRE
E1b
LUC
20% O2
<1% O2
DMOG
RELATIVE LUCIFERASE UNITS
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
pGal4DBD
pGal4DBD/ HIF-1αCAD
pGal4DBD/ HIF-1αCAD N803A
CONSTRUCT


B
pGal4DBD
pGal4DBD/ HIF-1αCAD
pGal4DBD/ HIF-1αCAD N803A
20% O2
<1% O2
DMOG
20% O2
<1% O2
DMOG
20% O2
<1% O2
DMOG
37 kDa
Gal4DBD/ HIF-1α CAD

ablating HIF-1α asparaginyl hydroxylase activity, whether via hypoxic treatment, 2-oxoglutarate competition or N803A mutation and not by any changes in protein levels.

With respect to full length HIF-α, its accumulation and transcriptional potency can be invoked in normoxia via the forced expression of the relevant introduced gene, probably as a consequence of the saturation of the HIF-α prolyl-4- and asparaginyl hydroxylases that would otherwise regulate the function of the endogenous protein. This concept formed the basis of the system adopted in the previous chapter to analyse differential target gene specificity of HIF-1α and HIF-2α in the PC12 cell line. Indeed, HIF-2α overexpression elicited both selective stabilisation and HRE reporter activity of the product (see chapter 3.3, 3.4.1 and 3.4.3). Interestingly, this was not the case with HIF-1α where an N803A mutation was required to ensure that the overexpressed protein stabilised in normoxia still displayed a constitutive activation of an HRE reporter (chapter 3.4.5). This was not altogether surprising since it has been reported that, unlike HIF-2α, HIF-1α protein which has been overexpressed and stabilised in normoxia still requires hypoxia for full transcriptional activity. More specifically, in Hep3B cells, at high doses of transfected plasmid (which would probably reflect what is observed in the best expressing doxycycline inducible stable PC12 cell line), Gal4DBD/HIF-2α 19-870 demonstrated higher normoxic and lower hypoxia inducible (almost none) GRE mediated reporter activity than the equivalent Gal4DBD/HIF-1α 28-826, which displays almost no activity at normal oxygen levels but shows a marked increase in reporter when O_2 is reduced (O'Rourke *et al.*, 1999). A possible explanation for this is the reduced ability of FIH-1 to hydroxylate asparagines within peptides derived from the HIF-2α sequence containing the crucial N when compared to the corresponding residues from HIF-1α (Koivunen *et al.*, 2004).

Therefore, the fact that HIF-1α protein which has been overexpressed and stabilised in normoxia still displays a high hypoxia inducibility of its transcriptional activity (O'Rourke *et al.*, 1999) may explain the poor doxycycline inducibility of HRE reporter activity in PC12/TetON/HIF-1α/EYFP cells described in the previous chapter. In light of its effect on Gal4DBD/HIF-1α CAD, it was proposed that cotreatment with DMOG would inhibit FIH-1 hydroxylation of the doxycycline inducible HIF-1α protein, resulting in enhanced transcriptional potency. It was also expected that protein levels would not change as DMOG does not appear to stabilise either HIF-α in PC12 cells.

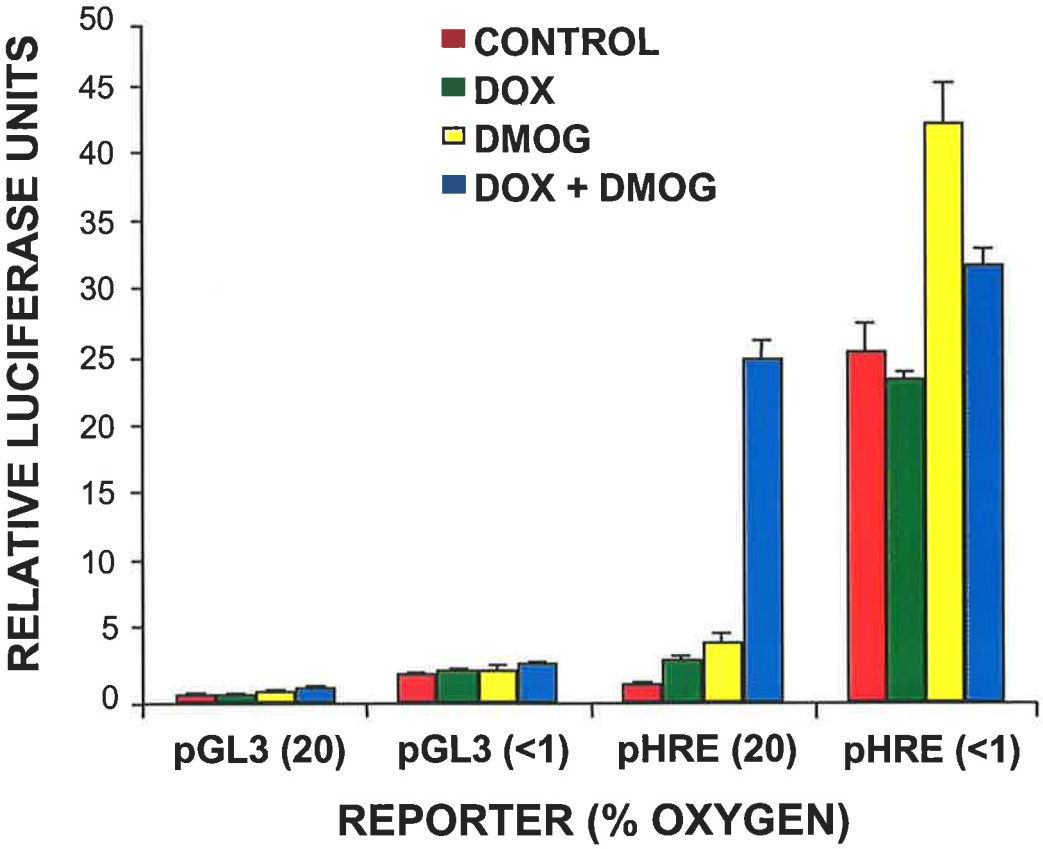
To test this, PC12/TetON/HIF-1α/EYFP cells were transfected with pHRE or pGL3 reporters. 6 hours post transfection, cells were treated with 2 μg/mL doxycycline and/or 1mM DMOG or left untreated and exposed to normoxia or hypoxia. After 16 hours, cells were assayed for firefly luciferase activity against that from cotransfected pRLTK (figure 4.5.A). As expected, addition of doxycycline or DMOG resulted in only slightly enhanced HRE reporter activity in normoxia (compare pHRE + DOX or pHRE + DMOG versus pHRE at 20% O_2). However, cotreatment with both doxycycline and DMOG caused a superinduction of HRE mediated reporter activity (pHRE + DOX + DMOG at 20%O_2). The results of the reporter assay could most likely be explained by DMOG's ability to enhance the transactivation of the doxycycline inducible HIF-1α rather than protein levels. To confirm this, whole cell extracts from similarly treated cells were immunoblotted with antibodies to HIF-1α or HIF-2α (figure 4.5.B). HIF-2α protein was induced only as a consequence of exposure to hypoxia (lower panel), regardless of doxycycline and/or DMOG exposure. This reconfirmed the previous conclusions that neither doxycycline (chapter 3.4.1) nor DMOG stabilised endogenous HIF-α nor could the accumulation of one overexpressed HIF-α result in the residual stabilisation of the other (chapter 4.1). Most importantly, any HRE reporter activity in the doxycycline inducible HIF-1α PC12 line in normoxia could not be attributed to HIF-2α. Regarding HIF-1α (upper panel), doxycycline caused a marked accumulation of the doxycycline inducible HIF-1α gene product to a point where levels were equivalent to those of endogenous hypoxically inducible HIF-1α (compare + DOX with untreated and <1% O_2). The poor doxycycline inducible HRE mediated reporter activity displayed by this derivative of PC12 cells can therefore not be accredited to an inability to accumulate sufficient quantities of HIF-1α. As expected, DMOG caused no stabilisation of HIF-1α. Also, cotreatment with doxycycline and DMOG caused no change in doxycycline inducible HIF-1α levels (compare + DOX versus + DOX + DMOG). As the same cotreatment resulted in a superinduction of HRE reporter activity when compared to individual exposure of PC12/TetON/HIF-1α/EYFP cells to these agents, it confirms that the low activity of the doxycycline inducible HIF-1α is due to poor transactivation and not impaired protein stabilisation. In other words, the inhibitors of transcriptional potency (it appears only FIH-1) are not saturated, whereas the degradative machinery is. Whether this consists of the PHD enzymes is still unclear as it does not appear to be inhibited by 2-oxoglutarate competition, despite the fact that 2-oxoglutarate competition can hinder the

Figure 4.5. In PC12/TetON/HIF-1α/EYFP cells, HIF-1α but not HIF-2α is stabilised by the addition of doxycycline, although there is only a negligible increase in HRE reporter activity. However, treatment with the 2-oxoglutarate competitor DMOG causes a superinduction of doxycycline inducible HRE reporter activity without any change in HIF-1α stability.

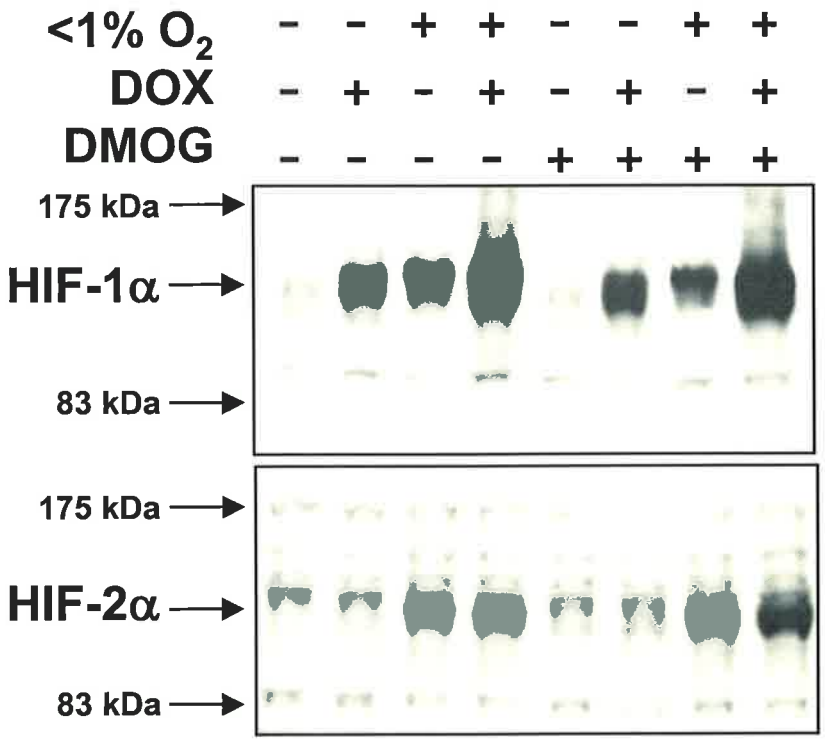
A. Cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX) with or without additional treatment with 1 mM DMOG. After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of two separate experiments.

B. Whole cell extracts were prepared from cells exposed to 20% O_2 or <1% O_2 in the presence or absence of 2 μg/mL doxycycline (DOX) with or without additional treatment with 1 mM DMOG for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to HIF-1α (upper panel) or HIF-2 α (lower panel). Results are representative of two separate experiments.

A


RELATIVE LUCIFERASE UNITS
50
45
40
35
30
25
20
15
10
5
0
CONTROL
DOX
DMOG
DOX + DMOG
pGL3 (20)
pGL3 (<1)
pHRE (20)
pHRE (<1)
REPORTER (% OXYGEN)

B


<1% O2
DOX
DMOG
- - + + - - + +
- + - + - + - +
- - - - + + + +
175 kDa
HIF-1α
83 kDa
175 kDa
HIF-2α
83 kDa

function of another HIF-α hydroxylase in PC12 cells (as exemplified by DMOG induced activation of HIF-1α activity presumably via ablated FIH-1 function).

In summary, it appears that in PC12 cells DMOG can inhibit FIH-1 by competing with 2-oxoglutarate, resulting in no hydroxylation of N803. This permits association of the HIF-1α CAD with CBP/p300 and subsequent transactivation. The classical FIH-1 initiated inhibition of HIF-α transcriptional activity is thus conserved in PC12 cells. It is also possible to conclude that DMOG is functional in PC12 cells. Its failure, along with that of iron chelation, to stabilise HIF-2α protein thus reconfirms the suspicion of the absence of a role for PHD activity in initiating the destruction of HIF-2α. With regards to HIF-1α, this leaves two possibilities. Either an iron dependent mechanism separate from prolyl-4-hydroxylation is involved in its oxygen dependent degradation or PHD activity is involved but is not dependent on 2-oxoglutarate. If the latter is the case, then this suggests that a cosubstrate other than 2-oxoglutarate is involved.

4.4. EFFECT OF HIF-α PROLYL-4-HYDROXYLASES ON HIF-1α EXPRESSION IN PC12 CELLS

4.4.1. HIF-α PROLYL-4-HYDROXYLASE 1, 2 AND 3 mRNA ARE EXPRESSED IN PC12 CELLS

Before further exploring the relevance of HIF-α prolyl-4-hydroxylase activity to PC12 cells, it was necessary to demonstrate whether or not PHD1, PHD2 and/or PHD3 were expressed in this line. Primers were designed to amplify nucleotides 32-591 of rat PHD1 (GenBank accession number AY229997), 113-666 of rat PHD2 (GenBank accession number AY228140), 191-879 of rat PHD3 (GenBank accession number NM_019371) and β actin (see chapter 3.5.2). These were employed to perform RTPCR on mRNA derived from PC12 cells. In short, cDNA was derived from reverse transcription of poly A RNA purified from PC12 cells exposed to normoxia or hypoxia for 16 hours. The cDNA (or water as a negative control) provided the template for PCR reactions employing the above primers (figure 4.6.A). PHD1 mRNA was expressed, although its levels were not altered by hypoxia when compared to those of β actin. This reflects what has been seen in previous studies, albeit into other cell lines (Epstein *et al.*, 2001; Metzen *et al.*, 2003a; del Peso *et al.*, 2003; D'Angelo *et al.*, 2003). However, PHD2 and PHD3 showed hypoxic induction in their mRNA levels when compared to β actin (compare <1% O_2 versus 20%

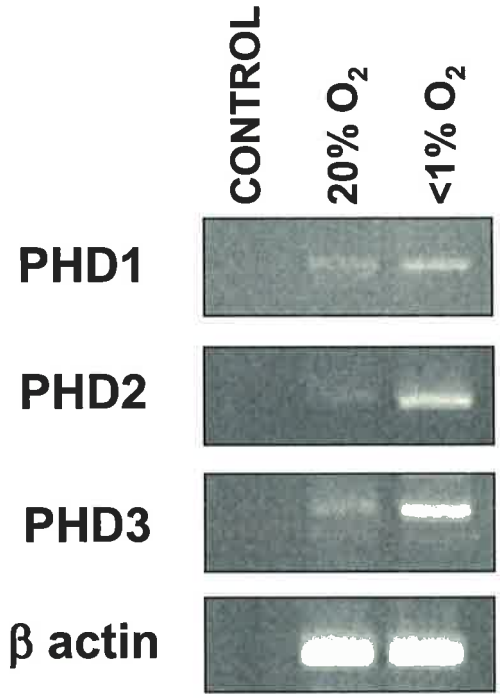
Figure 4.6. HIF-α prolyl-4-hydroxylase (PHD) 1 mRNA and protein remains at constant levels in PC12 cells regardless of oxygen levels, whereas those of PHD2 and PHD3 are increased in hypoxia.

A. cDNA was prepared from reverse transcription of 200 ng of poly A RNA extracted from PC12 cells exposed to 20% O_2 or <1% O_2 for 16 hours. 1 μL of this cDNA (20 μL total) was used as a template in a PCR reaction employing primers designed to amplify a portion of the sequences of PHD1, PHD2, PHD3 or β actin. Reactions were electrophoresed through a 2% agarose/TBE/ethidum bromide gel. Results are representative of two independent experiments.

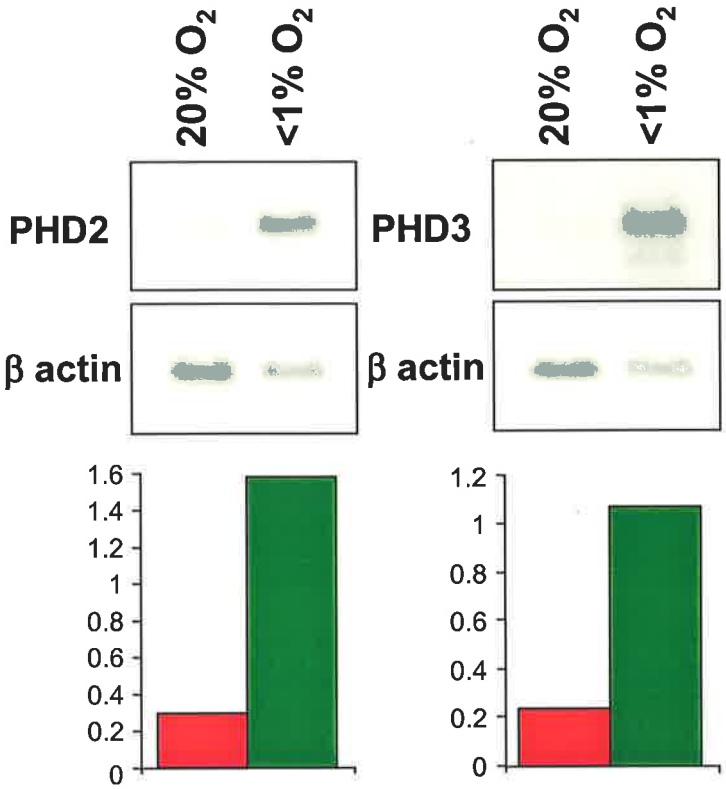
B. Poly A RNA was prepared from PC12 cells exposed to 20% O_2 or <1% O_2 for 16 hours. 3 μg of each were analysed by blotting with a ^{32}P labelled cDNA probe to PHD2 or PHD3. Hybridisation was normalised against that of a cDNA probe for β actin.

C. Whole cell extracts were prepared from cells exposed to 20% O_2 or <1% O_2 for 16 hours. 10 μg of each were analysed by immunoblotting with antibodies to PHD1, PHD2, PHD3 or ARNT. Results are representative of two independent experiments.

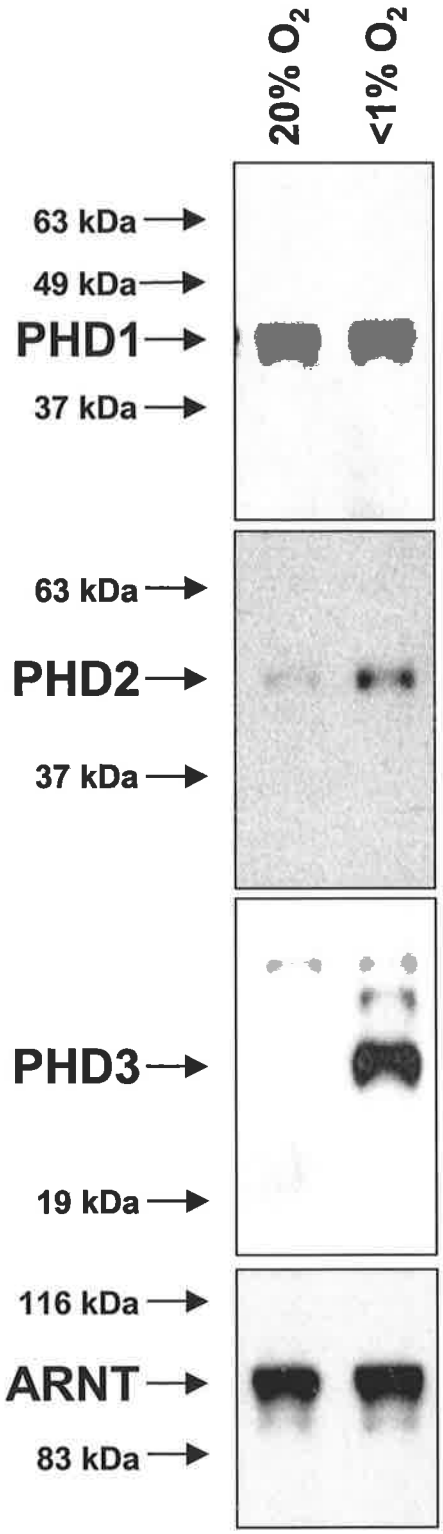
A


CONTROL
20% O2
<1% O2
PHD1
PHD2
PHD3
β actin

B


20% O2
<1% O2
20% O2
<1% O2
PHD2
PHD3
β actin
β actin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.2
1
0.8
0.6
0.4
0.2
0

C


20% O2
<1% O2
63 kDa
49 kDa
PHD1
37 kDa
63 kDa
PHD2
37 kDa
PHD3
19 kDa
116 kDa
ARNT
83 kDa

O_2). Again, this has been recorded in previously published studies (Epstein *et al.*, 2001; Metzen *et al.*, 2003a; del Peso *et al.*, 2003). Finally, all reaction products were of the estimated size when compared to the DNA markers. However, to confirm that the fragments were the coding regions that the primers were designed to amplify, the bands were excised from the gel, purified, cloned into pGEMTEASY (hereafter referred to as pGEM/PHD1, pGEM/PHD2 and pGEM/PHD3, respectively) and sequenced. Indeed, all were of the expected sequence corresponding to the relevant PHD. The rat PHD1 and rat PHD2 sequences in GenBank are unfortunately not of the complete cDNA. Using the known sequences, however, rat PHD1 has a cDNA sequence similarity of 85% and an amino acid sequence similarity of 86% when compared with the human sequence using ClustalW (GenBank accession number NM_053056). For PHD2, this is 88% and 93%, respectively when compared with the human variant (GenBank accession number NM_022051) and for rat PHD3 it is 91% and 96% (GenBank accession number NM_022073).

To validate the results obtained from the RTPCR with respect to the hypoxia inducibility of PHD2 and PHD3, the poly A RNA prepared from PC12 cells treated as described above was analysed by blotting with ^{32}P labelled cDNA probes to PHD2 or PHD3, which in turn were generated from the insert released from the *Eco*RI digestion of pGEM/PHD2 and pGEM/PHD3, respectively. Hybridisation was normalised against that of a cDNA probe for β actin (figure 4.6.B). When contrasted with β actin levels, PHD2 and PHD3 mRNA were hypoxically inducible (compare <1% O_2 versus 20% O_2). The results from the Northern analysis thus replicated those from the RTPCR, as well as those presented in a recent study involving quantitative RTPCR of total RNA from hypoxic and normoxic PC12 cells (Naranjo-Suárez *et al.*, 2003).

Finally, whole cell extracts were prepared from PC12 cells exposed to normoxia or hypoxia for 16 hours and analysed via immunoblotting with antibodies to PHD1, PHD2, PHD3 or the aryl hydrocarbon receptor nuclear translocator (ARNT) (4.6.C). ARNT is ubiquitously expressed in mammalian cells and maintained at constant levels regardless of oxygen tension (Kallio *et al.*, 1997). When contrasted with ARNT, PHD1 levels were not influenced by a reduction in oxygen, whereas PHD2 and PHD3 protein were markedly increased in hypoxia (compare <1% O_2 versus 20% O_2).

Collectively, these results demonstrate that PHD1, PHD2 and PHD3 mRNA and protein are present in PC12 cells and that PHD2 and PHD3 are hypoxically inducible.

4.4.2. RNA INTERFERENCE OF HIF-α PROLYL-4-HYDROXYLASE ACTIVITY STABILISES HIF-1α PROTEIN IN PC12 CELLS

Silencing of PHD2 via RNA interference (RNAi) alone has been shown to be sufficient to induce HIF-1α protein and activity in a number of cell lines, whereas this was not the case with PHD1 or PHD3 (Berra *et al.*, 2003). It was decided therefore to demonstrate whether or not this was also the case in the PC12 cell line.

RNAi is a term used to describe a method of posttranscriptional gene silencing. It is a sequence specific mechanism initiated by double stranded RNA (dsRNA) which is homologous to the targeted gene (Hannon and Rossi, 2004). The effective mediators of RNAi are 21 to 22 nucleotide dsRNA oligonucleotides which themselves can be generated from the cleavage of longer dsRNA molecules (Elbashir *et al.*, 2001a). Indeed, in mammalian cells, RNAi can be elicited via the introduction of exogenous 21 nucleotide dsRNA with 2 nucleotide 3' overhangs (Elbashir *et al.*, 2001b). With this in mind, it was decided to utilise the mammalian expression plasmid pSUPER into which can be subcloned annealed oligonucleotides. These consist of a common hairpin loop which in turn divides two self complementary sequences derived from the mRNA of the gene to be targeted, the second of which is immediately followed by a transcription stop sequence. In this situation, rather than introducing synthesised RNA, plasmid DNA is transiently transfected into mammalian cells. The introduced oligonucleotide is transcribed under the control of the H1 RNA promoter. The selfcomplementary sequences hybridise to each other and result in the formation of a short hairpin RNA which is processed to form the effector dsRNA molecule (Hannon and Rossi, 2004).

The rat PHD2 (GenBank accession number AY228140) sequences were analysed by using an RNAi designing program (http://sirna.qiagen.com). Criteria include a lack of homology to any other rat sequence and the presence of two sequential adenines in the 5' region. In total, four pSUPER constructs were designed: pSUPER/PHD2 112 (to target nucleotides 112-132), pSUPER/PHD2 126 (to target nucleotides 126-146), pSUPER/PHD2 542 (to target 542-562) and pSUPER/PHD2 554 (to target 554-574). In addition, a negative control whose self complementary regions demonstrated no

significant homology to any known rat, human or mouse sequence was also generated (pSUPER/SCR). The purpose of this construct was to ensure that any changes in PHD2 levels and/or any downstream effects (notably changes in HIF-α levels) were not due to nonspecific effects of dsRNA. Once constructed, it was of prime importance to first test whether any of these upon transient transfection were sufficient to downregulate PHD2 protein expression and if so which were the most efficient.

PC12 cells were transfected with the negative control mammalian expression plasmid pSUPER/SCR (SCR) or the PHD2 targeted variants (PHD2 112, 126, 542 or 554). Whole cell extracts were prepared 48 hours post transfection and were analysed via immunoblotting with antibodies to PHD2 or ARNT (figure 4.7.A). In addition to the fact that ARNT mRNA should not serve as a target of the PHD2 RNAi sequences, its protein levels should not alter as a consequence of any changes in PHD2 activity. Transfection with the PHD2 directed constructs caused at least some PHD2 protein downregulation (compare SCR versus PHD2 112, 126, 542 and 554 on the PHD2 immunoblot and with the ARNT immunoblot). Introduction of the pSUPER/PHD2 542 and 554 into PC12 cells mediated the most marked reduction in PHD2 protein levels, so these were used in the subsequent experiment which was designed to test for the effect of PHD2 downregulation on HIF-1α and HIF-2α protein levels.

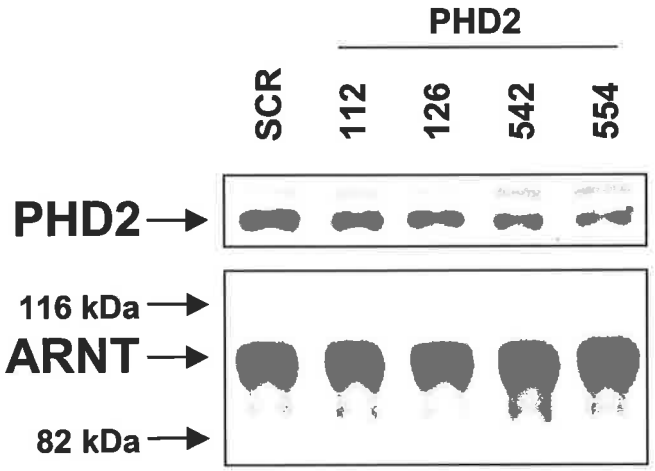
Therefore, PC12 cells were transfected with the negative control mammalian expression plasmid SCR or one of the two selected PHD2 targeted variants (PHD2 542 or 554). 32 hours post transfection cells were exposed to normoxia or hypoxia. 16 hours later, whole cell extracts were prepared and analysed via immunoblotting with antibodies to PHD2, HIF-1α, HIF-2α or ARNT (figure 4.7.B). PHD2 was again demonstrated to be hypoxically inducible (compare SCR at 20% O_2 versus SCR at <1% O_2 on the PHD2 immunoblot). Transfection with the PHD2 directed constructs resulted in PHD2 protein downregulation regardless of oxygen tension (compare SCR versus PHD2 542 and 554 on the PHD2 immunoblot and with the ARNT immunoblot, both at 20% O_2 and <1% O_2). Furthermore, it resulted in a significant (albeit modest) increase in HIF-1α (compare SCR versus PHD2 542 and 554 at 20% O_2 on the HIF-1α immunoblot) but not HIF-2α protein in normoxia (compare the same lanes on the HIF-2α immunoblot) or ARNT. The RNAi of PHD2 and resulting decrease in its protein caused no apparent change in either HIF-α protein in hypoxia, presumably because there was inadequate oxygen to permit PHD2

Figure 4.7. Inhibition of PHD2 via RNAi interference promotes HIF-1α but not HIF-2α protein stabilisation.

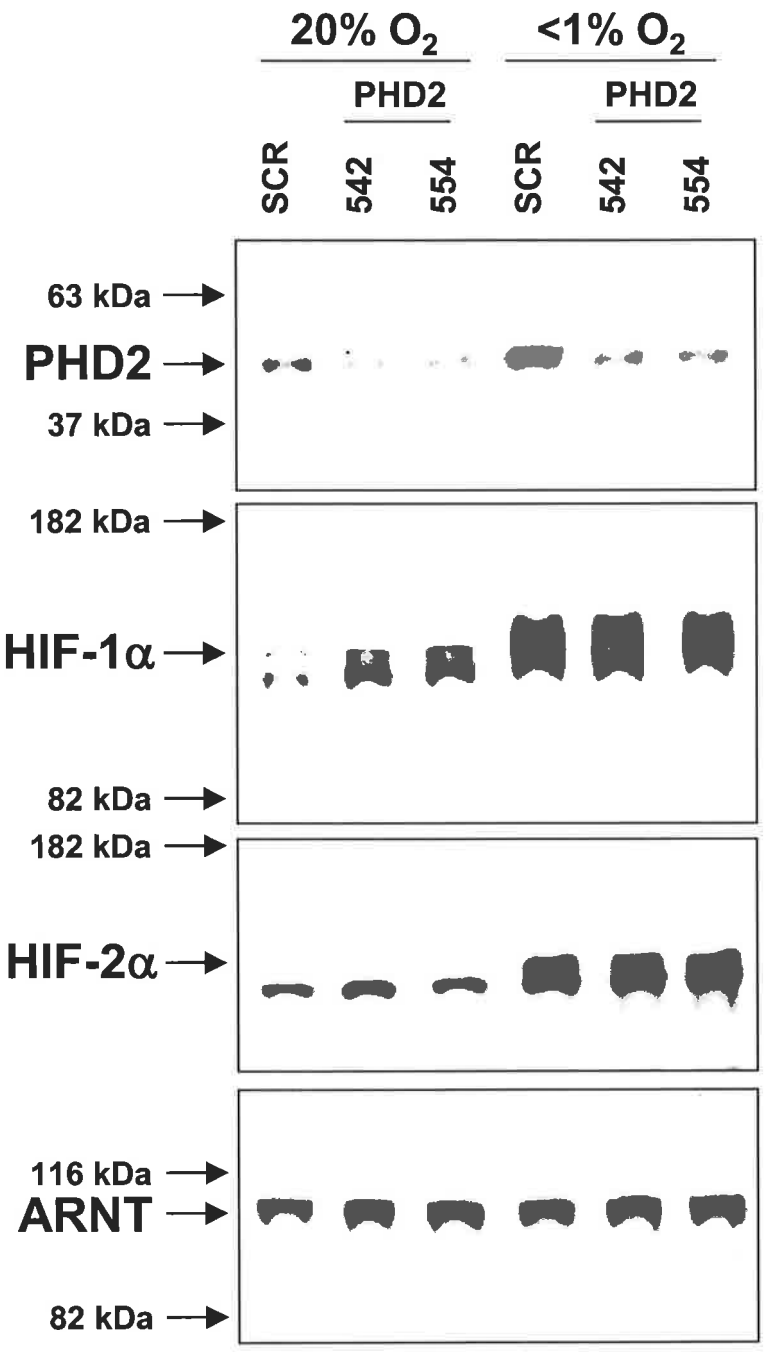
A. PC12 cells were transiently transfected with either a mammalian expression construct encoding a selfcomplementary transcript that is not homologous to any sequence in the rat genome (SCR) or that which is specific to sequences within PHD2 (PHD2 112, 126, 542 or 554). 48 hours post transfection, whole cell extracts were prepared. 10 μg of each were analysed by immunoblotting with antibodies to either PHD2 (upper panel) or the arylhydrocarbon receptor nuclear translocator (ARNT) (lower panel). Results are representative of two independent experiments.

B. PC12 cells were transiently transfected with either a mammalian expression construct encoding a selfcomplementary transcript that is not homologous to any sequence in the rat genome (SCR) or that which is specific to sequences within PHD2 (PHD2 542 or 554). 32 hours post transfection, cells were exposed to 20% O_2 or <1% O_2. 16 hours later, whole cell extracts were prepared. 20 μg of each were analysed by immunoblotting with antibodies to either PHD2 (top panel), HIF-1α (second panel), HIF-2α (third panel) or ARNT (bottom panel). Results are representative of three independent experiments.

A



B


20% O2
<1% O2
PHD2
PHD2
SCR
542
554
SCR
542
554
63 kDa
PHD2
37 kDa
182 kDa
HIF-1α
82 kDa
182 kDa
HIF-2α
116 kDa
ARNT
82 kDa

activity (compare SCR versus PHD2 542 and 554 at <1% O_2 on the HIF-1α and HIF-2α immunoblots).

In short, PHD2 appears to contribute to the degradation of HIF-1α but not HIF-2α in PC12 cells. In other words, PHD activity (as exemplified by that of PHD2, itself the proposed master regulator HIF-1α activity (Berra *et al.*, 2003)) is required for the oxygen dependent degradation of only HIF-1α in PC12 cells, presumably via its subsequent association with the von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex and destruction by the ubiquitin proteasome pathway (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001; Epstein *et al.*, 2001; Bruick and McKnight, 2001). But what of the significance of PHD1 and PHD3 in the regulation of HIF-2α, particularly given that PHD3 has been proposed as the preferential regulator of HIF-2α (Appelhof *et al.*, 2004)? PHD1 and PHD3 activity, like that of PHD2, is still published as iron dependent (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001; Epstein *et al.*, 2001; Bruick and McKnight, 2001). Both iron chelation and PHD2 downregulation can allow for the accumulation of endogenous HIF-1α protein in PC12 cells, so it is unlikely that PHD1 and PHD3 mediate HIF-2α degradation in an iron independent manner. In addition, the ability of all three PHD enzymes when overexpressed to degrade similarly introduced HIF-1α in PC12 cells is iron dependent (chapter 4.4.4). The observation that iron chelation inhibits the activity of all PHD enzymes but fails to induce HIF-2α protein thus provides good evidence that prolyl-4-hydroxylation does not promote HIF-2α destruction in PC12 cells. That PHD2 downregulation does not stabilise HIF-2α is consistent with the lack of a role for prolyl-4-hydroxylation in the degradation of HIF-2α. However, pertaining to HIF-1α, HIF-α prolyl-4-hydroxylase activity (as exemplified by that of PHD2) does indeed appear to be involved in its oxygen dependent degradation. Furthermore, it appears that a cosubstrate other than 2-oxoglutarate is involved, given the observations in this thesis where the treatment of PC12 cells with DMOG fails to result in the accumulation of HIF-1α protein.

4.4.3. OVEREXPRESSED HIF-α PROLYL-4-HYDROXYLASES CAN INHIBIT THE ACCUMULATION OF OVEREXPRESSED HIF-1α

As explained above, HIF-α stability in normoxia can be attained as a consequence of its forced expression. It is assumed that the PHD enzymes or other crucial factors that mediate the rapid destruction of the endogenous protein in normoxia become saturated.

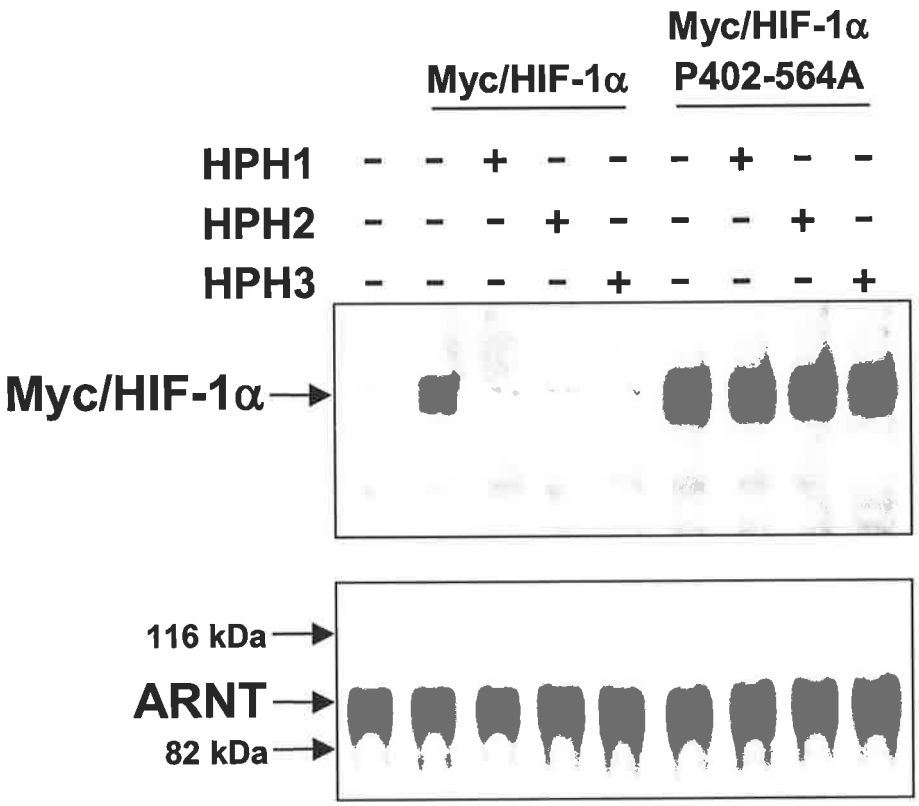
Consequently, some protein escapes targeting for degradation. This balance can be reversed by overexpressing any of PHD enzymes. To further test the functionality of the HIF-α prolyl-4-hydroxylases in PC12 cells with respect to reducing HIF-1α levels in normoxia, it was decided to force the accumulation of HIF-1α by overexpression and to examine the effects of coexpression of either of the PHD enzymes.

This entailed the employment of mammalian expression vectors capable of expressing either wildtype HIF-1α or a variant where the two prolines were mutated to prevent hydroxylation mediated degradation (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001; Masson *et al.*, 2001). One encoding wildtype HIF-1α (pEF/HisMycHIF-1α/IRES/PURO) was at our disposal, although a similar construct from which the mutant HIF-1α could be expressed was still required. HIF-1α P402-564A was thus subcloned into the mammalian expression vector pEF/IRES/PURO, resulting in the generation of pEF/HIF-1αP402-564A/IRES/PURO. This allows for control of the transcription of the HIF-1α cDNA by the elongation factor 1α (EF-1α) promoter. To facilitate its detection, HIF-1α P402-564A was fused to 6 copies of the epitope to the anti Myc monoclonal antibody 9E10 (as well as 6 consecutive histidines to facilitate purification if necessary for future studies). This gave rise to pEF/HisMycHIF-1αP402-564A/PURO. With regards to the Myc tag, although adequate HIF-1α polyclonal antibodies were at our disposal, it was deemed necessary to permit the differentiation between introduced and endogenous HIF-1α, particularly given that it was intended that DP, which induces endogenous HIF-1α in PC12 cells, was to be used.

To test PHD activity on introduced HIF-1α, PC12 cells were transfected with either pEF/IRES/PURO, pEF/HisMycHIF-1α/IRES/PURO or pEF/HisMycHIF-1αP402-564A/PURO. With regards to the latter two, these were cotransfected with the mammalian expression vector (pcDNA3.1) into which was subcloned the human cDNA for HPH1, HPH2 or HPH3 (corresponding to PHD3, PHD2 and PHD1, respectively (Seta *et al.*, 2002)). 24 hours post transfection, whole cell extracts derived from these cells were analysed by immunoblotting with antibodies to either the Myc epitope or ARNT (figure 4.8). As expected, Myc/HIF-1α was upregulated upon its overexpression in normoxia, although this was not so when Myc/HIF-1α was cotransfected with constructs encoding either HPH (compare Myc/HIF-1α versus Myc/HIF-1α + either HPH1, HPH2 or HPH3). However, levels of overexpressed Myc/HIF-1α P402-564A were not altered via the

Figure 4.8. HIF-α prolyl-4-hydroxylases introduced into PC12 cells can inhibit the accumulation of cotransfected HIF-1α in normoxia but not when the target prolines are mutated to alanines.

PC12 cells were transiently transfected with either pEF/IRES/PURO, pEF/HisMycHIF-1α/IRES/PURO (Myc/HIF-1α) or pEF/HisMycHIF-1αP402-564A/PURO (Myc/HIF-1α P402-564A). With regards to the latter two, these were cotransfected with mammalian expression vectors pcDNA3.1/HPH1, (HPH1) pcDNA3.1/HPH2 (HPH2) or pcDNA3.1/HPH3 (HPH3) (which encode PHD3, PHD2 and PHD1, respectively). 24 hours post transfection, whole cell extracts were prepared. 10 μg of these were analysed by immunoblotting with antibodies to either the Myc epitope (upper panel) or the arylhydrocarbon receptor nuclear translocator (ARNT) (lower panel). Results are representative of two independent experiments.


Myc/HIF-1α
P402-564A
Myc/HIF-1α
HPH1 - - + - - - + - -
HPH2 - - - + - - - + -
HPH3 - - - - + - - - +
Myc/HIF-1α
116 kDa
ARNT
82 kDa

introduction of HPH activity (compare Myc/HIF-1α P402-564A versus Myc/HIF-1α P402-564A + either HPH1, HPH2 or HPH3). Collectively, this data suggests that the PHD enzymes can initiate the degradation of HIF-1α in PC12 cells via hydroxylation of the two prolines known to serve as targets of PHD activity.

4.4.4. IRON CHELATION BUT NOT 2-OXOGLUTARATE COMPETITION INHIBITS OVEREXPRESSED HIF-α PROLYL-4-HYDROXYLASE MEDIATED DEGRADATION OF OVEREXPRESSED HIF-1α

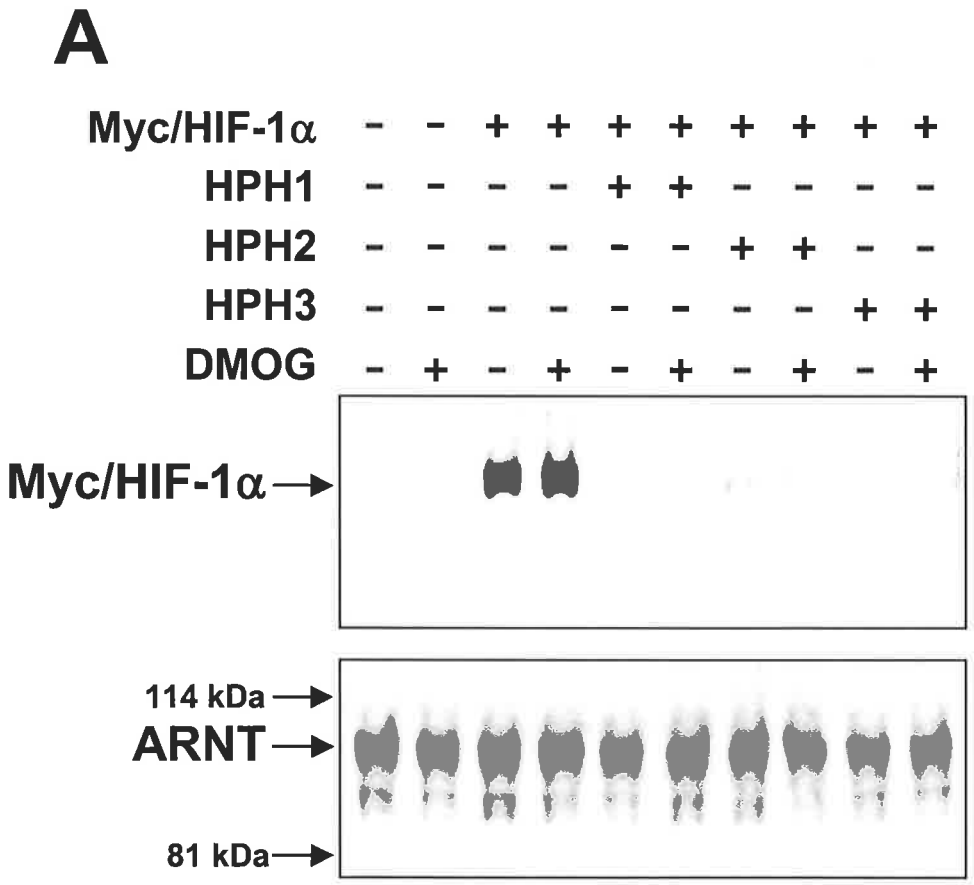
The preceding results allow for two possible scenarios. One is that HIF-α prolyl-4-hydroxylase activity can be introduced into PC12 cells although the endogenous PHDs are themselves inactive. If this is the case, it would be expected that DMOG should reverse the reduction of overexpressed Myc/HIF-1α levels by introduced PHD. However, in this scenario, it is unlikely that HIF-1α accumulation would have occurred as a consequence of the RNAi of PHD2 (figure 4.7.B). Conversely, as suggested previously, it is plausible that PHD activity can function independently of 2-oxoglutarate. In this situation, 2-oxoglutarate competition via DMOG treatment should not inhibit the reduction of Myc/HIF-1α levels by cotransfected or endogenous PHD.

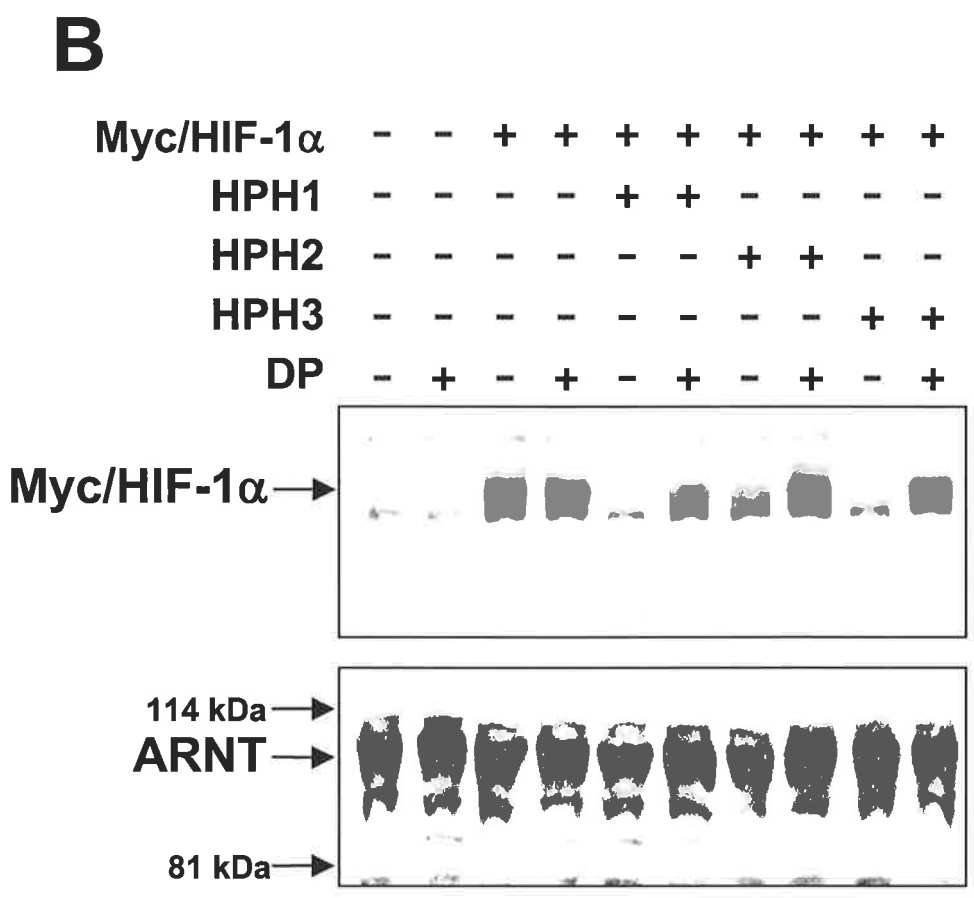
To ascertain which situation was occurring, PC12 cells were transfected with either pEF/IRES/PURO or pEF/HisMycHIF-1α/IRES/PURO, with the latter being cotransfected with mammalian expression vectors pcDNA3.1/HPH1, pcDNA3.1/HPH2 or pcDNA3.1/HPH3. 6 hours post transfection, cells were treated with 1mM DMOG or 100 μM DP or left untreated. After 16 hours, whole cell extracts derived from these cells were analysed by immunoblotting with antibodies to either the Myc epitope or ARNT. (Results from DMOG treated cells are shown in figure 4.9.A, whereas those from DP treated cells are displayed in 4.9.B). DMOG was clearly unable to alter the levels of Myc/HIF-1α that is accumulated in these cells (compare Myc/HIF-1α versus Myc/HIF-1α + DMOG). Furthermore, DMOG was unable to reverse the degradation of Myc/HIF-1α resulting from the introduction of prolyl-4-hydroxylase activity (compare Myc/HIF-1α + HPH1, 2 or 3 versus Myc/HIF-1α + HPH1, 2 or 3 + DMOG). However, although iron chelation in the form of DP treatment was also unable to further stabilise overexpressed Myc/HIF-1α (compare Myc/HIF-1α versus Myc/HIF-1α + DP), it did have the capacity to enhance the stabilisation of Myc/HIF-1α in the presence of cotransfected prolyl-4-hydroxylase (compare Myc/HIF-1α + HPH1, 2 or 3 versus Myc/HIF-1α + HPH1, 2 or 3 + DP).

Figure 4.9. Treatment of PC12 cells with the iron chelator DP but not the 2-oxoglutarate competitor DMOG reverses HIF-α prolyl-4-hydroxylase mediated initiated degradation of cotransfected HIF-1α in normoxia.

A. PC12 cells were transiently transfected with either pEF/IRES/PURO or pEF/HisMycHIF-1α/IRES/PURO (Myc/HIF-1α). The latter was also cotransfected with mammalian expression vectors pcDNA3.1/HPH1, (HPH1) pcDNA3.1/HPH2 (HPH2) or pcDNA3.1/HPH3 (HPH3) (which encode PHD3, PHD2 and PHD1, respectively). 6 hours post transfection, cells were left untreated or exposed to 1 mM DMOG. 16 hours later, whole cell extracts were prepared. 20 μg of these were analysed by immunoblotting with antibodies to either the Myc epitope (upper panel) or the arylhydrocarbon receptor nuclear translocator (ARNT) (lower panel). Results are representative of three independent experiments.

B. As above, except cells were exposed to 100 μM DP. Results are representative of three independent experiments.


A
Myc/HIF-1α - - + + + + + + + +
HPH1 - - - - + + - - - -
HPH2 - - - - - - + + - -
HPH3 - - - - - - - - + +
DMOG - + - + - + - + - +
Myc/HIF-1α
114 kDa
ARNT
81 kDa


B
Myc/HIF-1α - - + + + + + + + +
HPH1 - - - - + + - - - -
HPH2 - - - - - - + + - -
HPH3 - - - - - - - - + +
DP - + - + - + - + - +
Myc/HIF-1α
114 kDa
ARNT
81 kDa

The ability of DP to interfere with the overexpressed prolyl-4-hydroxylase mediated degradation of Myc/HIF-1α reflects the ability of iron chelation to cause the stabilisation of endogenous HIF-1α in PC12 cells. Furthermore, the fact that DMOG treatment does not reverse Myc/HIF-1α destruction resulting from cotransfection with the PHD enzymes suggests a peculiar reason for DMOG's incapacity to induce endogenous HIF-1α protein in PC12 cells. That is, although prolyl-4-hydroxylation appears to still target HIF-1α for eradication, a 2-oxoglutarate analogue is unable to inhibit these hydroxylases. A potential scenario is that a cosubstrate other than 2-oxoglutarate is involved. One also cannot rule out the possibility that the PHD enzymes have a substantially higher affinity for 2-oxoglutarate than does FIH-1, although this is unlikely to be of a significant difference to account for DMOG's ability to inhibit FIH-1 but not PHD function (see below). Therefore, the evidence suggests that, in the PC12 cell line, oxygen dependent prolyl-4-hydroxylation of two conserved proline residues within HIF-1α permits its association with the pVHL E3 ubiquitin ligase complex and subsequent destruction by the ubiquitin proteasome pathway. Although this process appears to be iron dependent, it is surprisingly 2-oxoglutarate independent.

4.5. DISCUSSION OF HIF-α PROLYL-4-HYDROXYLASE ACTIVITY IN PC12 CELLS

In summary, the data collectively indicates that in the PC12 cell line, oxygen and iron dependent prolyl-4-hydroxylation of two conserved proline residues within HIF-1α permits its association with the pVHL E3 ubiquitin ligase complex and subsequent destruction by the ubiquitin proteasome pathway. The fact that this process is 2-oxoglutarate independent appears to be peculiar to PC12 cells.

A potential technical reason for this is that DMOG has not been used at a sufficiently high concentration. Clearly the 1 mM concentration is adequate to inhibit asparaginyl hydroxylation in PC12 cells as demonstrated by its ability to induce HIF-1α transactivation (figure 4.4 and figure 4.5). However, could it be that FIH-1 has a significantly higher affinity for DMOG than does the HIF-α prolyl-4-hydroxylases? There is some conjecture with regards to the relative affinities of FIH-1 and PHD for NOG (the cell impermeable variant of DMOG). Originally it was shown in *in vitro* kinetic assays of

recombinant FIH-1 that the IC_{50} for NOG is 25 μM (Hewitson *et al.*, 2002), although another group determined the K_i as 2 μM (Koivunen *et al.*, 2004). K_i values of recombinant prolyl-4-hydroxylases for NOG are 50, 8 and 10 μM for PHD1, PHD2 and PHD3, respectively (Hirsilä *et al.*, 2003). The 1 order of magnitude difference between the two FIH-1 studies means that it is difficult to make any conclusions regarding what is seen in the PC12 cell line. However, it is of interest that it has been postulated that PHD2 serves as the master HIF-α prolyl-4-hydroxylase (Berra *et al.*, 2003). PHD2's K_i value for NOG as determined by Hirsilä and coworkers is four times higher than that for FIH-1 as determined by the same group, but about 3 times lower than that measured in the other FIH-1 study (Hirsilä *et al.*, 2003; Koivunen *et al.*, 2004; Hewitson *et al.*, 2002). In other words, one may expect DMOG to be a worse or better inhibitor of PHD2 than FIH-1 depending on the study. At any rate, 1 mM DMOG is recommended for use in cell culture and well above either of the predicted K_i values and the activation of HIF-1α has been seen with 0.1 mM treatment of Hep3B and U2OS cells (Jaakkola *et al.*, 2001). A higher concentration (10 mM DMOG) was attempted in this study, although this caused total cell death (data not shown). Interestingly, DMOG has been used to enhance mRNA levels of the hypoxia inducible genes of the T type voltage gated calcium channel (Del Toro *et al.*, 2003) and glucose-6-phosphate dehydrogenase (Gao *et al.*, 2004) in PC12 cells, but has not been demonstrated to induce HIF-1α itself.

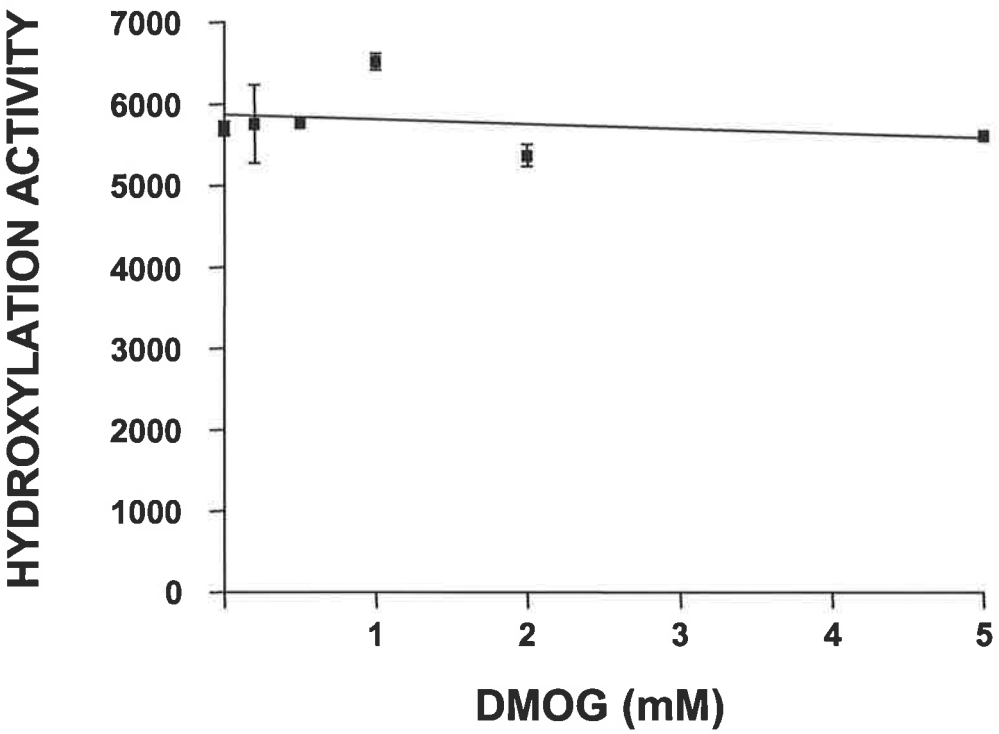
It must once again be mentioned that DMOG is not itself the inhibitor of prolyl-4-hydroxylase activity. In fact, DMOG was originally shown to be able to suppress the generation of hydroxyproline in cells but not the activity of purified prolyl-4-hydroxylases, thus suggesting that it is most probably converted to NOG in the cell prior to its acting as a competitor of 2-oxoglutarate (Baader *et al.*, 1994). Indeed, NOG was used in all studies whereby the relative affinities of purified FIH-1, PHD1, PHD2 and PHD3 for various inhibitors were determined (Hewitson *et al.*, 2002; Hirsilä *et al.*, 2003; Koivunen *et al.*, 2004). Is it possible that FIH-1 but not the HIF-α prolyl-4-hydroxylases can be directly inhibited by DMOG? If so, could DMOG enter PC12 cells but not be converted to NOG and as a consequence be able to inhibit FIH-1 but not PHD activity? Unlike that with respect to prolyl-4-hydroxylases (Baader *et al.*, 1994), the data to suggest or refute this has not been published, but it is important to note that the binding site for 2-oxoglutarate in FIH-1 differs from that of other 2-oxoglutarate dioxygenases, such as the HIF-α prolyl-4-hydroxylases. Normally these enzymes have an RXS motif with which

hydrogen bonds can be formed with the C5 carbon of 2-oxoglutarate, although in FIH-1 this motif is absent and the role of forming the hydrogen bonds is adopted by T196 and K214 (Dann *et al.*, 2003; Lee *et al.*, 2003; Elkins *et al.*, 2003). Therefore, it is possible that there is a sufficient structural difference in FIH-1 compared with the PHD enzymes for 2-oxoglutarate to be competed out with unmodified DMOG. Indeed, FIH-1 treatment inhibited the interaction between ^{35}S labelled HIF-2α 774-874 and the CH1 domain of CBP unless DMOG had been added or the crucial N851 had been mutated (Lando *et al.*, 2002b), although it is possible that in this situation DMOG was converted to NOG due to the presence of reticulocyte lysate. However, in an experiment performed by Wiltiana Barak in our research group (Barak *et al.*, 2004, data not published) employing as substrates bacterially expressed proteins, it was shown that the *in vitro* hydroxylation of HIF-1α 737-826 by FIH-1 was not inhibited by various concentrations of DMOG (figure 4.10). Therefore, a few conclusions can be made. One is that NOG is the effective inhibitor of both FIH-1 and PHD activity, regardless of the context. Secondly, it appears that 2-oxoglutarate competition is capable of inhibiting HIF-α asparaginyl but not prolyl-4-hydroxylase function in PC12 cells.

Given that the inhibition of HIF-α prolyl-4-hydroxylase activity whether by iron chelation or 2-oxoxglutarate competition was not able to promote the accumulation of endogenous HIF-2α in PC12 cells, it appears that the PHDs are not responsible for initiating the degradation of HIF-2α in this line. Somewhat surprisingly, both DFO and DMOG have been shown to promote HIF-2α accumulation in PC12 cells to levels similar to those of hypoxically inducible HIF-2α protein, although the degree of HIF-2α accumulation as a consequence of reduced oxygen was itself quite modest and also difficult to ascertain due to a background band present at the same molecular weight in the immunoblot (Naranjo-Suárez *et al.*, 2003). In this particular study, cells were treated for only 4 hours prior to whole cell extraction as opposed to the 16 hours used in the research described in this thesis. It is thus possible that after 16 hours, DFO and DMOG induced HIF-2α protein accumulation is downregulated once again, which is itself unlikely given the duration of hypoxic treatment necessary for HIF-2α to accrue and reach maximum levels in the cells used in this thesis (chapter 4.2). However, shorter time periods of DP and DMOG treatment were attempted and there was no apparent increase in HIF-2α as identified by immunoblotting (data not shown). Therefore, it is proposed that the inhibition of another

Figure 4.10. DMOG does not inhibit the ability of MBP/FIH-1 to hydroxylate trx/his/HIF-1α CAD in an *in vitro* hydroxylation assay.

An *in vitro* hydroxylation assay was performed by Wiltiana Barak using a standard method (Linke *et al.*, 2004) to detect the ability of purified maltose binding protein/FIH-1 fusion protein (MBP/FIH-1) to hydroxylate a thioredoxin/histidine$_6$/HIF-1α 737-826 (trx/his/HIF-1α CAD) in conditions where all substrates and cofactors are saturating and thus MBP/FIH-1 is operating at V_{max}. Various concentrations of dimethyloxalylglycine (DMOG) were added to the reactions. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of three independent experiments.


HYDROXYLATION ACTIVITY
7000
6000
5000
4000
3000
2000
1000
0
1
2
3
4
5
DMOG (mM)

mechanism is predominant for the oxygen dependent degradation of HIF-2α in the PC12 cell line.

The two crucial prolines within HIF-1α (P402 and P564) are conserved in HIF-2α as P405 and P531 (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Masson *et al.*, 2001). Therefore, it has generally been assumed that, like HIF-1α, oxygen dependent prolyl-4-hydroxylation of two conserved proline residues within HIF-2α permits its association with the pVHL E3 ubiquitin ligase complex and subsequent destruction by the ubiquitin proteasome pathway. Studies into the RCC line 786-O which expresses a non functioning pVHL and constitutively upregulated HIF-2α but no HIF-1α (Maxwell *et al.*, 1999) support this presumption. As mentioned above, in a derivative stably expressing wildtype pVHL and therefore where normoxic downregulation of HIF-2α is restored, hypoxia caused an upregulation of wildtype HIF-2α , but no change in a transfected HIF-2α where P405 and P531 were mutated to alanines (Kondo *et al.*, 2003). In fact, the mutant's normoxic levels in the pVHL expressing line were equivalent to those of wildtype HIF-2α in the parental non functioning pVHL 786-O line (Kondo *et al.*, 2003). This implicates PHD activity in initiating the degradation of HIF-2α. Even in the context of the PC12 cell line where one other study has implicated a degree of differential regulation of the HIF-α proteins, there is no reason to suggest that prolyl-4-hydroxylation is excluded a role in controlling HIF-2α protein levels. That is, it has been shown that treatment of PC12 cells with nerve growth factor (NGF) results in a reduction in normoxic and hypoxic HIF-2α but not HIF-1α protein levels (Naranjo-Suárez *et al.*, 2003). However, this is due to a specific decrease in HIF-2α mRNA stability (Naranjo-Suárez *et al.*, 2003). Therefore, the fact that different oxygen dependent posttranslational modifications may be involved in targeting the respective HIF-α proteins for destruction is itself novel. Interestingly, there is one controversial study using immortalised mouse embryo fibroblasts (MEFs), where HIF-2α was easily detectable regardless of oxygen levels or pVHL presence and primarily cytoplasmic, unlike HIF-1α which demonstrated the classical qualities of hypoxically inducible protein accumulation and nuclear translocation (Park *et al.*, 2003). Other data in the same study suggest that the capacity of HIF-2α to function as a transcription factor in MEFs involves an as yet unknown mechanism to induce its translocation to the nucleus and induce target gene expression (Park *et al.*, 2003). Therefore, the suggestion that HIF-2α protein maintenance involves a peculiar mechanism of regulation is not novel. However, the study by Park and coworkers differs from that described in this thesis in two

respects. Firstly with regards to the cell line (non cancerous mouse fibroblasts versus rat pheochromocytoma), but perhaps most importantly pertaining to the fact that in PC12 cells, unlike the MEFs, HIF-2α is still a hypoxia inducible protein (figure 3.1.A). Therefore, that HIF-2α protein degradation is dependent on oxygen but apparently not prolyl-4-hydroxylation is itself conceptually unusual.

4.6. EFFECT OF TYROSINE KINASE INHIBITORS ON HIF-α EXPRESSION IN PC12 CELLS

It became imperative that other potential forms of oxygen dependent mediated degradation of HIF-2α be examined. Various agents such as the tyrosine kinase inhibitor genistein and the serine/threonine kinase inhibitor 2-aminopurine ablate at least HIF-1α function (Wang *et al.*, 1995a). It has been shown that both HIF-1α (Richard *et al.*, 1999) and HIF-2α (Conrad *et al.*, 1999) are phosphoproteins, although little is known with regards to which residues are phosphorylated and the significance of any of these post translational modifications (chapter 1.5.3). Furthermore, nothing is known with respect to whether phosphorylation plays a role in the differential regulation of HIF-1α and HIF-2α in any context. Given that it appears that HIF-2α protein degradation in the PC12 cell line requires O_2 but not HIF-α prolyl-4-hydroxylation, the question was raised as to whether phosphorylation may contribute to the oxygen dependent regulation of either HIF-α. This is of particular interest as HIF-2α was shown to be a phosphoprotein in PC12 cells (Conrad *et al.*, 1999).

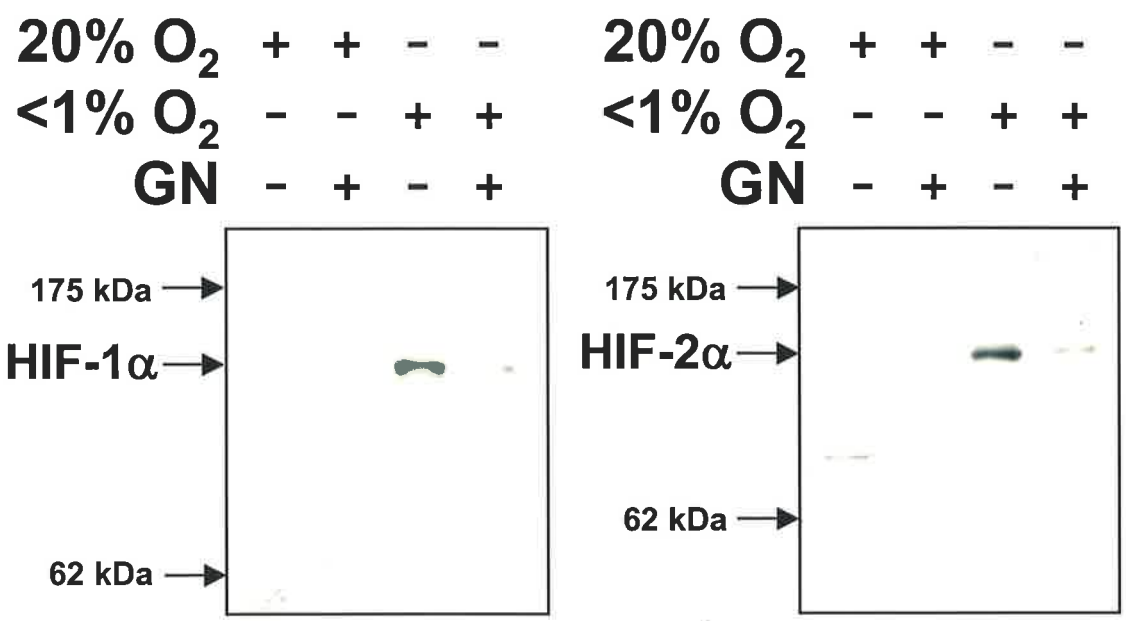
To that end, whole cell extracts were prepared from PC12 cells exposed to normoxia or hypoxia with or without additional exposure to 100 μM genistein for 16 hours. These were analysed by immunoblotting with antibodies to either HIF-1α or HIF-2α (figure 4.11.A). Genistein markedly reduced the levels of both hypoxically induced HIF-1α and HIF-2α protein (compare <1% O_2 + GN versus <1% O_2 in both immunoblots) Therefore, inhibition of tyrosine phosphorylation appears to cause a reduction in HIF-1α and HIF-2α protein in hypoxic PC12 cells. This was further confirmed by the following experiment where PC12 cells were transfected with pHRE or pGL3 reporters. 6 hours post transfection, cells were treated with 100 μM genistein or left untreated and exposed to normoxia or hypoxia. After 16 hours, cells were assayed for firefly luciferase activity against cotransfected pRLTK (figure 4.11.B). Genistein treatment clearly inhibited

Figure 4.11. Tyrosine phosphorylation is required for the activation of both HIF-1α and HIF-2α.

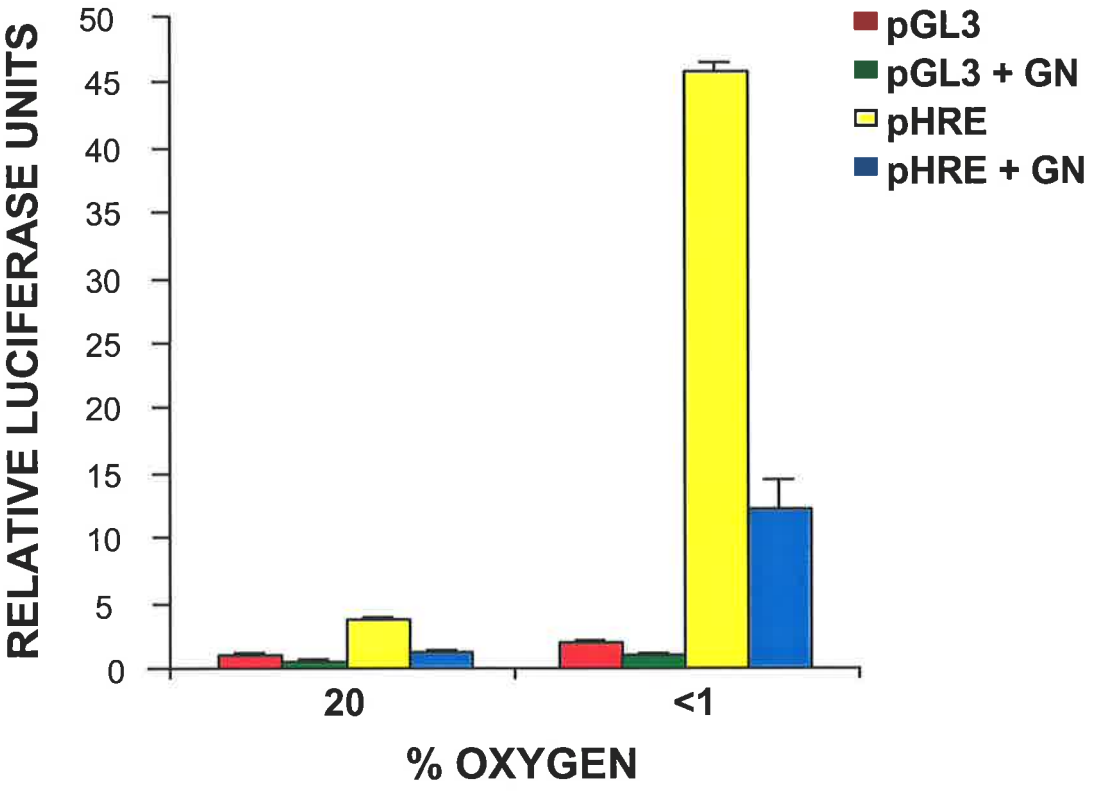
A. Whole cell extracts were prepared from cells exposed to 20% O_2 or <1% O_2 in the presence or absence of 100 μM genistein (GN) for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to HIF-1α (left panel) or HIF-2 α (right panel). Results are representative of two independent experiments.

B. Cells were transiently transfected with a firefly luciferase reporter gene containing 4 copies of the HRE (pHRE) or a control construct which lacks the HRE (pGL3). 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2 in the presence or absence of 100 μM genistein (GN). After 16 hours, cells were assayed for firefly luciferase activity against a cotransfected internal renilla luciferase control. Experiments were performed in triplicate. Error bars represent -/+ standard deviation. Results are representative of two independent experiments.

A


20% O2 + + - -
<1% O2 - - + +
GN - + - +
175 kDa
HIF-1α
62 kDa
20% O2 + + - -
<1% O2 - - + +
GN - + - +
175 kDa
HIF-2α
62 kDa

B


RELATIVE LUCIFERASE UNITS
50
45
40
35
30
25
20
15
10
5
0
20
<1
% OXYGEN
pGL3
pGL3 + GN
pHRE
pHRE + GN

hypoxically induced HRE reporter activity (compare pHRE + GN versus pHRE at <1%O_2). This was almost certainly due to the reduction in both the HIF-1α and HIF-2α protein that otherwise would have accumulated in hypoxia if it were not for the genistein treatment (figure 4.11.A).

In short, tyrosine phosphorylation appears to be crucial for the full upregulation and subsequent activity of both HIF-1α and HIF-2α in hypoxic PC12 cells. As mentioned previously (chapter 1.5.3) little is known pertaining to whether HIF-α phosphorylation is regulated by oxygen levels or whether it occurs to all translated protein. However, a significance of threonine 796 of HIF-1α (844 of HIF-2α) has been implicated in enhancing the transcriptional potency of HIF-α by providing the correct structure to maximise the association with CBP/p300 (Gradin *et al.*, 2002) and disrupting the interaction between HIF-1α and FIH-1 (Lancaster *et al.*, 2004). Phosphorylation of this threonine is unlikely to explain any of detected differences between HIF-1α and HIF-2α in the PC12 cells as these are due to distinct mechanisms of degradation and not inactivation of transcriptional ability.

4.7. EFFECT OF DEACETYLASE INHIBITORS ON HIF-α EXPRESSION IN PC12 CELLS

The mammalian homologue of the N-acetyltransferase ARD1 in bacteria and yeast (Park and Szostak, 1992; Tribioli *et al.*, 1994) has been characterised as an acetylase involved in accelerating the destruction of HIF-α. ARD1 is reported to acetylate a crucial lysine which is conserved in both HIF-α variants (K532 in HIF-1α, K497 in HIF-2α) and consequently enhance pVHL's interaction with the prolyl-4-hydroxylated oxygen dependent degradation domain (ODD) of HIF-α (Jeong *et al.*, 2002). It was proposed that HIF-1α acetylation is reduced in hypoxia via downregulation of the ARD1 transcript (and subsequently protein) and inhibition of the interaction between ARD1 and HIF-α (Jeong *et al.*, 2002). As prolyl-4-hydroxylation of HIF-2α does not seem to predominate in PC12 cells, it may seem illogical to analyse whether ARD1 performs a role in HIF-2α degradation given that acetylation evidently enhances the effects of hydroxylation. However, overexpression of ARD1 in hypoxia (where there is little O_2 to permit prolyl-4-hydroxylation) has also been shown to downregulate endogenous HIF-1α (Jeong *et al.*, 2002). Lysyl acetylation of HIF-α may mediate its degradation via mechanisms not

requiring concurrent prolyl-4-hydroxylation. It was thus decided to perform preliminary experiments to provide clues as to any possible function of ARD1 in PC12 cells. It is of note that not only did ARD1 overexpression cause a reduction of the hypoxically stabilised HIF-1α, but so did treatment of HT1080 cells with the histone deacetylase inhibitor trichostatin A (TSA) or the protein deacetylase inhibitor sodium butyrate (NaB) (Jeong *et al.*, 2002). It is possible that another mechanism of stabilisation in hypoxia involves deacetylation of the crucial lysine, thus interfering with this process may accelerate HIF-α degradation in hypoxia.

To investigate a possible role for acetylation in the regulation of HIF-2α stability, PC12 cells were exposed to normoxia or hypoxia with or without subsequent treatment with 30 ng/mL TSA or a 10 mM NaB for 16 hours. Whole cell extracts prepared from these cells were then analysed by immunoblotting with antibodies to either HIF-1α or HIF-2α (figure 4.12). TSA (figure 4.12.A) or NaB (figure 4.12.B) treatment markedly reduced the levels of HIF-2α that accumulated in hypoxia (compare <1% O_2 + TSA or NaB versus <1% O_2, lower panel) although this was not the case with HIF-1α (compare <1% O_2 + TSA or NaB versus <1% O_2, upper panel). Therefore, inhibition of deacetylation appears to cause a reduction in HIF-2α but not HIF-1α levels in hypoxic PC12 cells. Lysyl acetylation of HIF-2α by ARD1 and not prolyl-4-hydroxylation may be the means by which HIF-2α is targeted for destruction in the PC12 line.

4.8. EFFECT OF THE HIF-α ACETYLASE ARD1 ON HIF-α EXPRESSION IN PC12 CELLS

4.8.1. THE HIF-α ACETYLASE mRNA AND PROTEIN IS EXPRESSED IN PC12 CELLS

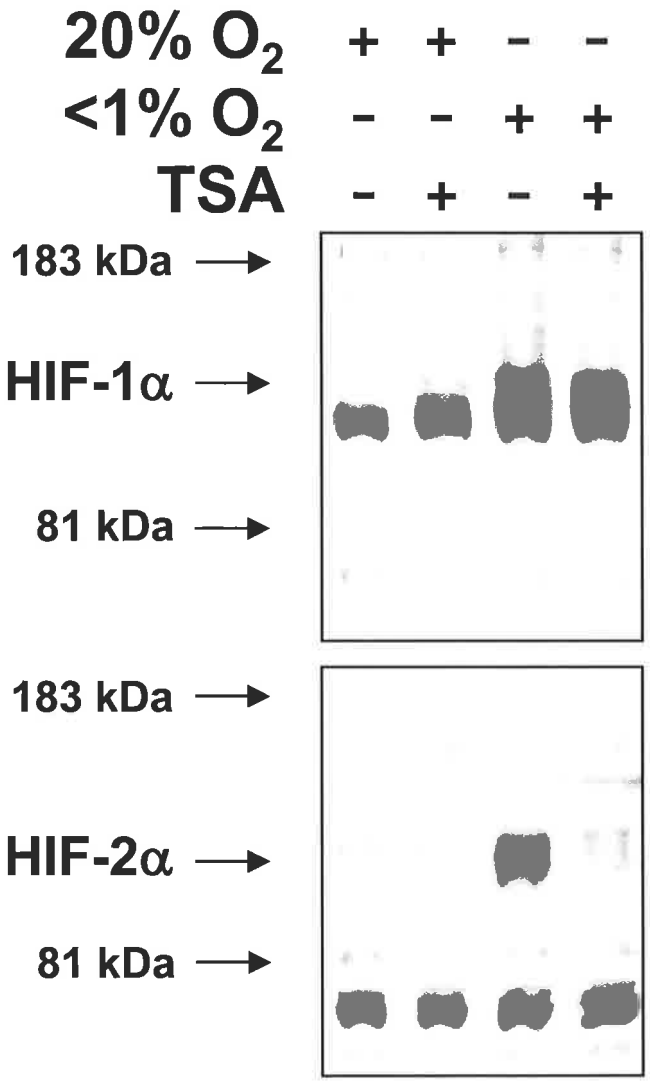
It is not known whether ARD1 is expressed in PC12 cells. Experiments were thus formulated to answer this question. Primers were designed to amplify the entire coding sequence of rat ARD1 (nucleotides 1-708 of rat ARD1 (GenBank accession number XM_343842)) and used to perform RTPCR on mRNA derived from PC12 cells. In short, cDNA was derived from reverse transcription of poly A RNA purified from PC12 cells exposed to normoxia or hypoxia for 16 hours. The cDNA (or water as a negative control) provided the template for PCR reactions employing the above primers (figure 4.13.A). ARD1 was expressed in PC12 cells and its levels were not altered by hypoxic treatment

Figure 4.12. Treatment of PC12 cells with the deacetylase inhibitors TSA or NaB inhibits hypoxic accumulation of HIF-2α but not HIF-1α.

A. Whole cell extracts were prepared from PC12 cells exposed to 20% O_2 or <1% O_2 with or without additional treatment with a 30 ng/mL concentration of the deacetylase inhibitor trichostatin A (TSA) for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (upper panel) or HIF-2α (lower panel). Results are representative of two independent experiments.

B. Whole cell extracts were prepared from PC12 cells exposed to 20% O_2 or <1% O_2 with or without additional treatment with a 10 mM concentration of the deacetylase inhibitor sodium butyrate (NaB) for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to either HIF-1α (upper panel) or HIF-2α (lower panel). Results are representative of two independent experiments.

A



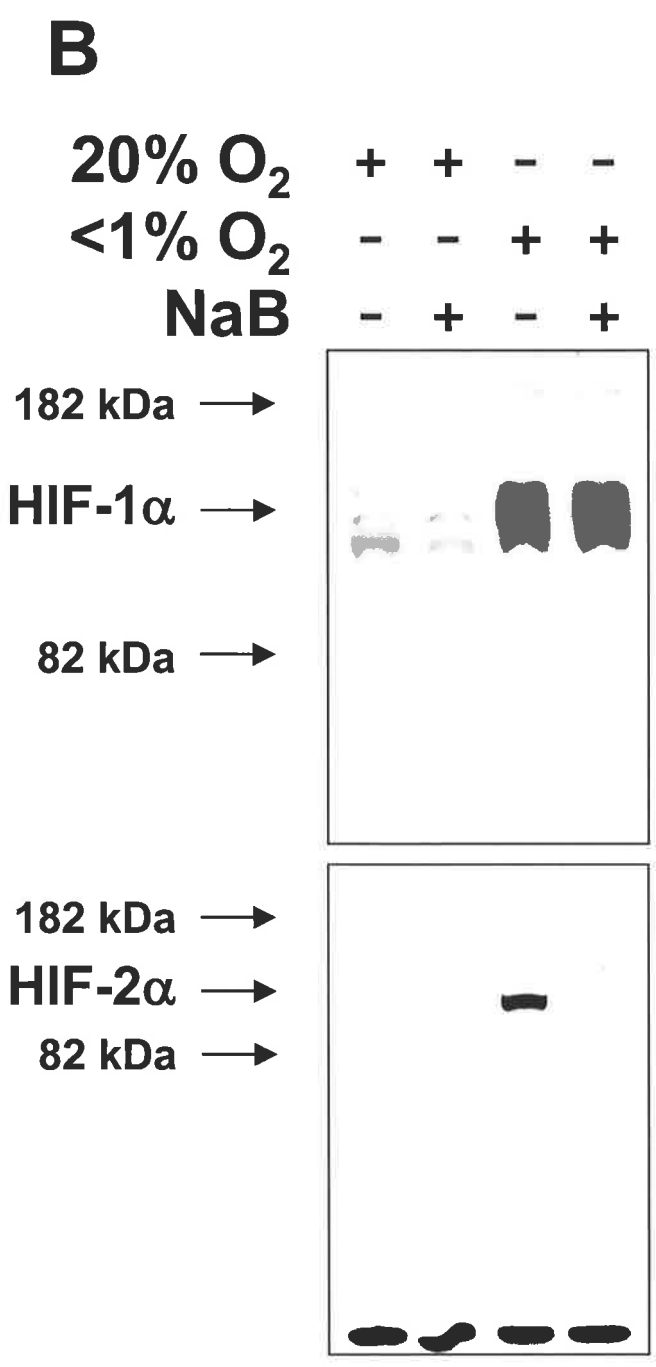
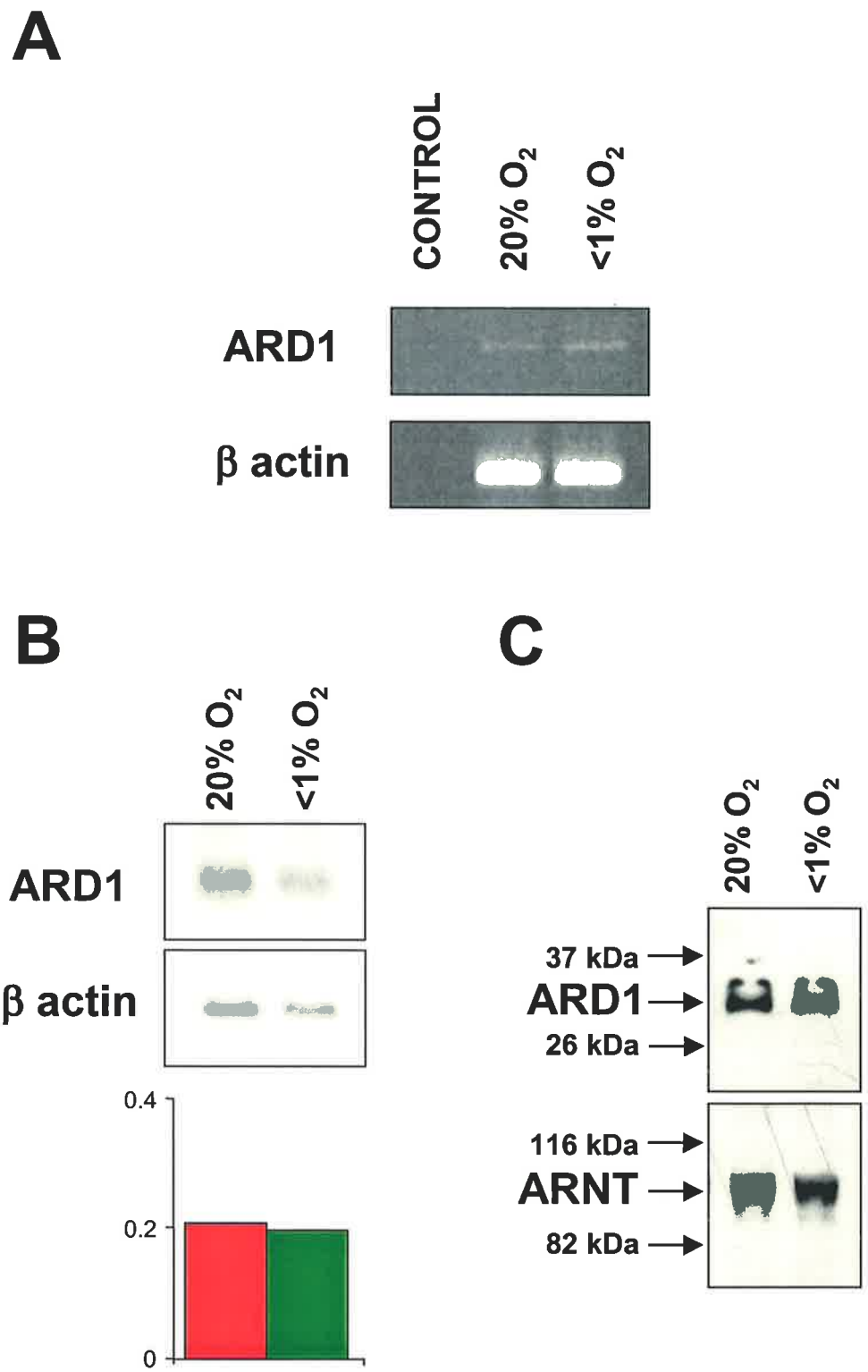

B
20% O2 + + - -
<1% O2 - - + +
NaB - + - +
182 kDa
HIF-1α
82 kDa
182 kDa
HIF-2α
82 kDa

Figure 4.13. HIF-α acetylase ARD1 mRNA and protein is expressed in PC12 cells.

A. cDNA was prepared from reverse transcription of 200 ng of poly A RNA extracted from PC12 cells exposed to 20% O_2 or <1% O_2 for 16 hours in a 20 μL reaction. 1 μL of this cDNA was used a template in a PCR reaction employing primers designed to amplify the entire coding sequence of ARD1 (upper panel) or β actin (lower panel, please note that this is the same as the β actin RTPCR from figure 4.6 as the reactions were performed at the same time and on the same samples). Reactions were electrophoresed through a 2% agarose/TBE/ethidum bromide gel. Results are representative of two independent experiments.

B. Poly A RNA was prepared from PC12 cells exposed to 20% O_2 or <1% O_2 for 16 hours. 3 μg of each were analysed by blotting with a ^{32}P labelled cDNA probe to ARD1 (upper panel). Hybridisation was normalised against that of a cDNA probe for β actin (lower panel).

C. Whole cell extracts were prepared from PC12 cells exposed to 20% O_2 and <1% O_2 for 16 hours. 20 μg of each were analysed by immunoblotting with antibodies to either ARD1 (upper panel) or ARNT (lower panel). Results are representative of two independent experiments.


A
CONTROL
20% O2
<1% O2
ARD1
β actin
B
20% O2
<1% O2
ARD1
β actin
0.4
0.2
0
C
20% O2
<1% O2
37 kDa
ARD1
26 kDa
116 kDa
ARNT
82 kDa

when compared to those of β actin. To validate that the amplified fragment corresponded to the complete coding region of ARD1, it was excised from the gel, purified, cloned into pGEMTEASY (hereafter referred to as pGEM/ARD1) and sequenced. When compared to GenBank accession number XM_343842, it could be concluded that the entire cDNA for rat ARD1 had been cloned. A comparison between rat ARD1 and that of human (NM_003491) using ClustalW showed that there was a 91% cDNA sequence similarity and 96% amino acid sequence similarity.

To validate the results of the RTPCR, the same poly A RNA detailed above was analysed by blotting with ^{32}P labelled cDNA probe to ARD1 (generated from the insert released from pGEM/ARD1) and was normalised against the hybridisation of a cDNA probe for β actin (figure 4.13.B). Again, ARD1 appeared to be unaltered by oxygen levels when contrasted with β actin levels (compare <1% O_2 versus 20% O_2).

Finally, whole cell extracts were prepared from PC12 cells exposed to normoxia or hypoxia for 16 hours. These were analysed via immunoblotting with antibodies to ARD1 or ARNT (4.13.C). When contrasted with ARNT, the levels of ARD1 protein were not altered by a reduction in oxygen (compare <1% O_2 versus 20% O_2).

These results are of interest as the only other study in which ARD1 mRNA was measured suggested that it was downregulated in hypoxia. In short, RTPCR and Northern analysis of normoxic and hypoxic HT1080 cells showed a reduction in ARD1 mRNA when compared with β actin and the hypoxic reduction of ARD1 transcript and subsequently protein was touted as a mechanism by which a reduction in oxygen levels results in reduced acetylation and decreased destruction of HIF-α (Jeong *et al.*, 2002). However, it was also shown that the interaction between ARD1 and HIF-1α is inhibited in hypoxia, even if the protein levels of the former are artificially maintained (Jeong *et al.*, 2002). Therefore, it is perhaps possible that in PC12 cells hypoxia causes a reduction in the acetylation of HIF-2α via somehow interfering with the ARD1/HIF-2α association, although ARD1 protein levels remain unaltered.

In short, the HIF-α acetylase ARD1 is expressed in PC12 cells. This further supports a possible role for ARD1 in this cell line with respect to regulating HIF-α (and in particular HIF-2α) protein levels in both normoxia and hypoxia, given that ARD1 appears to

function in hypoxia (Jeong *et al.*, 2002) and that HIF-2α degradation may be initiated via its lysyl acetylation by ARD1 in hypoxic PC12 cells (figure 4.12). If ARD1 downregulates HIF-2α in normoxia or hypoxia, it would be expected that inhibiting ARD1 protein expression would enhance HIF-2α levels.

4.8.2. RNA INTERFERENCE OF THE HIF-α ACETYLASE ARD1 STABILISES NEITHER HIF-α PROTEIN IN PC12 CELLS

In the other study where ARD1 downregulation was analysed with regards to its effects on HIF-α levels, transfection of 293 cells with a mammalian expression construct that expresses antisense ARD1 resulted in an upregulation of HIF-1α protein (HIF-2α was not measured) (Jeong *et al.*, 2002). Therefore, if ARD1 were to mediate the degradation of HIF-2α but not HIF-1α in the PC12 cell line, it would be expected that the inhibition of its expression would result in the stabilisation of the former but not the latter.

It was decided to employ RNAi in the same fashion as had been used for the downregulation of PHD2. The rat ARD1 (GenBank accession number XM_343842) specific sequences were selected upon criteria used previously. In total, four pSUPER constructs were designed: pSUPER/ARD1 144 (to target nucleotides 144-164), pSUPER/ARD1 150 (to target nucleotides 150-170), pSUPER/ARD1 552 (to target 552-572) and pSUPER/ARD1 627 (to target 627-647). It was imperative to test which of these were the most efficient in inhibiting ARD1 protein expression.

PC12 cells were transfected with the negative control mammalian expression plasmid pSUPER/SCR (SCR) or the ARD1 targeted variants (ARD1 144, 150, 552 or 627). Whole cell extracts were prepared 48 hours post transfection and were analysed via immunoblotting with antibodies to ARD1 or ARNT (figure 4.14.A). Transfection of PC12 cells with the ARD1 directed constructs resulted in a significant reduction in the levels of ARD1 protein, particularly pSUPER/ARD1 144 or pSUPER/ARD1 150 (compare SCR versus ARD1 144, 150, 552 and 627 on the ARD1 immunoblot and with the ARNT immunoblot). These two constructs were used for subsequent experiments.

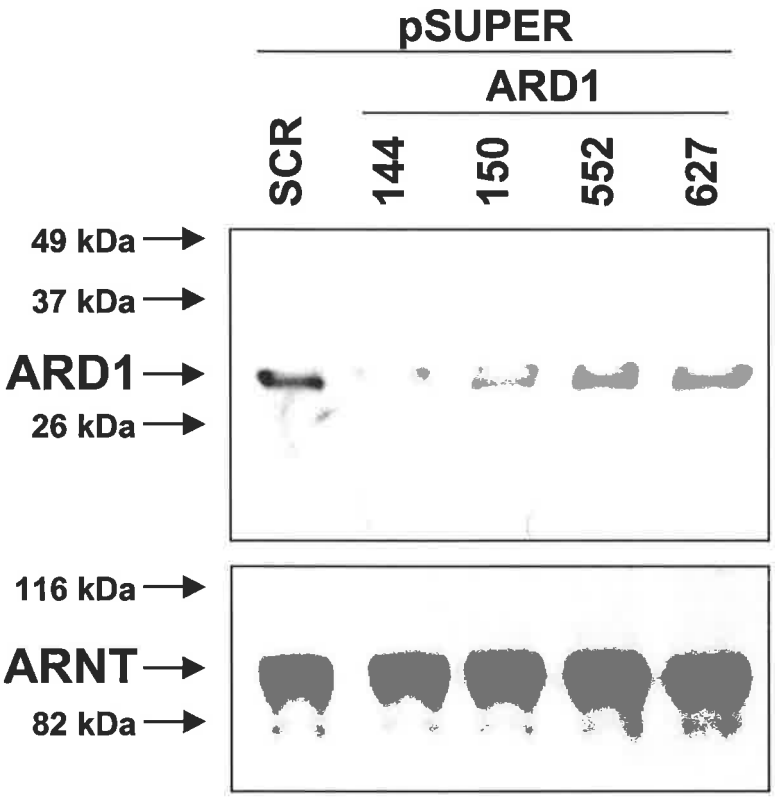
Therefore, PC12 cells were transfected with the negative control mammalian expression plasmid SCR or the two selected ARD1 targeted variants (ARD1 144 or 150). 32 hours post transfection cells were exposed to normoxia or hypoxia and 16 hours later, whole cell

Figure 4.14. Inhibition of ARD1 via RNA interference stabilises neither HIF-1α nor HIF-2α protein.

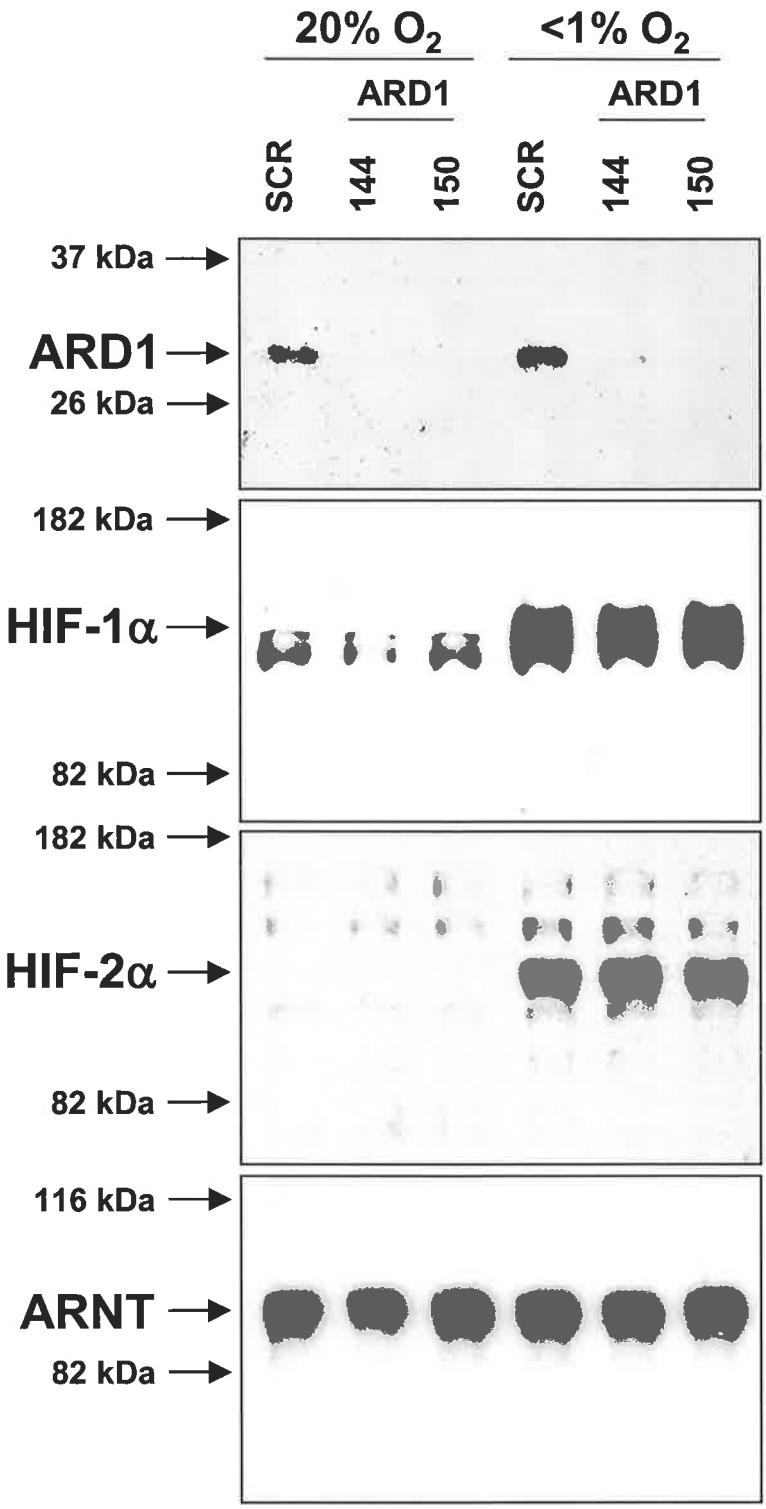
A. PC12 cells were transiently transfected with either a mammalian expression construct encoding a selfcomplementary transcript that is not homologous to any sequence in the rat genome (pSUPER/SCR) or that which is specific to sequences within ARD1 (pSUPER/ARD1 144, 150, 552 or 627). 48 hours post transfection, whole cell extracts were prepared. 20 μg of each were analysed by immunoblotting with antibodies to either ARD1 (upper panel) or the arylhydrocarbon receptor nuclear translocator (ARNT) (lower panel). Results are representative of two independent experiments.

B. PC12 cells were transiently transfected with either a mammalian expression construct encoding a selfcomplementary transcript that is not homologous to any sequence in the rat genome (SCR) or that which is specific to sequences within ARD1 (ARD1 144 or 150). 32 hours post transfection, cells were exposed to 20% O_2 or <1% O_2. After 16 hours, whole cell extracts were prepared. 10 μg of each were analysed by immunoblotting with antibodies to either ARD1 (top panel), HIF-1α (second panel), HIF-2α (third panel) or ARNT (bottom panel). Results are representative of two independent experiments.

A


pSUPER
ARD1
SCR
144
150
552
627
49 kDa
37 kDa
ARD1
26 kDa
116 kDa
ARNT
82 kDa

B


20% O2
<1% O2
ARD1
ARD1
SCR
144
150
SCR
144
150
37 kDa
ARD1
26 kDa
182 kDa
HIF-1α
82 kDa
182 kDa
HIF-2α
82 kDa
116 kDa
ARNT
82 kDa

extracts were prepared and analysed via immunoblotting with antibodies to ARD1, HIF-1α, HIF-2α or ARNT (figure 4.14.B). If ARD1 were to have a significant role in the destruction of either HIF-α, it would be expected that reducing ARD1 protein levels would cause an increase in HIF-α protein. Transfection with the ARD1 directed constructs caused ARD1 protein downregulation in normoxia and hypoxia (compare SCR versus ARD1 144 and 150 on the ARD1 immunoblot and with the ARNT immunoblot, both at 20% O_2 and <1% O_2). However, regardless of oxygen tension, it influenced neither HIF-1α (compare SCR versus PHD2 542 and 554 at both 20% O_2 and <1% O_2 on the HIF-1α immunoblot) nor HIF-2α protein levels (compare the same lanes on the HIF-2α immunoblot) when compared with ARNT.

In short, it appears that ARD1 has no evident role in the regulation of either endogenous HIF-α in PC12 cells and, of most relevance, no apparent influence on HIF-2α levels whether in normoxia or hypoxia. To confirm this, it was decided to examine the effects of forcing the expression of ARD1 in PC12 cells exposed to normoxia and hypoxia.

4.8.3. OVEREXPRESSION OF THE HIF-α ACETYLASE ARD1 DOES NOT ALTER THE PROTEIN LEVELS OF EITHER HIF-α IN PC12 CELLS

HIF-1α and HIF-2α are virtually undetectable in normoxic PC12 cells, so the transfection and overexpression of ARD1 may only reduce the levels of an already almost untraceable protein. However, the forced expression of ARD1 in HT1080 cells has been shown to reduce HIF-1α without any change in the latter's mRNA (Jeong *et al.*, 2002). Therefore, if ARD1 were to mediate the degradation of HIF-2α but not HIF-1α that has accumulated in PC12 cells exposed to hypoxia, it would be expected that its overexpression would inhibit the accumulation of the former but not the latter.

To demonstrate whether this was indeed the case, it was first required that the cDNA for ARD1 be subcloned in a mammalian expression construct. Therefore, the fragment generated from the insert released from the *Eco*RI digestion of pGEM/ARD1 was subcloned into the mammalian expression vector pEF/IRES/PURO, thus resulting in the construction of pEF/ARD1/IRES/PURO.

To test the effect of ARD1 overexpression on HIF-α protein levels, PC12 cells were transfected with pEF/IRES/PURO or pEF/ARD1/IRES/PURO. 6 hours post transfection,

cells were exposed to normoxia or hypoxia. After 16 hours, whole cell extracts were prepared and analysed via immunoblotting with antibodies to ARD1, HIF-1α or HIF-2α (figure 4.15). ARD1 overexpression caused no change in endogenous HIF-1α or HIF-2α levels, regardless of oxygen tension.

Therefore, these experiments confirm that ARD1 appears to play no apparent role in PC12 cells with respect to the regulation of either HIF-1α or HIF-2α by reflecting what has already been seen in the RNAi of ARD1 (figure 4.14.B). The proposed downregulation of HIF-2α by lysyl acetylation at least in hypoxia must be by an enzyme other than ARD1.

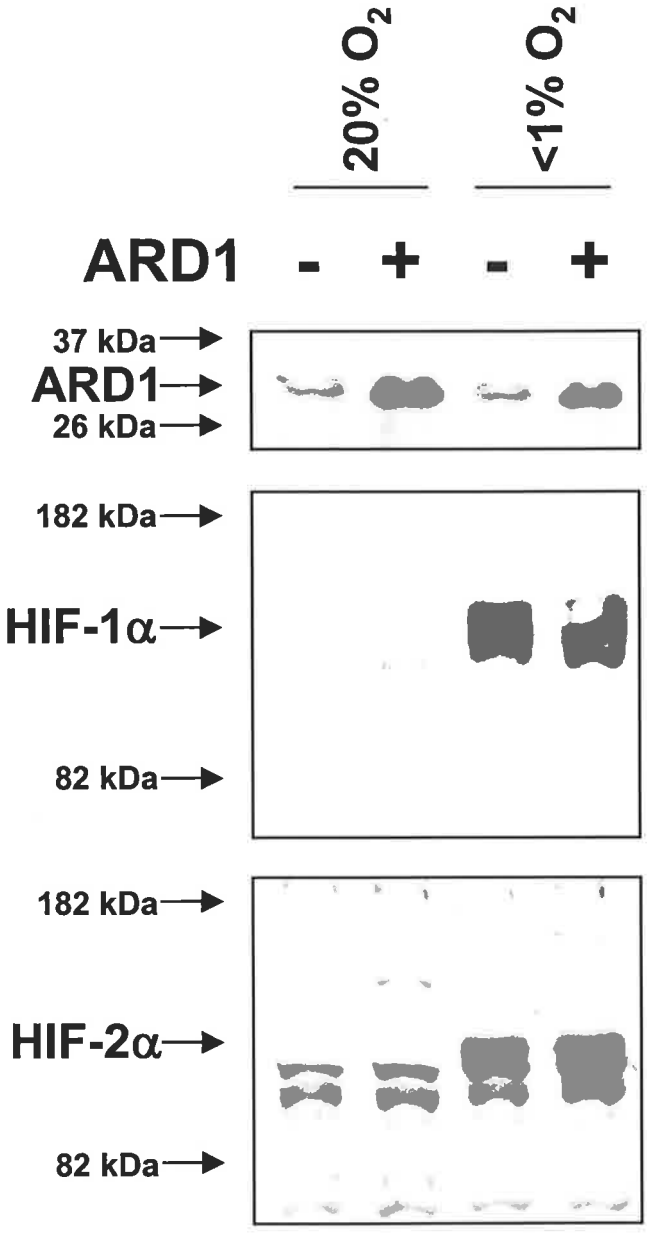
4.9. DISCUSSION OF ALTERNATIVE MECHANISMS OF OXYGEN DEPENDENT HIF-2α REGULATION IN PC12 CELLS

That all the data collected through the course of these studies suggested HIF-2α protein degradation is dependent on oxygen, but apparently not HIF-α prolyl-4-hydroxylation, in the PC12 cell line was surprising. In truth, prolyl-4-hydroxylation is perhaps the most effective and well characterised, but not necessarily the only, process which is known to regulate HIF-α protein levels in response to changes in oxygen levels. Reactive oxygen species, nitric oxide, phosphorylation, p53 and lysyl acetylation have all been implicated, even if in some cases there exists major conjecture between different research groups. This has been described in some detail previously (chapter 1.5). In short, there was no shortage of alternative mechanisms that could have been involved in the oxygen dependent destruction of HIF-2α. However, there were few clues as to which one of those mentioned above could actually be involved except that it is iron and 2-oxoglutarate independent (which rules in most processes) and, given that HIF-2α protein accumulation in response to hypoxia was not instantaneous, that it was perhaps a multistep process, far removed and potentially more complex than mere prolyl-4-hydroxylation, whereby in the simplest model, the enzyme involved serves as both the sensor and the effector of HIF-α function.

Tyrosine phosphorylation was initially investigated as a process that may influence HIF-2α levels in PC12 cells, with the use of a tyrosine kinase inhibitor (genistein) demonstrating that tyrosine phosphorylation was necessary for the accumulation of both

Figure 4.15. ARD1 overexpression does not influence endogenous HIF-1α or HIF-2α protein levels in PC12 cells.

PC12 cells were transfected with either pEF/IRES/PURO or pEF/ARD1/IRES/PURO (ARD1). 6 hours post transfection, cells were exposed to 20% O_2 or <1% O_2 for 16 hours. 10 μg of whole cell extracts derived from these cells were analysed by immunoblotting with antibodies to either ARD1 (top panel) HIF-1α (middle panel) or HIF-2α (bottom panel). Results are representative of three independent experiments.


20% O2
<1% O2
ARD1 - + - +
37 kDa
ARD1
26 kDa
182 kDa
HIF-1α
82 kDa
182 kDa
HIF-2α
82 kDa

HIF-α proteins and the hypoxic induction of HRE reporter activity (figure 4.11). Consequently, attention was drawn to another process recently described as contributing to at least HIF-1α destruction in normoxia, namely lysyl acetylation by the HIF-α acetylase ARD1 (Jeong *et al.*, 2002). Indeed, TSA and NaB treatment of PC12 cells resulted in the selective abolition of HIF-2α, but not HIF-1α, protein accumulation in response to hypoxia (figure 4.12). Therefore, as inhibition of deacetylation resulted in decreased HIF-2α but not HIF-1α levels in hypoxic PC12 cells, lysyl acetylation of HIF-2α by ARD1 became a proposed means by which HIF-2α is targeted for destruction in the PC12 cell line. Indeed, ARD1 was shown to be present in PC12 cells, although neither its transcript nor protein appear to be influenced by oxygen partial pressures (figure 4.13), which was of interest as the hypoxic reduction of ARD1 transcript and subsequently protein was hypothesised as a means by which hypoxia causes reduced acetylation and increased stabilisation of HIF-α (Jeong *et al.*, 2002). Furthermore, neither a decrease in ARD1 levels via RNAi (figure 4.14) nor an increase of its levels via overexpression (figure 4.15) altered HIF-2α (nor for that matter HIF-1α) protein. Therefore, if HIF-2α levels are indeed controlled by lysyl acetylation, then an enzyme other than ARD1 must be involved.

Reversible lysyl acetylation has been almost exclusively associated with that of histones, resulting in chromatin remodelling and enhanced transcription. The significance of protein acetylation as a more general protein modification and its involvement in a large array of cellular processes has only recently begun to emerge (Kouzarides, 2000; Cohen and Yao, 2004), with NF-κB transcriptional activity being one such example. NF-κB is a family of transcription factors which mediate the expression of a range of genes involved in cell proliferation, as well as inflammation and the immune system, the most abundant isoform of which consists of the p50 and p65 heterodimer (Hayden and Ghosh, 2004). Both of these have been demonstrated to be directly lysyl acetylated by p300. The lysyl acetylation of p50 enhances its DNA binding activity, whereas there is some discrepancy with regards to the function of the p65 modification (Quivy and Van Lint, 2004). With respect to protein stability, direct acetylation has been shown to enhance the levels of a number of other transcription factors by inhibiting their ubiquitin proteasome pathway mediated degradation. These include E2F1 (Martínez-Balbás *et al.*, 2000), p53 (Ito *et al.*, 2002) Smad7 (Gronroos *et al.*, 2002), SREBP family proteins (Giandomenico *et al.*, 2003), NF-E4 (Zhao *et al.*, 2004) and p73 (Bernassola *et al.*, 2004). However, that

acetylation may result in enhancing a protein's ubiquitination and degradation, as may be the case with HIF-α, appears to be unique to this transcription factor.

The evidence in the PC12 cell line suggests that HIF-2α levels may be controlled by lysyl acetylation but that this involves an enzyme other than ARD1. One method by which to find this protein would be to perform a database search for other acetyltransferases similar to ARD1, other proteins possessing a putative acetyltransferase catalytic domain or to acquire those implicated in mediating protein stability, such as P/CAF, even if in this case it inhibits the destruction of its target, NF-E4 (Zhao *et al.*, 2004). Once found and cloned, experiments similar to those for testing ARD1 activity can be performed in PC12 cells. That is, investigating the effects of either the RNAi or overexpression of the putative HIF-2α acetylase on HIF-α (particularly HIF-2α) protein levels in normoxia and hypoxia.

Whether HIF-2α is itself directly acetylated can be determined by immunoprecipitating HIF-2α which has been stabilised via an agent or process which should inhibit destruction but not acetylation (for instance, by a proteasome inhibitor, assuming that HIF-2α is still degraded by the ubiquitin proteasome pathway in PC12 cells) and immunoblotting for lysyl acetylated protein. This was not attempted due to the lack of an antibody sufficient for immunoprecipitation (data not shown). It must therefore be remembered that other mechanisms cannot be ruled out. Perhaps the function of other factors specifically involved in the regulation of HIF-2α protein levels but not those of HIF-1α are directly regulated by oxygen dependent acetylation, so the inhibition of their deacetylation in hypoxia causes a maintenance of their ability to degrade HIF-2α. Alternatively, the TSA or NaB treatment may enhance the expression of these same putative factors whose transcription is somehow otherwise downregulated in hypoxia or whose protein levels are as a consequence so relatively induced that their high levels prevail over any direct control by O_2 tension. Finally, although it is unlikely to that deacetylase inhibition would directly cause reduced HIF-2α mRNA (hyperacetylation is after all associated with enhanced transcription), the possibility that a factor capable of specific transcriptional repression of HIF-2α becomes upregulated upon deacetylase inhibition (that in other circumstances would be suppressed in hypoxia or would not be expressed to any significant degree regardless of the conditions) cannot be ruled out. The measurement of the mRNA levels of HIF-1α and HIF-2α in PC12 cells resulting from TSA or NaB treatment and/or hypoxia was attempted, but without success (data not shown).

Regardless, lysyl acetylation appears to be involved in a process responsible for the regulation of HIF-2α but not HIF-1α protein in the PC12 cell line.

A final point to consider is whether in addition to the apparent non PHD mediated degradation of HIF-2α PC12 cell line, is it possible that, once posttranslationally modified for degradation, another mechanism is involved that does not require association with the pVHL E3 ubiquitin ligase and destruction via the ubiquitin proteasome pathway? As mentioned previously, prolyl-4-hydroxylation within the ODD is responsible for pVHL's association with HIF-1α and HIF-2α (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001), so, in theory, HIF-2α cannot be directed to the proteasome in PC12 cells as it does not appear to be significantly prolyl-4-hydroxylated by virtue of the fact that inhibition of the PHD activity does little to stabilise HIF-2α. Is pVHL indeed present? Briefly, all evidence points to a functional pVHL in PC12 cells. The observations of the behaviour of HIF-1α described (stabilisation by iron chelation and PHD2 downregulation and a reversal in the accumulation of overexpressed protein upon the coincident forced expression of PHD activity) is consistent with the assumption that the oxygen dependent prolyl-4-hydroxylation of HIF-1α results in its association with the pVHL E3 ubiquitin ligase complex. Furthermore, published data has shown that pVHL is expressed in PC12 cells and regulates both HIF-1α and HIF-2α levels (Kroll *et al.*, 1999; Schnell *et al.*, 2003). Interestingly, ubiquitination of HIF-2α itself in PC12 cells has been demonstrated to correlate with pVHL levels (Schnell *et al.*, 2003). How can the lack of prolyl-4-hydroxylation of HIF-2α be reconciled with the capacity of pVHL and HIF-2α to interact? In short, although theoretically unlikely, it is possible that another as yet unknown oxygen dependent posttranslational modification (such as lysyl acetylation, despite what is proposed in the original ARD1 paper (Jeong *et al.*, 2002)) structurally permits a pVHL/HIF-α interaction. As an extension of this, it is equally impossible to exclude the suggestion that this same novel alteration recruits another E3 ubiquitin ligase that does not require recognition by hydroxyproline. Nonetheless, what is clear is that HIF-2α levels do not appear to be significantly mediated by prolyl-4-hydroxylation.

4.10. SUMMARY

Of the two hypoxia inducible factors in the PC12 cell line, initial experiments involving the analysis of HIF-1α protein suggested that its degradation was initiated by prolyl-4-hydroxylation. The subsequent research that branched from these first observations

suggests that HIF-1α and HIF-2α undergo oxygen dependent degradation via mechanisms that are mutually exclusive (figure 4.16). It seems that HIF-1α destruction is initiated by the activity of the PHD enzymes on P402 and P564. Surprisingly, despite requiring iron, it appears that the prolyl-4-hydroxylation is not dependent on 2-oxoglutarate as competition with the analogue DMOG does not seem to inhibit PHD activity in the PC12 cells. Conversely, and perhaps in itself even more strange, the data suggests that HIF-2α protein levels are not significantly mediated by prolyl-4-hydroxylation. Rather, it appears that it is targeted for degradation by acetylation, at least in hypoxia, but via a mechanism that does not involve the known HIF-α lysyl acetylase, ARD1.

Figure 4.16. Differential regulation of HIF-1α and HIF-2α in the PC12 cell line. The destruction of HIF-1α is initiated by prolyl-4-hydroxylation, albeit without an apparent requirement for 2-oxoglutarate. In contrast, HIF-2α levels are not mediated by prolyl-4-hydroxylation. Rather, preliminary data suggest that lysyl acetylation is involved in regulating HIF-2α protein stability, but by a mechanism which is independent of the function of the only known HIF-α lysyl acetylase, ARD1.

HIF-1α

Hypoxia causes instantaneous stabilisation

Degradation initiated by P hydroxylation

Fe^{2+} dependent

2-oxoglutarate independent

HIF-2α

Stabilisation requires long duration of hypoxia

P hydroxylation not involved

Targeted for destruction by acetylation

ARD1 independent

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

GENERAL DISCUSSION

5.1. INTRODUCTION

Many of the responses of mammals to a reduced availability of oxygen (hypoxia) are controlled by the transcription factors hypoxia inducible factor 1α (HIF-1α) and HIF-2α. Despite the biochemical similarities that are evident between HIF-1α and HIF-2α, gene targeting experiments show major differences in developmental abnormalities between HIF-1α$^{-/-}$ and HIF-2α$^{-/-}$ mice and thus suggest distinct physiological roles and target gene specificities (Iyer *et al.*, 1998; Ryan *et al.*, 1998; Carmeliet *et al.*, 1998; Tian *et al.*, 1997; Peng *et al.*, 2000; Compernolle *et al.*, 2002; Scortegagna *et al.*, 2003a; Scortegagna *et al.*, 2003b). A number of HIF-1α target genes have been documented, but there are currently none that have been conclusively identified for HIF-2α. The aim of this project was thus to determine specific gene targets and differential regulation of HIF-1α and HIF-2α in the rat pheochromocytoma cell line PC12.

5.2. HIF-2α SPECIFIC TARGET GENES

Both HIF-1α and HIF-2α proteins accumulate during hypoxia in PC12s (Conrad *et al.*, 1999; Agani *et al.*, 2000). Monoclonal PC12 cell lines capable of induced and selective HIF-1α and HIF-2α expression and function upon doxycycline treatment were established. Northern analysis of these lines with cDNA probes to a number of known HIF-1α target genes indicates that these are essentially HIF-1α specific and are not significantly induced by HIF-2α. Nonetheless, it is clear that inducing HIF-1α transcriptional potency in normoxia is sufficient to upregulate the genes for which it serves as a hypoxia inducible transactivator. Therefore, the levels of any HIF-2α targets should be enhanced via the forced expression of this HIF-α in normoxia. An initial screen was performed whereby the expression profile of the inducible HIF-2α PC12 derivative exposed to doxycycline was compared with that of the similarly treated inducible HIF-1α line. A number of genes known to be targets of HIF-1α and in some cases already analysed as such via Northern blotting were indeed induced in the latter. However, a number of genes shown to be

relatively induced in the former were ultimately shown to be constitutively upregulated in the HIF-2α line regardless of doxycycline administration and thus the induction of HIF-2α. Therefore, they were not deemed to be transactivated by HIF-2α. An alternative experiment had to be conducted. To that end, a screen determining differential expression between doxycycline treated and untreated inducible HIF-2α PC12 cells revealed a few (although unfortunately not many) potential HIF-2α target genes, particularly as these same genes were not upregulated in a similar experiment comparing the expression profile of a control line with or without exposure to doxycycline. Of those further investigated, preliminary experiments suggested that the mRNA of vitronectin and regulator of G protein signalling 4 (Rgs4) were hypoxically inducible, however it appears that they may be targeted for transcriptional upregulation not only by HIF-2α but by HIF-1α as well. The voltage gated sodium channel β3 subunit, despite also appearing to display hypoxia inducible expression, appeared to be regulated by neither HIF-1α nor HIF-2α. Further experiments will be required in order to validate whether this is indeed the case and also to verify the characteristics of other genes that emerged as putative targets of HIF-2α in the microarray.

Given the ever expanding list of direct HIF-1α target genes (table 1.1), it is difficult to fathom precisely why none have been found for HIF-2α. This has not been through a lack of trying and a number of cell types and systems have been employed, including in a limited capacity PC12 cells (chapter 1.7.6 and chapter 3.7). Indeed, there are some proposed targets, but in each case HIF-2α cannot be definitely described as the direct transcription factor responsible for mediating mRNA expression, or the gene described is also a characterised HIF-1α target. There is a possibility that HIF-2α has no specific target genes and serves to transactivate only a subset of HIF-1α target genes. Indeed, there is much evidence to suggest this (chapter 3.7). However, this is somewhat difficult to reconcile with the fact that HIF-1α cannot completely compensate for HIF-2α, and *vice versa*, in the null mice, so a more likely possibility is that, in addition to transactivating a subset HIF-1α target genes, there also a limited number of HIF-2α specific targets, which substantially increases the difficulty of discovering and characterising them. Indeed, the profile of genes upregulated in the doxycycline inducible HIF-2α PC12 line when compared with untreated cells demonstrated that there were very few putative targets of HIF-2α and further analysis of some of these via RTPCR suggested that only two of these (vitronectin and Rgs4) may in fact be HIF-2α targets, and even then, these may be

transactivated by HIF-1α as well (chapter 3.6.4). Moreover, it is possible that complete HIF-2α activity is also dependent on other cellular factors and is thus dependent on cell type and context. In short, there is perhaps little wrong in the conception and execution of the search for HIF-2α target genes as described in this thesis as a first approach, but other complementary methods will be required to uncover the full complement of HIF-2α target genes.

5.3. DIFFERENTIAL REGULATION OF HIF-1α AND HIF-2α

One also cannot discount the physiological relevance of any differential mechanisms of regulation of HIF-1α and HIF-2α protein in determining any downstream effects, namely the induction of their respective target genes. This is of particular interest in a therapeutic sense if a selective inhibition or enhancement of HIF-1α and HIF-2α is desired.

A surprising feature of the monoclonal PC12 cell lines that were developed during the course of this work is that the selective upregulation of HIF-1α does not induce HIF-2α, and *vice versa*. This suggested uncharacterised differences in their degradation mechanisms. Subsequent research stemming from these original observations indicated that HIF-1α and HIF-2α undergo oxygen dependent degradation via mechanisms that are mutually exclusive. It was clear that the degradation of HIF-1α is initiated by the enzymatic activity of the HIF-α prolyl-4-hydroxylases on P402 and P564. Interestingly, although iron dependent, it appears that the prolyl-4-hydroxylation reaction may not require 2-oxoglutarate as competition with the analogue DMOG does not appear to inhibit PHD activity in the PC12 cell line. Conversely, HIF-2α levels do not appear to be controlled by prolyl-4-hydroxylation at all, which is in itself of greater interest. Rather, it appears that it is targeted for degradation by acetylation, at least in hypoxia, but via a mechanism which does not involve the known HIF-α lysyl acetylase, ARD1.

There is some limited insight into differences between the regulation of HIF-1α and HIF-2α stability and activity, although in general it has been assumed that oxygen dependent prolyl-4-hydroxylation of two conserved proline residues within both HIF-1α and HIF-2α permits their association with the von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex and subsequent destruction by the ubiquitin proteasome pathway (chapter 4.5). For example in immortalised mouse embryo fibroblasts, stable HIF-2α protein has been demonstrated to be present regardless of oxygen levels and its ability to function as a

transcription factor may involve an as yet unknown mechanism to induce its translocation to the nucleus and induce target gene expression (Park *et al.*, 2003). However, the study described in this thesis is the first to indicate that the oxygen dependent mediation of HIF-2α stability in PC12 cells is via a mechanism other than proly-4-hydroxylation. One could question the significance of such a finding if it were limited to one cancerous cell type but even in the context of whole organs there is some data to suggest that there may be differences in the regulation of HIF-1α and HIF-2α protein stability, even if these mechanisms are somewhat less well characterised than what has been presented in this thesis. Of interest is one study where the expression of HIF-1α and HIF-2α in hypoxic and ischaemic rat kidneys is examined via immunohistochemistry (Rosenberger *et al.*, 2002). Not surprisingly, some cell types demonstrate the exclusive expression of either HIF-1α or HIF-2α protein regardless of the stimulus (ischaemia, the hypoxia mimetic $CoCl_2$ or CO which results in functional anaemia). For instance, HIF-2α was exclusive to glomerular and peritubular endothelial cells, whereas only HIF-1α could be detected in connecting tubules and collecting ducts (Rosenberger *et al.*, 2002). However, it is also clear that some cell types can express both HIF-1α and HIF-2α protein but in response to different stimuli. For instance, in glomeruli, $CoCl_2$ caused an accumulation of HIF-2α but not HIF-1α protein, whereas ischaemia caused that of both (Rosenberger *et al.*, 2002). It is proposed, therefore, that characteristics of each cell type within the kidney can either determine which of the HIF-α proteins is expressed or, in situations where both can be upregulated, the conditions required for the upregulation of either, such as the level of oxygen required (Rosenberger *et al.*, 2002). If what is observed in the PC12 cells provides any indication, differences in the degradative mechanisms of HIF-1α and HIF-2α may also be of significance in the latter scenario. It will be interesting to examine whether this is indeed also the case in other cell types, including primary and non cancerous lines, in order to ascertain the significance of the apparent differences in the mechanisms by which a reduction in oxygen levels results in an increase in HIF-1α and HIF-2α protein and how widespread this phenomenon actually is.

Inhibitors and activators of HIF-α are actively being sought (chapter 1.6). That HIF-1α and HIF-2α may have differential oxygen dependent mechanisms of regulation provides disadvantages to the use of these as therapeutics, particularly if the targeted enzymes are the regulatory proteins themselves, such as the HIF-α prolyl-4- and asparaginyl hydroxylases. For instance, if indeed HIF-1α but not HIF-2α protein levels are controlled

in a given tumour type by the PHD enzymes, then the administration of an inhibitor of prolyl-4-hydroxylation may only serve to decrease HIF-1α function and not the general HIF-α response, so some of a cancer's adaptive responses to intratumoural hypoxia may remain intact. However, there may be advantages as well, and these will become more apparent once the exact details of differential roles of HIF-1α and HIF-2α are known. That is, it may be desirable to specifically target a HIF-1α regulated process, thus avoiding any deleterious effects of targeting both factors. Therefore, in the context of therapeutic manipulation of the hypoxia inducible factors, it is imperative that we have a better understanding of how they are differentially activated and the identity of any target genes which are unique to either. The work in this study has contributed significantly to both of these important questions.

5.4. CONCLUSION

It is clear that there are differences in both the function and regulation of HIF-1α and HIF-2α in the PC12 cell line. These may explain their distinct function in other cell lines and *in vivo*. Furthermore, an understanding of any distinguishing characteristics of how they are regulated and what they in turn control may provide processes to focus on in the development of therapeutics for the treatment of various hypoxically mediated diseases.

CHAPTER 6

REFERENCES

CHAPTER 6

REFERENCES

Agani, F.H., Pichiule, P., Chavez, J.C., LaManna, J.C. (2000). The role of mitochondria in the regulation of hypoxia inducible factor 1 expression during hypoxia. J. Biol. Chem. *275*: 35863-35867.

Ambrosini, G., Nath, A.K., Sierra-Honigmann, M.R. and Flores-Riveros, J. (2002). Transcriptional activation of the human leptin gene in response to hypoxia: involvement of hypoxia inducible factor 1. J. Biol. Chem. *277*: 34601-34609.

An, W.G., Kanekal, M., Celeste Simon, M., Maltepe, E., Blagosklonny, M.V. and Neckers, L.M. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1α. Nature. *392*: 405-408.

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). Differential functions of the prolyl hydroxylases PHD1, 2 and 3 in the regulation of hypoxia inducible factor (HIF). J. Biol. Chem. *279*: 38458-38465.

Aprelikova, O., Chandramouli, G.V.R., Wood, M., Vasselli, J.R., Riss, J., Maranchie, J.K., Linehan, W.M. and Barrett, J.C. (2004). Regulation of HIF prolyl hydroxylases by hypoxia inducible factors. J. Cell. Biochem. *92*: 491-501.

Arany, Z., Huang, L.E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M.A., Bunn, H.F. and Livingston, D.M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. Proc. Natl. Acad. Sci. U.S.A. *93*: 12969-12973.

Baader, E., Tschank, G., Baringhaus, K.H., Burghard, H. and Gunzler, V. (1994). Inhibition of prolyl-4-hydroxylase by oxalyl amino acid derivatives *in vitro*, in isolated microsomes and in embryonic chicken tissues. Biochem. J. *300*: 525-530.

Bajou, K., Noel, A., Gerard, R.D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N.E., Carmeliet, P., Collen, D., Foidart, J.M. (1998). Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularisation. Nat. Med. *4*: 923-928.

Battacharya, S., Michels, C.L., Leung, M.K., Arany, Z., Kung, A.L. and Livingston, D.M. (1999). Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. Genes Dev. *13*: 64-75.

Bauer, A.L., Paulding, W.R., Striet, J.B., Schnell, P.O. and Czyzyk-Krzeska, M.F. (2002). Endogenous von Hippel-Lindau tumour suppressor protein regulates catecholaminergic phenotype in PC12 cells. Cancer Res. *62*: 1682-1687.

Bazan, N.G. and Lukiw, W.J. (2002). Cyclooxygenase 2 and presinilin 1 gene expression induced by interleukin 1β and amyloid β 42 peptide is potentiated by hypoxia in primary human neural cells. J. Biol. Chem. *277*: 30359-30367.

Beck, I., Weinmann, R. and Caro, J. (1993). Characterisation of hypoxia responsive enhancer in the human erythropoietin gene shows presence of hypoxia inducible 120 Kd nuclear DNA binding protein in erythropoietin producing and nonproducing cells. Blood. *82*: 704-711.

Bernassola, F., Salomani, P., Oberst, A., Di Como, C.J., Pagano, M., Melino, G. and Pandolfi, P.P. (2004). Ubiquitin dependent degradation of p73 is inhibited by PML. J. Exp. Med. *199*: 1545-1557.

Berra, E., Benizri, E., Ginouvès, A., Volmat, V., Roux, D. and Pouysségur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady state levels of HIF-1α in normoxia. EMBO J. *22*: 4082-4090.

Berra, E., Richard, D.E., Gothié, E. and Pouysségur, J. (2001). HIF-1 dependent transcriptional activity is required for oxygen mediated HIF-1α degradation. FEBS Lett. *491*: 85-90.

Bianchi, L., Tacchini, L. and Cairo, G. (1999). HIF-1 mediated activation of transferrin receptor gene transcription by iron chelation. Nucleic Acids Res. *27*: 4223-4227.

Blagosklonny, M.V., Giannakakou, P., Romanova, L.Y., Ryan, K.M., Vousden, K.H. and Fojo, T. (2001). Inhibition of HIF-1 and wildtype p53 stimulated transcription by codon Arg^{175} p53 mutants with selective loss of functions. Carcinogenesis. *22*: 861-867.

Bruick, R.K. and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science. *294*: 1337-1340.

Bruick, R.K. (2000). Expression of the gene encoding Nip3 protein is induced by hypoxia. Proc. Natl. Acad. Sci. U.S.A. *97*: 9082-9087.

Brusselmans, K., Bono, F., Maxwell, P., Dor, Y., Dewerchin, M., Collen, D., Herbert, J.M. and Carmeliet, P. (2001). Hypoxia inducible factor 2α (HIF-2α) is involved in the apoptotic response to hypoglycaemia but not hypoxia. J. Biol. Chem. *276*: 39192-39196.

Buckler, K.J. and Vaughan-Jones, R.D. (1994). Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type I cells. J. Physiol. *476*: 423-428.

Bunn, H.F. and Poyton, R.O. (1996). Oxygen sensing and molecular adaptation to hypoxia. Physiol. Rev. *76*: 839-885.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D. and Keshet, E. (1998). Role of HIF-1α in hypoxia mediated apoptosis, cell proliferation and tumour angiogenesis. Nature. *394*: 485-490.

Caro, J., Zon, L., Silver, R., Miller, O. and Erslev, A.J. (1983). Erythropoietin in liver tissue extracts and in liver perfusates from hypoxic rats. Am. J. Physiol. *241*: E431-E434.

Carrero, P., Okamoto, K., Coumailleau. P., O'Brien, S, Tanaka, H. and Poellinger, L. (2000). Redox regulated recruitment of the transcriptional coactivators CREB binding protein and SRC-1 to hypoxia inducible factor 1α. Mol. Cell. Biol. *20*: 402-415.

Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P. and Gurtner, G.C. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SFD-1. Nat. Med. *10*: 858-864.

Chandel, N.S., McClintock, D.S., Feliciano, C.E., Wood, T.M., Melendez, J.A., Rodriguez, A.M. and Schumacker, P.T. (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia. J. Biol. Chem. *275*: 25130-25138.

Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C. and Schumacker, P.T. (1998). Mitochondrial reactive oxygen species trigger hypoxia induced transcription. Proc. Natl. Acad. Sci. U.S.A. *95*: 11715-11720.

Chauvet, C., Bois-Joyeux, B., Berra, E., Pouysségur, J. and Danan, J.L. (2004). The gene encoding human retinoic acid receptor related orphan receptor (ROR) α is a target for hypoxia inducible factor 1. Biochem. J. *384*: 79-85.

Chazaud, B., Ricoux, R., Christov, C., Plonquet, A., Gherardi, R.K., Barlovatz-Meimon, G. (2002). Promigratory effect of plasminogen activator inhibitor 1 on invasive breast cancer cell populations. Am. J. Pathol. *160*: 237-246.

Chen, D., Li, M., Luo, J. and Gu, W. (2003). Direct interactions between HIF-1α and Mdm-2 modulate p53 function. J. Biol. Chem. *273*: 11995-11998.

Cho, H.Y., Jedlicka, A.E., Reddy, S.P., Kensler, T.W., Yamamoto, M., Zhang, L.Y., Kleeberger, S.R. (2002). Role of Nrf2 in protection against hyperoxic lung injury in mice. Am. J. Respir. Cell Mol. Biol. *26*: 175-182.

Choi, J.W., Park, S.C., Kang, G.H., Liu, J.O. and Youn, H.D. (2004). Nur77 activated by hypoxia inducible factor 1 overproduces proopiomelanocortin in von Hippel-Lindau mutated renal cell carcinoma. Cancer Res. *64*: 35-9.

Clerch, L.B. (2000). Posttranscriptional regulation of lung antioxidant enzyme gene expression. Ann. N.Y. Acad. Sci. *899*: 103-111.

Clifford, S.C., Cockman, M.E., Smallwood, A.C., Mole, D.R., Woodward, E.R., Maxwell, P.H., Ratcliffe, P.J. and Maher E.R. (2001). Contrasting effects on HIF-1α regulation by disease causing pVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease. Hum. Mol. Genet. *10*: 1029-1038.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J. and Maxwell, P.H. (2000). Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumour suppressor protein. J. Biol. Chem. *275*: 25733-25741.

Cohen, Y. and Wao, T.P. (2004). AcK-knowledge reversible acetylation. Science's STKE: http://www.stke.org/cgi/content/full/sigtrans;2004/245/pe42.

Comerford, K.M., Wallace, T.J., Louis, N.A., Montalto, M.C. and Colgan, S.P. (2002). Hypoxia inducible factor 1 dependent regulation of the multidrug resistance (*MDR1*) gene. Cancer Res. *62*: 3387-3394.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D. and Carmeliet, P. (2002). Loss of HIF-2α and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat. Med. *8*: 702-710.

Conforti, L. and Millhorn, D.E. (1997). Selective inhibition of a slow inactivating voltage dependent K^+ channel in rat PC12 cells by hypoxia. J. Physiol. *502*: 293-305.

Conrad, P.W., Freeman, T.L., Beitner-Johnson, D. and Millhorn, D.E. (1999). EPAS1 transactivation during hypoxia requires p42/p44 MAPK. J. Biol. Chem. *274*: 33709-33713.

Cormier-Regard, S., Nguyen, S.V. and Claycomb, W.C. (1998). Adrenomedullin gene expression is developmentally regulated and induced by hypoxia in rat ventricular cardiac myocytes. J. Biol. Chem. *273*: 17787-17792.

Curcio-Morelli, C., Zavacki, A.M., Christofollete, M., Gereben, B., de Freitas, B.C.G., Harney, J.W., Li, Z. Wu, G. and Bianco, A.C. (2003). Deubiquitination of type 2 iodothryonine deiodinase by von Hippel-Lindau protein interacting deubiquitinating enzymes regulates thyroid hormone activation. J. Clin. Invest. *112*: 189-196.

Coulet, F., Nadaud, S., Agrapart, M. and Soubrier, F. (2003). Identification of hypoxia response element in the endothelial nitric oxide synthase gene promoter. J. Biol. Chem. *278*: 46230-46240.

Czyzyk-Krzeska, M.F. and Beresh, J.E. (1996). Characterization of the hypoxia inducible protein binding site within the pyrimidine rich tract in the 3' untranslated region of the tyrosine hydroxylase mRNA. J. Biol. Chem. *271*: 3293-3299.

Czyzyk-Krzeska, M.F., Dominski, Z., Kole, R. and Millhorn, D.E. (1994b). Hypoxia stimulates binding of a cytoplasmic protein to a pyrimidine rich sequence in the 3' untranslated region of rat tyrosine hydroxylase mRNA. J. Biol. Chem. *269*: 9940-9945.

Czyzyk-Krzeska, M.F., Furnari, B.A., Lawson, E.E. and Millhorn, D.E. (1994a). Hypoxia increases rate of transcription and stability of tyrosine hydroxylase mRNA in pheochromocytoma (PC12) cells. J. Biol. Chem. *269*: 760-764.

Czyzyk-Krzeska, M.F., Bayliss, D.A., Lawson, E.E. and Millhorn, D.E. (1992). Regulation of tyrosine hydroxylase gene expression in the rat carotid body by hypoxia. J. Neurochem. *58*: 1538-1546.

Dames, S.A., Martinez-Yamout, M., De Guzman, R.N., Dyson, J., and Wright, P.E. (2002). Structural basis for Hif-1α/CBP recognition in the cellular hypoxic response. Proc. Natl. Acad. Sci. U.S.A. *99*: 5271-5276.

D'Angelo, G., Duplan, E., Boyer, N., Vigne, P. and Frelin, C. (2003). Hypoxia upregulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J. Biol. Chem. *278*: 38183-38187.

Dann, C.E., Bruick, R.K. and Deisenhofer, J. (2003). Structure of factor inhibiting hypoxia inducible factor 1: an asparaginyl hydroxylase involved in the hypoxic response pathway. Proc. Natl. Acad. Sci. U.S.A. *99*: 15351-15356.

Datta, K., Li, J., Bhattacharya, R., Gasparian, L., Wang, E. and Mukhopadhyay, D. (2004). Protein kinase C ζ transactivates hypoxia inducible factor α by promoting its asscociation with p300 in renal cancer. Cancer Res. *64*: 456-462.

Declerck, P.J., De Mol, M., Alessi, M.C., Baudner, S., Pâques, E.P., Preissner, K.T., Müller-Berghaus, G. and Collen, D. (1988). Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. J. Biol. Chem. *263*: 15454-15461.

del Peso, L., Castellanos, M.C., Temes, E., Martín-Puig, S.M., Cuevas, Y., Olmos, G. and Landázuri, M.O. (2003). The von Hippel-Lindau/hypoxia inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen. J. Biol. Chem. *278*: 48690-48695.

Del Toro, R., Levitsky, K.L., López-Barneo, J and Chiara, M.D. (2003). Induction of T-type calcium channel gene expression by chronic hypoxia. J. Biol. Chem. *278*: 22316-22324.

Digicaylioglu, M. and Lipton, S.A. (2001). Erythropoietin mediated neuroprotection involves cross talk between Jak2 and NF-κB signalling cascades. Nature. *412*: 641-647.

Dinchuk, J.E., Focht, R.J., Kelley, J.A., Henderson, N.L., Zolotarjova, N.I., Wynn, R., Neff, N.T., Link, J., Huber, R.M., Burn, T.C., Rupar, M.J., Cunningham, M.R., Selling, B.H., Ma, J., Stern, A.A., Hollis, G.F., Stein, R.B. and Friedman, P.A. (2002). Absence of post-translational aspartyl β-hydroxylation of epidermal growth factor domains in mice leads to developmental defects and an increased incidence in intestinal neoplasia. J. Biol. Chem. *277*: 12970-12977.

Ebert, B.L. and Bunn, H.F. (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. Mol. Cell. Biol. *18*: 4089-4096.

Ebert, B.L., Firth, J.D. and Ratcliffe, P.J. (1995). Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter 1 via distinct *cis* acting sequences. J. Biol. Chem. *270*: 29083-29089.

Eckhart, A.D., Yang, N., Xin, X. and Faber, J.E. (1997). Characterisation of the α_{1B} adrenergic receptor gene promoter region and hypoxia regulatory elements in smooth muscle. Proc. Natl. Acad. Sci. U.S.A. *94*: 9487-9492.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalchin, A., Weber, K. and Tuschl, T. (2001b). Duplexes of 21 nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. *411*: 494-498.

Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001a). RNA interference is mediated by 21 and 22 nucleotide RNAs. Genes Dev. *15*: 188-200.

Elkins J.M., Hewitson, K.S., McNeill, L.A., Seibel, J.F., Sclemminger, I., Pugh, C.W., Ratcliffe, P.J. and Schofield, C.J. (2003). Structure of factor inhibiting hypoxia inducible factor (HIF) reveals mechanism of oxidative modification of HIF-1α. J. Biol. Chem. *278*: 1802-1806.

Elson, D.A., Thurston, G., Huang, L.E., Ginzinger, D.G., McDonald, D.M., Johnson, R.S. and Arbeit, J.M. (2001). Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia inducible factor-1α. Genes Dev. *15*: 2520-2532.

Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L. and Fujii-Kuriyama, Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF-1α in response to hypoxia: their stabilization and redox signal induced interaction with CBP/p300. EMBO J. *18*: 1905-1914.

Ema, M., Taya, S., Tokotani, N., Sogowa, K., Matsuda, Y. and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia inducible factor 1α regulates VEGF expression and is potentially involved in lung and vascular development. Proc. Natl. Acad. Sci. U.S.A. *94*: 4273-4278.

Epstein, A.C.R., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Barsteas, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. and Ratcliffe, P.J. (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. *107*: 43-54.

Estes, S.D., Stoler, D.L. and Anderson, G.R. (1995). Anoxic induction of a sarcoma virus related VL30 retrotransposon is mediated by a *cis* acting element which binds hypoxia inducible factor 1 and an anoxia inducible factor. J. Virol. *69*: 6335-6341.

Feldser, D., Agani, F., Iyer, N.V., Pak, B., Ferreira, G. and Semenza, G.L. (1999). Reciprocal positive regulation of hypoxia inducible factor 1α and insulin like growth factor 2. Cancer Res. *59*: 3915-3918.

Firth, J.D., Ebert, B.L. and Ratcliffe, P.J. (1995). Hypoxic regulation of lactate dehydrogenase A: interaction between hypoxia inducible factor 1 and cAMP response elements. J. Biol. Chem. *270*: 21021-21027.

Firth, J.D., Ebert, B.L., Pugh, C.W. and Ratcliffe, P.J. (1994). Oxygen regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. Proc. Natl. Acad. Sci. U.S.A. *91*: 6496-6500.

Fishman, M., Greene, W.L. and Platika, D. (1985). Oxygen chemoreception by carotid body cells in culture. Proc. Natl. Acad. Sci. U.S.A. *82*: 1448-1450.

Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A. and Risau, R. (1997). HRF, a putative basic helix-loop-helix-PAS domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. Mech. Dev. *63*: 51-60.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D. and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia inducible factor 1. Mol. Cell. Biol. *16*: 4604-4613.

Freedman, S.J., Sun, Z.Y.J., Poy, F., Kung, A.L., Livingston, D.M., Wagner, G. and Eck, M.J. (2002). Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1α. Proc. Natl. Acad. Sci. U.S.A. *99*: 5367-5372.

Furuta, G.T., Turner, J.R., Taylor, C.T., Hershberg, R.M., Comerford, K., Narravula, S., Podolsky, D.K. and Colgan, S.P. (2001). Hypoxia inducible factor 1 dependent induction of intestinal trefoil factor protects barrier function during hypoxia. J. Exp. Med. *193*: 1027-1034.

Gao, L., Mejías, R., Echevarría, M. and López-Barneo, J. (2004). Induction of the glucose-6-phosphatase dehydrogenase gene expression by chronic hypoxia in PC12 cells. FEBS Lett. *569*: 256-260.

Gerber, H.P., Condorelli, F., Park. J. and Ferrara, N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes: Flt-1, but not Flk-1/KDR, is upregulated by hypoxia. J. Biol. Chem. *272*: 23659-23667.

Gess, B., Hofbauer, K.H., Deutzman, R. and Kurtz, A. (2004). Hypoxia upregulates triosephosphate isomerase expression via a HIF dependent pathway. Eur. J. Physiol. *448*: 175-180.

Gess, B., Hofbauer, K.H., Wenger, R.H., Lohaus, C., Meyer, H.E. and Kurtz, A. (2003). The cellular oxygen tension regulates expression of the endoplasmic oxidoreductase ERO1-Lα. Eur. J. Biochem. *270*: 2228-2235.

Giandomenico, V., Simonsson, M., Gronroos, E. and Ericsson, J. (2003). Coactivator-dependent acetylation stabilizes members of the SREBP family of transcription factors. Mol. Cell. Biol. *23*: 2587-2599.

Gidday, J.M., Fitzgibbons, J.C., Shah, A.R. and Park, T.S. (1994). Neuroprotection from ischaemic brain injury by hypoxic preconditioning in the neonatal rat. Neurosci. Lett. *168*: 221-224.

Gnarra, J.R., Zhou, S., Merrill, M.J., Wagner, J.R., Krumm, A., Papavassiliou, E., Oldfield, E.H., Klausener, R.D. and Linehan, W.M. (1996). Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the *VHL* tumor suppressor gene. Proc. Natl. Acad. Sci. U.S.A. *93*: 10589-10594.

Goldberg, M.A. and Schneider, T.J (1994). Similarities between the oxygen sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J. Biol. Chem. *269*: 4355-4359.

Goldberg, M.A., Dunning, P. and Bunn, H.F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science. *242*: 1412-1415.

Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W. and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science. *268*: 1766-1769.

Gradin, K., Takasaki, C., Fujii-Kuriyama, Y. and Sogawa, K. (2002). The transcriptional activation function of the HIF-like factor requires phosphorylation at a conserved threonine. J. Biol. Chem. *277*: 23508-23514.

Graven, K.K., Yu, Q., Pan, D., Roncarati, J.S. and Farber, H.W. (1999). Identification of an oxygen response enhancer element in the glyceraldyde-3-phosphate dehydrogenase gene. Biochem. Biophys. Acta. *1447*: 208-218.

Greene, L.A. and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Nat. Acad. Sci. U.S.A. *73*: 2424-2428.

Grimm, C., Wenzel, A., Groszer, M., Mayser, H., Seeliger, M., Samardzija, M., Bauer, C., Gassmann, M. and Remé, C.E. (2002). HIF-1 induced erythropoietin in the hypoxic retina protects against light induced retinal degeneration. Nat. Med. *8*: 718-724.

Gronroos, E., Hellman, U., Heldin, C.H. and Ericsson, J. (2002). Control of Smad7 stability by competition between acetylation and ubiquitination. Mol. Cell. *10*: 483-493.

Grosfeld, A., André, J., Cauzac, M., Hauguel-de Mouzon, S., Berra, E., Pouysségur, J. and Guerre-Millo, M. (2002). Hypoxia inducible factor 1 transactivates the human leptin gene promoter. J. Biol. Chem. *277*: 42953-42957.

Gu, J., Milligan, J. and Huang, L.E. (2001). Molecular mechanism of hypoxia inducible factor 1α-p300 interaction: a leucine-rich interface regulated by a single cysteine. J. Biol. Chem. *276*: 3550-3554.

Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L. and Bradfield, C.A. (1998). Molecular characterisation and chromosomal localization of a third α class hypoxia inducible factor subunit, HIF-3α. Gene Expr. *7*: 205-213.

Guillemin, K. and Krasnow, M.A. (1997). The hypoxic response: huffing and HIFing. Cell. *89*: 9-12.

Hagen, T., Taylor, C.T., Lam, F. and Moncada, S. (2003). Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF-1α. Science. *302*: 1975-1978.

Hannon, G.J. and Rossi, J.J. (2004). Unlocking the potential of the human genome with RNA interference. Nature. *431*: 371-378.

Hara, S., Kobayashi, C. and Imura, N. (1999). Nuclear localisation of hypoxia inducible factor 2α in bovine arterial endothelial cells. Mol. Cell. Biol. Res. Commun. *2*: 119-123.

Hayden, M.S. and Ghosh, S. (2004). Signalling to NF-κB. Genes Dev. *18*: 2195-2224.

Hewitson, K.S., McNeill, L.A., Riordan, M.V., Tian, Y.M., Bullock, A.N., Welford, R.W., Elkins, J.M., Oldham, N.J., Bhattacharya, S., Gleadle, J.M., Ratcliffe, P.J., Pugh, C.W. and Schofield, C.J. (2002). Hypoxia inducible factor (HIF) asparaginyl hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J. Biol. Chem. *277*: 26351-26355.

Hinrichs, W., Kisker, C., Düvel, M., Müller, A., Tovar, K., Hillen, W. and Saenger, W. (1994). Structure of the Tet repressor tetracycline complex and regulation of antibiotic resistance. Science. *264*: 418-420.

Hirsilä, M., Koivunen, P., Günzler, V., Kivirikko, K.I. and Myllyharju, J. (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia inducible factor. J. Biol. Chem. *278*: 30772-30780.

Ho, Y.S., Dey, M.S. and Crapo, J.D. (1996). Antioxidant enzyme expression in rat lungs during hyperoxia. Am. J. Physiol. *270*: L810-L818.

Hoffman, M.A., Ohh, M., Yang, H., Klco, J.M., Ivan, M. and Kaelin, W.G. (2001). Von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. Hum. Mol. Genet. *10*: 1019-1027.

Hogenesch, J.B., Chan, W.K., Jackiw, V.H., Brown, R.C., Gu, Y.Z., Pray-Grant, M., Perdew, G.H. and Bradfield, C.A. (1997). Characterisation of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signalling pathway. J. Biol. Chem. *272*: 8581-8593.

Hon, W.C., Wilson, M.I., Harlos, K., Claridge, T.D.W., Schofield, C.J., Pugh, C.W., Maxwell, P.H., Ratcliffe, P.J., Stuart, D.I. and Jones, E.Y. (2002). Structural basis for the recognition of hydroxyproline in HIF-1α by pVHL. Nature. *417*: 975-978.

Horio, T., Nishikimi, T., Yoshihara, F., Nagaya, N., Matsuo, H., Takishita, S. and Kangawa, K. (1998). Production and secretion of adrenomedullin in cultured rat cardiac myocytes and non-myocytes: stimulation by interleukin 1β and tumour necrosis factor α. Endocrinology. *139*: 4576-4580.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B. and Celeste Simon, M. (2003). Differential roles of hypoxia inducible factor 1α (HIF-1α) and HIF-2α in hypoxic gene regulation. Mol. Cell. Biol. *23*: 9361-9374.

Hu, J., Discher, D.J., Bishopric, N.H. and Webster, K.A. (1998). Hypoxia regulates expression of the endothelin 1 gene through a proximal hypoxia inducible factor 1 binding site on the antisense strand. Biochem. Biophys. Res. Commun. *245*: 894-899.

Huang, J., Zhao, Q., Mooney, S.M. and Lee, F.S. (2002). Sequence determinants in hypoxia inducible factor 1α for hydroxylation by the prolyl hydroxylases PHD1, PHD2 and PHD3. J. Biol. Chem. *277*: 39792-39800.

Huang, L.E., Willmore, W.G., Gu, J., Goldberg, M.A. and Bunn, H.F. (1999). Inhibition of hypoxia inducible factor 1 activation by carbon monoxide and nitric oxide: implications for oxygen sensing and signalling. J. Biol. Chem. *274*: 9038-9044.

Huang, L.E., Gu, J., Schau, M. and Bunn, H.F. (1998). Regulation of hypoxia inducible factor 1α is mediated by an O_2 dependent degradation domain via the ubiquitin proteasome pathway. Proc. Natl. Acad. Sci. U.S.A. *95*: 7987-7992.

Huang, L.E., Arany, Z., Livingston, D.M. and Bunn, H.F. (1996). Activation of hypoxia inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. J. Biol. Chem. *271*: 32253-32259.

Iliopoulos, O., Levy, A.P., Jiang, C., Kaelin, W.G. and Goldberg, M.A. (1996). Negative regulation of hypoxia inducible genes by the von Hippel-Lindau protein. Proc. Natl. Acad. Sci. U.S.A. *93*: 10595-10599.

Ischiropoulos, H., Duran, D. and Horwitz, J. (1995). Peroxynitrite mediated inhibition of DOPA synthesis in PC12 cells. J. Neurochem. *65*: 2366-2372.

Ishii, T., Itoh, K,. Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S. and Yamamoto, M. (2000). Transcription factor Nrf2 coordinately regulates a group of oxidative stress inducible genes in macrophages. J. Biol. Chem. *275*: 16023-16029.

Ishiyama, Y., Kitamura, K., Ichiki, Y., Sakata, J., Kida, O., Kangawa, K. and Eto, T. (1995). Haemodynamic responses to rat adrenomedullin in anesthetised spontaneously hypertensive rats. Clin. Exp. Pharmacol. Physiol. *22*: 614-618.

Ito, A., Kawaguchi, Y., Lai, C.H., Kovacs, J.J., Higashimoto, Y., Appealla, E. and Yao, T.P. (2002). MDM2-HDAC1 mediated deactylation of p53 is required for its degradation. EMBO J. *21*: 6236-6245.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D. and Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant response element by Nrf2 through binding to the amino terminal Neh2 domain. Genes Dev. *13*: 76-86.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin, W.G. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science. *292*: 464-468.

Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R.C., Conaway, J.W., Klausener, R.D. and Pause, A. (1999). Identification of the von Hippel-Lindau tumor suppressor protein as part of an active E3 ubiquitin ligase complex. Proc. Natl. Acad. Sci. U.S.A. *96*: 12436-12441.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassman, M., Gearhart, J.D., Lawler, A.M. Yu, A.Y. and Semenza, G.L. (1998). Cellular and developmental control of O_2 homeostasis by hypoxia inducible factor 1α. Genes Dev. *12*: 149-162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., von Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science. *292*: 468-472.

Jacobsen, L.O., Goldwasser, E., Fried, W. and Plzak, L. (1957). Role of the kidney in erythropoiesis. Nature. *179*: 633-634

Jaiswal, A.K. (2004). Nrf signalling in coordinated activation of antioxidant gene expression. Free Radic. Biol. and Med. *36*: 1199-1207.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J. and Kim, K.W. (2002). Regulation and destabilization of HIF-1α by ARD1 mediated acetylation. Cell. *111*: 709-720.

Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H. and Gassman, M. (2001). Induction of HIF-1α in response to hypoxia is instantaneous. FASEB J. *15*: 1312-1314.

Jiang, H., Guo, R. and Powell-Coffman, J.A. (2001). The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc. Natl. Acad. Sci. U.S.A. *98*: 7916-7921.

Jiang, B.H., Zheng, J.Z., Leung, S.W., Roe, R. and Semenza, G.L. (1997). Transactivation and inhibitory domains of hypoxia inducible factor 1α: modulation of transcriptional activity by oxygen tension. J. Biol. Chem. *272*: 19253-19260.

Jiang, B.H., Semenza, G.L., Bauer, C. and Marti, H.M. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. Am. J. Physiol. Cell Physiol. *271*: C1172-C1180.

Kallio, P.J., Wilson, W.J., O'Brien, S., Makino, Y. and Poellinger, L. (1999). Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin-proteasome pathway. J. Biol. Chem. *274*: 6519-6525.

Kallio, P.J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia inducible factor-1α. EMBO J. *17*: 6573-6586.

Kallio, P.J., Pongratz, I., Gradin, K., McGuire, J. and Poellinger, L. (1997). Activation of the hypoxia inducible factor 1α: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc. Natl. Acad. Sci. U.S.A. *94*: 5667-5672.

Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R.C. and Conaway, J.W. (2000). Activation of HIF1α ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc. Natl. Acad. Sci. U.S.A. *97*: 10430-10435.

Kappel, A., Rönicke, V., Damert, A., Flamme, I., Risau, W. and Breier, G. (1999). Identification of vascular endothelial growth factor (VEGF) receptor 2 (*Flk-1*) promoter/enhancer sequences sufficient for angioblast and endothelial cell specific transcription in transgenic mice. Blood. *12*: 4284-4292.

Kageyama, Y., Koshiji, M., To, K.K.W., Tian, Y.M., Ratcliffe, P.J. and Huang, L.E. (2004). Leu-574 of human HIF-1α is a molecular requirement of prolyl hydroxylation. FASEB J. *18*: 1028-1030.

Kewley, R.J., Whitelaw, M.L. and Chapman-Smith, A. (2004). The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. Int. J. Biochem. Cell Biol. *36*: 189-204.

Kietzmann, T., Roth, U. and Jungermann, K. (1999). Induction of the plasminogen activator inhibitor 1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia inducible factor 1 in rat hepatocytes. Blood. *94*: 4177-4185.

Kim, J.Y., Ahn, H.J., Ryu, J.H., Suk, K. and Park, J.H. (2004). BH3 only protein Noxa is a mediator of hypoxic cell death induced by hypoxia factor 1α. J. Exp. Med. *199*: 113-123.

Kitamura, K., Kanagawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H. and Eto, T. (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. *192*: 553-560.

Kivirikko, K. and Myllyharju, J. (1998). Prolyl 4-hydroxylases and their protein disulfide isomerase unit. Matrix Biol. *16*: 357-368.

Kline, D.D., Peng, Y.J., Manalo, D.J., Semenza, G.L. and Prabhakar, N.R. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia inducible factor 1α. Proc. Natl. Acad. Sci. U.S.A. *99*: 821-826.

Koivunen, P., Hirsilä, M., Günzler, V., Kivirikko, K.I. and Myllyharju, J. (2004). Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of the prolyl-4-hydroxylases. J. Biol. Chem. *279*: 9899-9904.

Kojima, H., Gu, H., Nomura, S., Caldwell, C.C., Kobata, T., Carmeliet, P., Semenza, G.L. and Sitkovsky, M.V. (2002). Abnormal B lymphocyte development and autoimmunity in hypoxia inducible factor 1α deficient chimeric mice. Proc. Natl. Acad. Sci. U.S.A. *99*: 2170-2174.

Kondo, K., Kim, W.Y., Lechpammer, M. and Kaelin, W.G. (2003). Inhibition of HIF2α is sufficient to suppress pVHL defective tumour growth. PLOS Biol. *1*: 439-444.

Kondo, K., Klco, J., Nakamura, E., Lechpammer, M. and Kaelin, W.G. (2002). Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell. *1*: 237-246.

Kouzarides, T. Acetylation: a regulatory mechanism to rival phosphorylation? EMBO J. *19*: 1176-1179.

Krishnamachary, B., Berg-Dixon, S., Kelly, B., Agani, F., Feldser, D., Ferreira, G., Iyer, N., LaRusch, J., Pak, B., Taghavi, P. and Semenza, G.L. (2003). Regulation of colon carcinoma cell invasion by hypoxia inducible factor 1. Cancer Res. *63*: 1138-1143.

Kroll, S.L., Paulding, W.R., Schnell, P.O., Barton, M.C., Conaway, J.W., Conaway, R.C. and Czyzyk-Krzeska, M.F. (1999). von Hippel-Lindau protein induces hypoxia regulated arrest of tyrosine hydroxylase transcript elongation in pheochromocytoma cells. J. Biol. Chem. *274*: 30109-30114.

Kuhn, D.M., Aretha, C.W. and Geddes, T.J. (1999). Peroxynitrite inactivation of tyrosine hydroxylase; mediation by sulfhydryl oxidation, not tyrosine nitration. J. Neurosci. *19*: 10289-10294.

Kumar, G.K., Overholt, J.L., Bright, G.R., Hui, K.Y., Lu, H., Gratzl, M. and Prabhakar, N.R. (1998). Release of dopamine and norepinephrine by hypoxia from PC12 cells. Am. J. Physiol. Cell Physiol. *274*: C1592-C1600.

Kumer, S.C. and Vrana, K.E. (1996). Intricate regulation of tyrosine hydroxylase activity and gene expression. J. Neurochem. *67*: 443-462.

Kung, A.L., Zabludoff, S.D., France, D.S., Freedman, S.J., Tanner, E.A., Vieira, A., Cornell-Kennon, S., Lee, J., Wang, B., Wang, J., Memmert, K., Naegeli, H.U., Petersen, F., Eck, M.J., Bair, K.W., Wood, A.W. and Livingston, D.M. (2004). Small molecule blockade of transcriptional coactivation of the hypoxia inducible factor pathway. Cancer Cell. *6*: 33-43.

Kung, A.L., Wang, S., Klco, J.M., Kaelin, W.G. and Livingston, D.M. (2000). Suppression of tumour growth through disruption of hypoxia inducible transcription. Nat. Med. *6*: 1335-1340.

Kuznetsova, A.V., Meller, J., Schnell, P.O., Nash, J.A., Ignacak, M.L., Sanchez, Y., Conaway, J.W., Conaway, R.C. and Czyzyk-Krzeska, M.F. (2003). von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. Proc. Natl. Acad. Sci. U.S.A. *100*: 2706-2711.

Lancaster, D.E., McNeill, L.A., McDonough, M.A., Aplin, R.T., Hewitson, K.S., Pugh, C.W., Ratcliffe, P.J. and Schofield, C.J. (2004). Disruption of dimerisation and substrate phosphorylation inhibit factor inhibiting hypoxia inducible factor (FIH) activity. Biochem. J. *363*: 429-437.

Lando, D., Peet, D., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., Bruick, R.K. (2002b). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. *16*: 1466-1471.

Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J. and Whitelaw, M.L. (2002a). Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. Science. *295*: 858-861.

Lando, D., Pongratz, I., Poellinger, L. and Whitelaw, M.L. (2000). A redox mechanism controls differential DNA binding activities of hypoxia inducible factor (HIF) 1α and HIF-like-Factor. J. Biol. Chem. *275*: 4618-4627.

Lee, C., Kim, S.J., Jeong, D.G., Lee, S.M. and Ryu, S.E. (2003). Structure of human FIH-1 reveals a unique active site pocket and interaction sites for HIF-1 and von Hippel Lindau. J. Biol. Chem. *278*: 7558-7563.

Lee, M.J., Kim, J.Y., Suk, K. and Park, J.H. (2004). Identification of the hypoxia inducible factor 1α responsive *HGTD-P* gene as a mediator in the mitochondrial apoptotic pathway. Mol. Cell. Biol. *24*: 3918-3927.

Lee, P.J., Jiang, B.H., Chin, B.Y., Iyer, N.V., Alam, J., Semenza, G.L. and Choi, A.M. (1997). Hypoxia inducible factor 1 mediates transcriptional activation of the heme oxygenase 1 gene in response to hypoxia. J. Biol. Chem. *272*: 5375-5381.

Levy, A.P., Levy, N.S., Wegner, S. and Goldberg, M.A. (1995). Regulation of the rat vascular endothelial growth factor gene by hypoxia. J. Biol. Chem. *270*: 13333-13340.

Li, Z., Wang, D., Na, X. Schoen, S.R., Messing, E.M. and Wu, G. (2002b). Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumour suppressor. Biochem. Biophys. Res. Commun. *294*: 700-709.

Li, Z., Na, X., Wang, D., Schoen, S., Messing, E.M. and Wu, G. (2002a). Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von-Hippel Lindau tumor suppressor protein. J. Biol. Chem. *277*: 4656-4662.

Lin, S., Tsai, S.C., Lee, C.C., Wang, B.W., Liou, J.Y. and Shyu, K.G. (2004). Berberine inhibits HIF-1α expression via enhanced proteolysis. Mol. Pharmacol. *66*: 612-619.

Linke, S., Stojkoski, C., Kewley, R.J., Booker, G.W., Whitelaw, M.L. and Peet, D.J. (2004). Substrate requirements of the oxygen-sensing asparaginyl hydroxylase factor inhibiting hypoxia inducible factor. J. Biol. Chem. *279*: 14391-14397.

Lisztwan, J., Imbert, G., Wirbeleauer, C., Gstaiger, M. and Krek, W. (1999). The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin protein ligase activity. Genes Dev. *13*: 1822-1833.

Liu, Y., Cox, S.R., Morita, T. and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells: identification of a 5' enhancer. Circ. Res. *77*: 638-643.

Liu, Y.L., Ang, S.O., Weigent, D.A., Prchal, J.T. and Bloomer, J.R. (2004). Regulation of ferrochelatase gene expression by hypoxia. Life Sci. *75*: 2035-2043.

Löfstedt, T., Jögi, A., Sigvardsson, M., Gradin, K., Poellinger, L., Påhlman, S. and Axelson, H. (2004). Induction of *ID2* expression by hypoxia inducible factor 1: a role in dedifferentiation of hypoxic neuroblastoma cells. J. Biol. Chem. *279*: 39223-39231.

Lok, C.N. and Ponka, P. (1999). Identification of a hypoxia response element in the transferrin receptor gene. J. Biol. Chem. *274*: 24147-24152.

López-Barneo, J., Pardal, R. and Ortega-Sáenz, P. (2001). Cellular mechanism of oxygen sensing. Annu. Rev. Physiol. *63*: 259-287.

López-Barneo, J., López-López, J.R., Ureña, J. and González, C. (1988). Chemotransduction in the carotid body: K^+ current modulated by PO_2 in type I chemoreceptor cells. Science. *241*: 580-582.

Lu, H., Forbes, R.A. and Verma, A. (2002). Hypoxia-inducible 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J. Biol. Chem. *277*: 23111-23115.

Lukiw, W.J., Gordon, W.C., Rogaev, E.I., Thompson, H. and Bazan, N.G. (2001). Presinilin -2 (PS-2) expression upregulated in a model of retinopathy of prematurity and pathoangiogenesis. Neuroreport. *12*: 53-57.

Luo, J.C. and Shibuya, M. (2001). A variant of nuclear localisation signal bipartite type is required for the nuclear translocation of hypoxia inducible factors (1α, 2α and 3α). Oncogene. *20*: 1435-1444.

Mahon, P.C., Hirota, K. and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. *12*: 2675-2686.

Majid, D.S.A., Kadowitz, P.J., Coy, D.H. and Navar, L.G. (1996). Renal responses to intraarterial administration of adrenomedullin in dogs. Am. J. Physiol. *270*: F200-F205.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H. and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia inducible splice variant of the hypoxia inducible factor 3α locus. J. Biol. Chem. *277*: 32405-32408.

Makino, Y., Cao, R., Svensson, K., Bertilsson, G., Åsman, M., Tanaka, H., Cao, Y., Berkenstam, A. and Poellinger, L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature. *414*: 550-554.

Maranchie, J.K., Vasselli, J.R., Riss, J., Bonifacino, J.S., Linehan, W.M. and Klausener, R.D. (2002). The contribution of VHL substrate binding and HIF1-α to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell. *1*: 247-255.

Martínez-Balbás, M., Bauer, U.M., Nielsen, S.J., Brehm, A. and Kouzarides, T. (2000). Regulation of E2F1 activity by acetylation. EMBO J. *19*: 661-671.

Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation. EMBO J. *20*: 5197-5206.

Mateo, J., García-Lecea, M., Cadenas, S., Hernández, C. and Moncada, S. (2003). Regulation of hypoxia inducible factor 1α by nitric oxide through mitochondria dependent and independent pathways. Biochem. J. *376*: 537-544.

Mathupala, S.P., Rempel, A. and Pederson, P.L. (2001). Glucose catabolism in cancer cells: identification and characterisation of a marked activation response of the type II hexokinase gene to hypoxic conditions. J. Biol. Chem. *276*: 43407-43412.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wycoff, C.C., Pugh, C.W., Maher, E.R. and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia inducible factors for oxygen dependent proteolysis. Nature. *399*: 271-275 (1999).

Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (1993). Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen sensing mechanism. Proc. Natl. Acad. Sci. U.S.A. *90*: 2423-2427.

McMahon, M., Itoh, K., Yamamoto, M. and Hayes, J.D. (2003). Keap1 dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of ARE driven gene expression. J. Biol. Chem. *278*: 21592-21600.

McNeill, L.A., Hewitson, K.S., Claridge, T.D., Seibel, J.F., Horsfall, L.E. and Schofield, C.J. (2002). Hypoxia inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the β carbon of asparagines 803. Biochem. J. *367*: 571-575.

Mekhail, K., Gunaratnam, L., Bonicalzi, M.E. and Lee, S. (2004). HIF activation by pH dependent nucleolar sequestration of VHL. Nat. Cell Biol. *6*: 642-647.

Melillo, G., Musso, T., Sica, A., Taylor, L.S., Cox, G.W. and Varesio, L. (1995). A hypoxia response element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J. Exp. Med. *182*: 1683-1693.

Metzen, E., Stiehl, D.P., Doege, K., Marxsen, J.H., Hellwig-Bürgel, T. and Jelkmann, W. (2004). Regulation of the *prolyl hydroxylase domain protein 2* (*phd2/egln1*) gene: identification of a functional hypoxia response element. Biochem. J. In press.

Metzen, E., Zhou, J., Jelkmann, W., Fandrery, J. and Brüne, B. (2003b). Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. Mol. Biol. Cell. *14*: 3470-3481.

Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J.H., Stolze, I., Klinger, M., Huang, W.Q., Wotzlaw, C., Hellwig-Bürgel., Jelkmann, W., Acker, H. and Fandrey, J. (2003a). Intracellular localisation of human HIF1-α hydroxylases: implications for oxygen sensing. J. Cell Sci. *116*: 1319-1326.

Min, J.H., Yang, H., Ivan, M., Gertler, F., Kaelin, W.G. and Pavletich, N.P. (2002). Structure of an HIF-1α-pVHL complex: hydroxyproline recognition in signalling. Science. *296*: 1886-1889.

Minchenko, A., Leschinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V. and Caro, J. (2002). Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (*PFKFB3*) gene. J. Biol. Chem. *277*: 6183-6187.

Minchenko, A. and Caro, J. (2000). Regulation of endothelin 1 gene expression in human microvascular endothelial cells by hypoxia and cobalt: role of the hypoxia response element. Mol. Cell. Biochem. *208*: 53-62.

Miyazaki, K., Kawamoto, T., Tanimoto, K., Nishiyama, M., Honda, H. and Kato, Y. (2002). Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. J. Biol. Chem. *277*: 47014-47021.

Morita, M., Ohneda, O., Yamashita, T., Takahashi, S., Suzuki, N., Nakajima, O., Kawauchi, S., Ema, M., Shibahara, S., Udono, T., Tomita, K., Tamai, M., Sogawa, K., Yamamoto, M. and Fujii-Kuriyama, Y. (2003). HLF/HIF-2α is a key factor in retinopathy of prematurity in association with erythropoietin. EMBO J. *22*: 1134-1146.

Morris, M.L. and Millhorn, D.E. (1995). Hypoxia induced protein binding to O_2 responsive sequences on the tyrosine hydroxylase gene. J. Biol. Chem. *270*: 23774-23779.

Muallem, S. and Wilkie, T.M. (1999). G protein dependent Ca^{2+} signalling complexes in polarized cells. Cell Calcium. *26*: 173-180.

Mukhopadhyay, C.K., Mazumder, B. and Fox, P.L. (2000). Role of hypoxia inducible factor 1 in transcriptional activation of ceruloplasmin by iron deficiency. J. Biol. Chem. *275*: 21048-21054.

Na, X., Duan, H.O., Messing, E.M., Schoen, S.R., Ryan, C.K., di Sant'Agnese, P.A., Golemis, E.A. and Wu, G. (2003). Identification of the RNA polymerase II subunit hsRPB7 as a novel target of the von Hippel-Lindau protein. EMBO J. *22*: 4249-4259.

Nagaya, N., Satoh, T., Nishikimi, T., Uematsu, M., Fururichi, S., Sakamaki, F., Oya, H., Kyotani, S., Nakanishi, N., Goto, Y., Masuda, Y., Miyatake, K. and Kangawa, K. (2000). Hemodynamic, renal and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. Circulation. *101*: 498-503.

Naranjo-Suárez, S., Castellanos, M.C., Álvarez-Tejado, M., Vara, A., Landázuri, M.O. and del Peso, L. (2003). Down-regulation of hypoxia inducible factor 2 in PC12 cells by nerve growth factor stimulation. J. Biol. Chem. *278*: 31895-31901.

Naughton, B.A., Kaplan, S.M., Roy, M., Burdowski, A.J., Gordon, A.S. and Piliero, S.J. (1977). Hepatic regeneration and erythropoietin production in the rat. Science. *196*: 301-302

Nguyen, S.V. and Claycomb, W.C. (1999). Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem. Biophys. Res. Commun. *265*: 382-386.

Nikitenko, L.L., Smith, D.M., Bicknell, R. and Rees, M.C.P. (2003). Transcriptional regulation of the CRLR gene in human microvascular endothelial cells by hypoxia. FASEB J. *17*: 1499-1501.

Nishi, H., Nakada, T., Kyo, S., Inoue, M., Shay, J.W. and Isaka, K. (2004). Hypoxia inducible factor 1 mediates upregulation of telomerase (hTERT). Mol. Cell. Biol. *24*: 6076-6083.

Oehme, F., Ellinghaus, P., Kolkhof, P., Smith, T.J., Ramakrishnan, S., Hütter, J., Schramm, M. and Flamme, I. (2002). Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia inducible transcription factors. Biochem. Biophys. Res. Commun. *296*: 343-349.

Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V. and Kaelin, W.G. (2000). Ubiquitination of hypoxia inducible factor requires direct binding to the β domain of the von Hippel-Lindau protein. Nat. Cell Biol. *2*: 423-427.

Ohh, M., Yauch, R.L., Lonergan, K.M., Whaley, J.M., Stemmer-Rachamimov, A.O., Louis, D.N., Gavin, B.J., Kley, N., Kaelin, W.G. and Iliopoulos, O. (1998). The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol. Cell. *1*: 959-968.

Oikawa, M., Abe, M., Kurosawa, H., Hida, W., Shirato, K. and Sato, Y. (2001). Hypoxia induces transcription factor ETS-1 via the activity of hypoxia inducible factor 1. Biochem. Biophys. Res. Commun. *289*: 39-43.

Okuda, H., Saitoh, K., Hirai, S., Iwai, K., Takaki, Y., Baba, M., Minato, N., Ohno, S. and Shuin, T. (2001). The von Hippel-Lindau tumour suppressor protein mediates ubiquitination of activated atypical protein kinase C. J. Biol. Chem. *276*: 43611-43617.

Okuda, H., Hirai, S., Takaki, Y., Kamada, M., Baba, M., Sakai, N., Kishida, T., Kaneko, S., Yao, M., Ohno, S. and Shuin, T. (1999). Direct interaction of the β domain of VHL tumour suppressor protein with the regulatory domain of atypical PKC isotypes. Biochem. Biophys. Res. Commun. *263*: 491-497.

O'Rourke, J.F., Tian, Y.M., Ratcliffe, P.J. and Pugh, C.W. (1999). Oxygen regulated and transactivation domains in endothelial PAS protein 1: comparison with hypoxia inducible factor 1α. J. Biol. Chem. *274*: 2060-2071.

O'Rourke, J.F., Pugh, C.W., Bartlett, S.M. and Ratcliffe, P.J. (1996). Identification of hypoxically inducible mRNAs in HeLa cells using differential display PCR: role of hypoxia inducible factor 1. Eur. J. Biochem. *241*: 403-410.

Pal, S., Datta, K., Khosravi-Far, R. and Mukhopadhyay, D. (2001). Role of protein kinase Cζ in Ras-mediated transcriptional activation of vascular permeability factor/vascular endothelial growth factor expression. J. Biol. Chem. *276*: 2395-2403.

Pal, S., Claffey, K.P., Dvorak, H.F. and Mukhopadhyay, D. (1997). The von Hippel-Lindau gene product inhibits vascular permeability factor/vascular endothelial growth factor expression in renal cell carcinoma by blocking protein kinase C pathways. J. Biol. Chem. *272*: 27509-27512.

Palmer, L.A., Semenza, G.L., Stoler, M.H. and Johns, R.A. (1998). Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am. J. Physiol. *274*: L212-219.

Park, E.C. and Szostak, J.W. (1992). ARD1 and NAT1 proteins form a complex that has N terminal acteyltransferase activity. EMBO J. *11*: 2087-2093.

Park, S.K., Dadak, A.M., Haase, V.H., Fontana, L., Giaccia, A.J. and Johnson, R.S. (2003). Hypoxia induced expression gene expression occurs solely through the action of hypoxia inducible factor 1- (HIF-1α): role of cytoplasmic trapping of HIF-2α. Mol. Cell. Biol. *23*: 4959-4971.

Paulding, W.R., Schnell, P.O., Bauer, A.L., Striet, J.B., Nash, J.A., Kuznetsova, A.V., Czyzyk-Krzeska, M.F. (2002). Regulation of gene expression for neurotransmitters during adaptation to hypoxia in oxygen sensitive neuroendocrine cells. Microsc. Res. Tech. *59*: 178-187.

Paulding, W.R. and Czyzyk-Krzeska, M.F. (1999). Regulation of tyrosine hydroxylase mRNA stability by protein binding, pyrimidine rich sequence in the 3' untranslated region. J. Biol. Chem. *274*: 2532-2538.

Peng, J., Zhang, L., Drysdale, L. and Fong, G.H. (2000). The transcription factor EPAS-1/hypoxia inducible factor 2α plays an important role vascular remodeling. Proc. Natl. Acad. Sci. U.S.A. *97*: 8386-8391.

Pierce, E.A., Avery, R.L., Foley, E.D., Aiello, L.P. and Smith, L.E.H. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularisation. Proc. Natl. Acad. Sci. U.S.A. *92*: 905-909.

Plate, K.H., Breier, G., Weich, H.A. and Risau, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. Nature. *359*: 845-848.

Poellinger, L. and Johnson, R.S. (2004). HIF-1 and hypoxic reponse: the plot thickens. Curr. Opin. Genet. Dev. *14*: 81-85.

Preissner, K.T. and Seiffert, D. (1998). Role of vitronectin and its receptors in haemostasis and vascular remodelling. Thromb Res. *89*: 1-21.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M. and Ratcliffe, P.J. (1997). Activation of the hypoxia inducible factor 1: definition of regulatory domains within the α subunit. J. Biol. Chem. *272*: 11205-11214.

Quivy, V. and Van Lint, C. (2004). Regulation of multiple levels of NF-κB mediated transactivation by protein acetylation. Biochem Pharmacol. *68*: 1221-1229.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L. and Bedi, A. (2000). Regulation of tumor angiogenesis by p53 induced degradation of hypoxia-inducible factor 1α. Genes Dev. *14*: 34-44.

Reyes, H., Reisz-Porszasz, S. and Hankinson, O. (1992). Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science. *256*: 1193-1195.

Reynolds, P.R., Mucenski, M.L., Le Cras, T.D., Nichols, W.C. and Whitsett, J.A. (2004). Midkine is regulated by hypoxia and causes pulmonary vascular remodelling. J. Biol. Chem. *279*: 37124-37132.

Richard, D.E., Berra, E., Gothié, E., Roux, D and Pouysségur, J. (1999). p42/p44 mitogen activated protein kinases phosphorylate hypoxia inducible factor 1α (HIF-1α) and enhance the transcriptional activity of HIF-1. J. Biol. Chem. *274*: 32631-32637.

Rolfs, A., Kvietikova, I., Gassman, M. and Wenger, R.H. (1997). Oxygen regulated transferring expression is mediated by hypoxia inducible factor 1. J. Biol. Chem. *272*: 20055-20062.

Rosenberger, C., Mandriotta, S., Jürgensen, J.S., Wiesner, M.S., Hörstrup, J.H., Frei, U., Ratcliffe, P.J., Maxwell, P.H., Bachman, S. and Eckardt, K.U. (2002). Expression of hypoxia inducible factor 1α and 2α in hypoxic and ischemic rat kidneys. J. Am. Soc. Nephrol. *13*: 1721-1732.

Ryan, H.E., Lo, J., Johnson, R.S. (1998). HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J. *17*: 3005-3015.

Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Ichiki, Y., Kangawa, K., Matsuo, H. and Eto, T. (1994). Distribution and characterisation of immunoreactive rat adrenomedullin in tissue and plasma. FEBS Lett. *352*: 105-108.

Salceda, S. and Caro, J. (1997). Hypoxia inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin proteasome system under normoxic conditions. J. Biol. Chem. *272*: 22642-22647.

Sandau, K.B., Fandrey, J. and Brüne, B. (2001). Accumulation of HIF-1α under the influence of nitric oxide. Blood. *97*: 1009-1015.

Sato, M., Tanaka, T., Maemura, K., Uchiyama, T., Sato, H., Maeno, T., Suga, T., Iso, T., Ohyama, Y., Arai, M., Tamura, J., Sakamoto, H., Nagai, R. and Kurabayashi, M. (2004). The PAI-1 gene as a direct target of endothelial PAS domain protein 1 in adenocarcinoma A549 cells. Am. J. Respir. Cell. Mol. Biol. *31*:209-15.

Sang, N., Fang, J., Srinivas, V., Leshchinsky, I. and Caro J. (2002). Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1α is governed by a von Hippel-Lindau protein-independent, hydroxylation regulated association with p300/CBP. Mol. Cell. Biol. *22*: 2984-2992.

Scheid, A., Wenger, R.H., Schaffer, L., Camensich, I., Distler, O., Ferenc, A, Cristina, H., Ryan, H.E., Johnson, R.S., Wagner, K.F., Stauffer, U.S., Bauer, C., Gassman, M. and Meuli, M. (2002). Physiologically low oxygen concentrations in fetal skin regulate hypoxia inducible factor 1 and transforming growth factor β3. FASEB J. *16*: 411-413.

Schmid, T., Zhou, J., Köhl, R. and Brüne, B. (2004). p300 relieves p53 evoked transcriptional repression of hypoxia inducible factor 1 (HIF-1). Biochem. J. *380*: 289-295.

Schnell, P.O., Ignacak, M.L., Bauer, A., Striet, J.B., Paulding, W.R., and Czyzyk-Krzeska, M.F. (2003). Regulation of tyrosine hydroxylase promoter activity by the von Hippel-Lindau tumor suppressor protein and hypoxia inducible transcription factors. J. Neurochem. *85*: 483-491.

Schoefield, C.J. and Ratcliffe, P.J. (2004). Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell. Biol. *5*: 343-354.

Schvartz, I., Seger, D. and Shaltiel, S. (1999). Vitronectin. Int. J. Biochem. Cell Biol. *31*: 539-544.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A., Bennett, M.J. and Garcia, J.A. (2003b). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *Epas*$^{-/-}$ mice. Nat. Genet. *35*: 331-340.

Scortegagna, M., Morris, M., Oktay, Y., Bennett, M. and Garcia, J.A. (2003a). The HIF family member EPAS/HIF-2α is required for normal hematopoiesis in mice. Blood. *102*: 1634-1640.

Semenza, G.L. (2002). HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol. Med. *8*: S62-S67.

Semenza, G.L. (2000). HIF-1 and human disease: one highly involved factor. Genes Dev. *14*: 1983-1991.

Semenza, G.L. (1998). Hypoxia inducible factor 1 and the molecular physiology of oxygen homeostasis. J. Lab. Clin. Med. *131*: 207-214.

Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P. and Gillongo, A. (1996). Hypoxia response elements in the in the aldolase A, enolase 1 and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia inducible factor 1. J. Biol. Chem. *270*: 32529-32537.

Semenza, G.L., Roth, P.H., Fang, H.M. and Wang, G.L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia inducible factor 1. J. Biol. Chem. *269*: 23757-23763.

Semenza, G.L. and Wang, G.L. (1992). A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. *12*: 5447-5454.

Semenza, G.L., Nejfelt, M.K., Chi, S.M. and Antonarakos, S.E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc. Natl. Acad. Sci. U.S.A. *88*: 5680-5684.

Seta, K.A., Spicer, Z., Yuan, Y., Lu, G. and Millhorn, D.E. (2002). Responding to hypoxia: lessons from a model cell line. Science's STKE: http://www.stke.org/cgi/content/full/sigtrans;2002/146/re11.

Shockett, P.E. and Schatz, D.G. (1996). Diverse strategies for tetracycline regulated inducible gene expression. Proc. Natl. Acad. Sci. U.S.A. *93*: 5173-5176.

Shoshani, T., Faerman, A., Mett, I., Zelin, E., Tenne, T., Gorodin, S., Moshel, Y., Elbaz, S., Budanov, A., Chajut, A., Kalinski, H., Kamer, I., Rozen, A., Mor, O., Keshet, E., Leshkowitz, D., Einat, P., Skaliter, R. and Feinstein, E. (2002). Identification of a novel hypoxia inducible factor 1 responsive gene, *RTP801*, involved in apoptosis. Mol. Cell. Biol. *22*: 2283-2293.

Shweiki, D.A., Itin, A., Soffer, D. and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia initiated angiogenesis. Nature. *359*: 843-845.

Smith, L.E., Wesolowski, E., McLellan, A., Kostyk, S.K., D'Amato, R., Sullivan, R. and D'Amore, P.A. (1994). Oxygen induced retinopathy in the mouse. Invest. Opthalmol. Vis. Sci. *35*: 101-111.

Sogawa, K., Numayama-Tsuruta, K., Ema, M., Abe, M., Abe, H. and Fujii-Kuriyama, F. (1998). Inhibition of hypoxia inducible factor 1 activity by nitric oxide donors in hypoxia. Proc. Natl. Acad. Sci. U.S.A. *95*: 7368-7373.

Sowter, H.M., Raval, R., Moore, J., Ratcliffe, P.J. and Harris, A.L. (2003). Predominant role of the hypoxia inducible transcription factor (Hif) 1α versus HIF-2α in regulation of the transcriptional response to hypoxia. Cancer Res. *63*: 6130-6134.

Sowter, H.M., Ratcliffe, P.J., Watson, P., Greenburg, A.H. and Harris, A.L. (2001). HIF-1 dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumours. Cancer Res. *61*: 6669-6673.

Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E. and Krek, W. (2003). Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour repressor pVHL. Nature. *425*: 307-311.

Stenflo, J., Holme, E., Lindstedt, S., Chandramouli, N., Tsai Huang, L.H., Tam, J.P. and Merrifield, R.B. (1989). Hydroxylation of aspartic acid in domains homologous to the epidermal growth factor precursor is catalysed by a 2-oxoglutarate-dependent dioxygenase. Proc. Natl. Acad. Sci. U.S.A. *86*: 444-447.

Stolze, I.P., Tian, Y.M., Appelhoff, R.J., Turley, H., Wykoff, C.C., Gleadle, J.M. and Ratcliffe, P.J. (2004). Genetic analysis of the role of the asparaginyl hydroxylase factor inhibiting hypoxia inducible factor (HIF) in regulating HIF transcriptional target genes. J. Biol. Chem. *279*: 42719-42725.

Synnestvedt, K., Furuta, G.T., Comerford, K.M., Louis, N., Karhausen, J., Eltzschig, H.K., Hansen, K.R., Thompson, L.F. and Colgan, S.P. (2002). Ecto-5'-nucleotidase (CD73) regulation by hypoxia inducible factor 1 mediates permeability changes in intestinal epithelia. J. Clin. Invest. *110*: 993-1002.

Tacchini, L., Bianchi, L., Bernelli-Zazzera, A. and Cairo, G. (1999). Transferrin receptor induction by hypoxia. HIF-1 mediated transcriptional activation and cell-specific post transcriptional regulation. J. Biol. Chem. *274*: 21142-24146.

Tajima, M., Katayose, D., Bessho, M. and Isoyama, S. (1994). Acute ischaemic preconditioning and chronic hypoxia independently increase myocardial tolerance to ischaemia. Cardiovasc. Res. *28*: 312-319.

Takahashi, Y., Takahashi, S., Shiga, Y., Yoshimi, T. and Miura, T. (2000). Hypoxic induction of prolyl 4-hydroxylase α(I) in cultured cells. J. Biol. Chem. *275*: 14139-14146.

Takeda, N., Maemura, K., Imai, Y., Harada, T., Kawanami, D., Nojiri, T., Manabe, I. and Nagai, R. (2004). Endothelial PAS domain protein 1 gene promotes angiogenesis through the transactivation of both vascular endothelial growth factor and its receptor, Flt-1. Circ. Res. *95*: 146-153.

Talks, K.L., Turley, H., Gatter, K.C., Maxwell, P.H., Pugh, C., Ratcliffe, P.J. and Harris, A.L. (2000). The expression and distribution of the hypoxia inducible factors HIF-1α and HIF-2α in normal human tissues, cancers and tumour associated macrophages. Am. J. Pathol. *157*: 411-421.

Tanaka, T., Akiyama, H., Kanai, H., Sato, M., Takeda, S., Sekiguchi, K., Yokoyama, T. and Kurubayashi, M. (2002). Endothelial PAS domain 1 (EPAS1) protein induces adrenomedullin gene expression in cardiac myocytes: role of EPAS1 in an inflammatory response in cardiac myocytes. J. Mol. Cell. Cardiol. *34*: 739-748.

Tanimoto, K., Makino, Y., Pereira, T. and Poellinger, L. (2000). Mechanism of regulation of the hypoxia inducible factor 1α by the von Hippel-Lindau tumour suppressor protein. EMBO J. *19*: 4298-4309.

Taylor, S.C. and Peers, C. (1998). Hypoxia evokes catecholamine secretion from rat pheochromocytoma PC12 cells. Biochem. Biophys. Res. Commun. *248*: 13-17.

Tazuke, S.I., Mazure, N.M., Sugawara, J., Carland, G., Faessen, G.H., Suen, L.F. Irwin, J.C., Powell, D.R., Giaccia, A.J. and Giudice, L.C. (1998). Hypoxia stimulates insulin like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia. Proc. Natl. Acad. Sci. U.S.A. *95*: 10188-10193.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W. and McKnight, S.L. (1998). The hypoxia responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev. *12*: 3320-3324.

Tian, H., McKnight, S.L. and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev. *11*: 72-82.

Tribioli, C., Mancini, M., Plassart, E., Bione, S., Rivella, S., Sala, C., Torri, G. and Toniolo, D. (1994). Isolation of new genes in distal Xq28: transcriptional map and identification of a human homologue of the ARD1 N-acetyltransferase of *Saccharomyces cerevisiae*. Hum. Mol. Genet. *3*: 1061-1067.

Trent, C., Tsung, N. and Horvitz, H.R. (1983). Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. Genetics. *104*: 619-647.

Wagner, K.D., Wagner, N., Wellman, S., Schley, G., Bondke, A., Theres, H. and Scholz, H. (2003). Oxygen regulated expression of the Wilms' tumour suppressor *Wt1* involves hypoxia inducible factor 1 (HIF-1). FASEB J. *17*: 1364-1266.

Wang, G.L., Jiang, B.H., Rue, E.A. and Semenza, G.L. (1995b). Hypoxia inducible factor is a basic helix loop helix PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. U.S.A. *92*: 5510-5514.

Wang, G.L., Jiang, B.H. and Semenza, G.L. (1995a). Effect of protein kinase and phosphatase inhibitors on the expression of hypoxia inducible factor 1. Biochem. Biophys. Res. Commun. *216*: 669-675.

Wang, G.L. and Semenza, G.L. (1993b). Desferrioxamine induces erythropoietin gene expression and hypoxia inducible factor DNA binding activity: implications for models of hypoxia signal transduction. Blood. *82*: 3610-3615.

Wang, G.L. and Semenza, G.L. (1993a). General involvement of hypoxia inducible factor 1 in transcriptional response to hypoxia. Proc. Natl. Acad. Sci. U.S.A. *90*: 4304-4308.

Wang, Q., VanDusen, W.J., Petroski, C.J., Garsky, V.M., Stern, A.M. and Friedman, P.A. (1991). Bovine liver aspartyl β-hydroxylase: purification and characterisation. J. Biol. Chem. *266*: 14004-14010.

Warburg, O. (1956). On the origin of cancer cells. Science. *123*: 309-314.

Warnecke, C., Zabrowska, Z., Kurreck, J., Erdmann, V.A., Frei, U., Wiesener, M. and Eckardt, K.U. (2004). Differentiating the role of hypoxia inducible factor (HIF) 1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells. FASEB J. *18*: 1462-1464.

Wiesener, M.S., Turley, H., Allen, W.E., William, C., Eckardt, K.U., Talks, K.L., Wood, S.M., Gatter, K.C., Harris, A.L., Pugh, C.W., Ratcliffe, P.J. and Maxwell, P.H. (1998). Induction of endothelial PAS domain protein 1 by hypoxia: characterisation and comparison with hypoxia inducible factor 1□. Blood. *92*: 2260-2268.

Wood, S.M., Wiesener, M.S., Yeates, K.M., Okada, N., Pugh, C.W., Maxwell, P.H. and Ratcliffe, P.J. (1998). Selection and analysis of a mutant cell line defective in the hypoxia inducible 1α subunit (HIF-1α). J. Biol. Chem. *273*: 8360-8368.

Wykoff, C.C., Beasley, N.J., Watson, P.H., Turner, K.H., Pastorek, J., Sibtain, A., Wilson, G.D., Turley, H., Talks, K.L., Maxwell, P.H., Pugh, C.W., Maxwell, P.J. and Harris, A.L. (2000). Hypoxia inducible expression of tumor associated carbonic anhydrases. Cancer Res. *60*: 7075-7083.

Yamashita, K., Discher, D.J., Hu, J., Bishopric, N.H. and Webster, K.A. (2001). Molecular regulation of the endothelin 1 gene by hypoxia: contributions of hypoxia inducible protein 1, activator protein 1, GATA-2 and p300/CBP. J. Biol. Chem. *276*: 12645-12653.

Yu, F., White, S.B., Zhao, Q. and Lee, F.S. (2001). HIF-1α binding to VHL is regulated by stimulus sensitive proline hydroxylation. Proc. Natl. Acad. Sci. U.S.A. *98*: 9630-9635.

Yun, Z, Maecker, H.L., Johnson, R.S. and Giaccia, A. (2002). Inhibition of *PPARγ2* expression by the HIF-1 regulated gene *DEC1/Stra13*: a mechanism for regulation of adipogenesis by hypoxia. Dev. Cell. *2*: 331-341.

Zelzer, E., Wappner, P. and Shilo, B.Z. (1997). The PAS domain confers target gene specificity of *Drosophila* bHLH/PAS proteins. Genes Dev. *11*: 2079-2089.

Zhang, D.D. and Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1 dependent ubiquitination of Nrf2 and for stabilisation of Nrf2 by chemoreceptive agents and oxidative stress. Mol. Cell Biol. *23*: 8137-8151.

Zhang, Z., Ren. J., Harlos, K., McKinnon, C.H., Clifton, I.J. and Schofield, C.J. (2002). Crystal structure of a clavaminate synthase-Fe(II)-2-oxoglutarate-substrate-NO complex: evidence for metal centred rearrangements. FEBS Lett. *517*: 7-12.

Zhao, Q., Cumming, H., Cerruti, L., Cunningham, J.M. and Jane, S.M. (2004). Site specific acetylation of the fetal globin activator NF-E4 prevents its ubiquitination and regulates its interaction with the histone deacetylase, HDAC1. J. Biol. Chem. *279*: 41477-41486.

Zhu, W.H., Conforti, L., Czyzyk-Krzeska, M.F. and Millhorn, D.E. (1996). Membrane depolarization in PC12 cells in PC12 cells during hypoxia is regulated by an O_2 sensitive K^+ current. Am. J. Physiol. Cell Physiol. *271*: C658-C665.