Elucidation of Oxygen Sensing Mechanisms in Human and Animal Cells

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INTRODUCTION

Oxygen is absolutely required for most forms of life. It is the terminal electron acceptor in energy-generating electron transport systems and deployed in a wide range of other metabolic, biosynthetic and detoxification processes. All such life requires the defence of oxygen homeostasis against variation in environmental concentrations of oxygen. Delivering oxygen homeostasis is more complicated in large, multi-cellular organisms, whose size precludes effective oxygen transport to the respiring cells by simple diffusion. The problem is most acute in higher animals that are not only large, but also use oxidative metabolism to generate the energy for rapid movement. In humans, the lungs, heart, and circulatory systems have all evolved to meet this challenge and many human diseases are complicated by hypoxia or inadequate tissue oxygen delivery. Thus, the physiology of oxygen homeostasis is of central importance to medicine. In this lecture I will outline how I came to be interested in the field and describe advances in the molecular under-

standing of the sensing and signalling of hypoxia that were made in my laboratory.

Homeostasis requires the detection and response to an error signal and I will refer to mechanisms of 'oxygen sensing' that underpin this process. The term itself requires some clarification. Since very many biological processes involve chemical reactions with oxygen, sufficiently low oxygen levels will cause the dysregulation of countless processes. All such systems will clearly be 'oxygen sensitive'. To distinguish *bona fide* homoeostatic pathways, I use the term 'oxygen sensor' to define systems whose prime function is in biological control. In theory, such control systems might respond to oxygen itself or to some metabolic consequence of hypoxia. These possibilities are difficult to distinguish in the intact animal and this difficulty appears to have held back understanding of oxygen homeostasis for much of the twentieth century. For instance, early studies of capillary density in different tissue beds recorded both the remarkable association with oxidative capacity, and dynamic responses to changes in oxygen demand or supply, but hypothesised mechanisms generally emphasised products of metabolism as candidates for transducing the observed effects. Thus, given the central importance of oxygen to metabolism, it was difficult to distinguish the direct sensing of oxygen from the sensing of metabolic signals. In fact, it is now clear that systems that sense and respond to different metabolic signals operate in parallel with the direct oxygen sensing mechanisms that I am going to describe.

ERYTHROPOIETIN AS A PARADIGM FOR OXYGEN-REGULATED GENE EXPRESSION

Despite the general emphasis on metabolic consequences of hypoxia, early studies of a few highly dynamic responses to hypoxia, including the control of breathing and regulation of blood red cell levels, suggested the existence of physiological systems that respond directly to oxygen availability. It was the study of one of these, the control of red cell production by oxygen-dependent production of the hormone erythropoietin, that led to the current work. Two aspects of this process supported the existence of a specific and direct 'oxygen sensing' process. First was the extraordinary sensitivity of erythropoietin production to alterations in blood oxygen content. This can be traced back to the pioneering work of Mabel FitzGerald, an unsung female scientist who accompanied J.S. Haldane and colleagues on the 1911 Oxford-Yale expedition to Pike's Peak, Colorado to study altitude acclimatisation. FitzGerald's key contribution to this story was to record changes in the blood haemoglobin levels and blood gas concentrations, in altitude-adapted men and women living at intermediate altitudes in the mining towns around Pike's Peak.[1] This work

revealed linear correlations with altitude, with even small increases in altitude (and hence decrease in pO2) being associated with a discernible increase in haemoglobin and decrease in blood carbon dioxide (representing increased breathing) (Figure 1). Later in the 20th century, Allan Elslev's plasma transfer experiments established the existence of a plasma factor (so-called erythropoietin) in the regulation of erythropoiesis, [2] paving the way for sensitive assays for erythropoietin in the blood that confirmed the extra-ordinary sensitivity of erythropoiesis and erythropoietin to very minor changes in blood oxygen availability.

Studies of erythropoiesis and erythropoietin also defined a second characteristic that suggested direct control by oxygen as opposed to secondary metabolic compromise. Early observations on poisoning by cobalt had identified increases in blood haemoglobin as an unusual toxicological phenotype.[3] Subsequent work demonstrated that cobalt induced erythropoietin production.[4] Since cobalt, at erythropoietic doses, had been shown not to be a 'metabolic' poison[5] and substances which are, such as cyanide, do not induce similar changes in erythropoietin and erythropoiesis,[6] it was concluded that this response was being governed by a specific 'oxygen sensing' process.
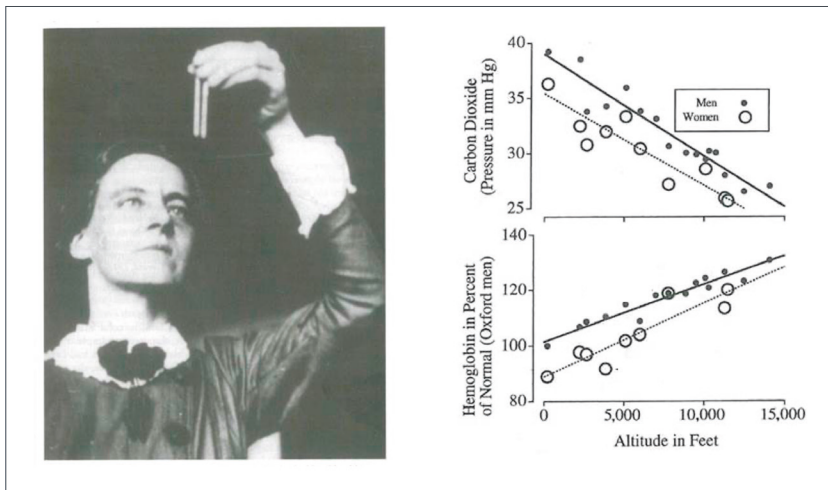

Figure 1. Mabel FitzGerald, the pioneering female scientist who accompanied J.S. Haldane's all male team to study adaptation to altitude on Pike's Peak, Colorado. (A) FitzGerald measuring haemoglobin concentration in the blood, by dilution and colour matching to a standard (photograph from the *Herald Telegraph*, Colorado Springs, 8th July 1911). (B) FitzGerald's measurements of haemoglobin (upper panel) and end expired carbon dioxide concentration (lower panel) in adapted subjects residing at different altitudes. The results demonstrate the effect of even small differences in altitude reflecting the high sensitivity of adaptive responses to hypoxia (adapted from[1]).

Erythropoietin is made in the kidneys (and to a lesser extent in the liver) and, as a recently trained clinical nephrologist, it was this aspect of renal physiology that drew me to the field. Many attempts to understand the process of erythropoietin regulation had been made, but as I completed my clinical training in the 1980s, a new opportunity for study arose. Following purification of erythropoietin from the urine of anaemic subjects,[7] the encoding cDNA and genetic locus had been identified.[8,9] It was established that erythropoietin is not stored at the site of production, but that oxygen-regulated production reflects changes in oxygen-regulated gene transcription. Broadly, our intended strategy was then to identify cis-acting sequences at the erythropoietin locus that mediate oxygen-regulated transcription, then follow the interactions of these sequences to what we believed would be a specific oxygen sensing mechanism lying within specialised cells of the kidney that produce erythropoietin.

ERYTHROPOIETIN-PRODUCING CELLS

The immediate obstacle to this approach was uncertainty as to the identity of the erythropoietin-producing cells in the kidneys and the non-availability in tissue culture of any kidney-derived cell line that produced erythropoietin. A friend at Oxford, John Bell (now Regius Professor) suggested that I tried to overcome this by expressing an oncogene, SV40 'T' antigen, under control of the erythropoietin cis-acting control sequences in transgenic mice. Since at the start of this work there was no knowledge of these sequences, I simply took the largest fragment of genomic DNA that I could isolate at the erythropoietin locus, and inserted 'T' antigen sequences into the erythropoietin 5' untranslated region. Several lines of transgenic mice were produced, and remarkably one of these contained the transgene recombined into the native erythropoietin locus. So we were confident of correct tissue-specific expression and initially optimistic that this would solve the problem. The mice expressed 'T' antigen in a renal interstitial cell population and developed an expansion of that cell population as they aged. Immuno-histochemical studies revealed that this population of cells was a subset of the renal interstitial fibroblasts, expressing 5' ectonucleotidase on the cell surface.[10] To our disappointment, no obvious special features were apparent, nor could we grow a 'T' antigen or erythropoietin expressing population after the explantation of cells into culture. Thus, the experiment failed in its primary intention, although together with work from Kai Eckardt's laboratory,[11] it did identify the erythropoietin producing cells, as a population of interstitial fibroblasts within the kidney cortex. The reasons why the erythropoietin gene (and our transgene) are extinguished in explanted kidney cells are, to my knowledge,

still not understood, but likely have a bearing on the mechanism(s) by which erythropoietin production fails in kidney disease.

At the time, the way forward was therefore dependent on two liver-derived hepatoma cell lines that had been demonstrated by Franklin Bunn and colleagues to produce erythropoietin in tissue culture, when ambient oxygen levels were lowered.[12] Not all the physiologists in the field were convinced that this properly represented the physiology of erythropoietin production in the kidneys, but we (my long-standing friend and colleague Chris Pugh and I) set about mapping cis-acting oxygen-regulated control sequences by transient transfection of mouse genomic DNA into these cells, taking advice from Richard Jones in David Weatherall's newly commissioned Institute of Molecular Medicine. This work identified a sequence lying 3' to the mouse erythropoietin gene as a transcriptional enhancer that conferred oxygen regulated expression on a heterologous reporter gene (Figure 2, upper panels).[13] The sequences were highly conserved and lay at exactly the same position with respect to the erythropoietin gene as human sequences identified in parallel work by both Gregg Semenza and Jaime Caro.[14,15]
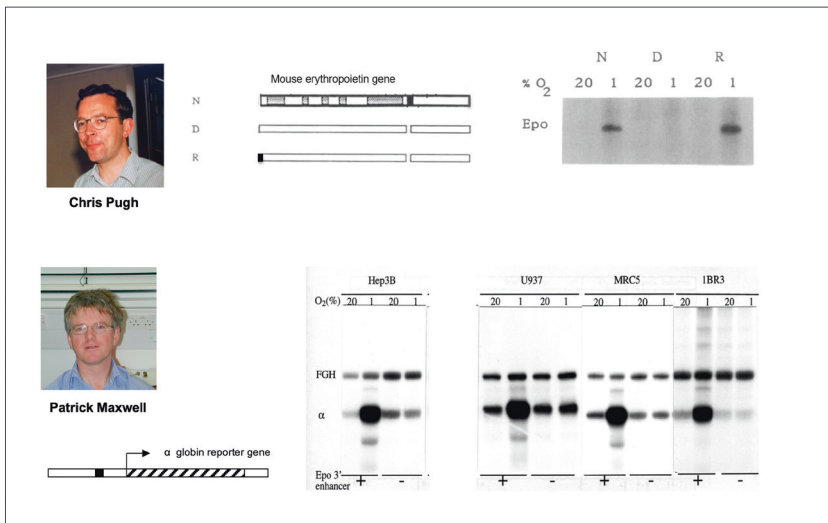
Figure 2. Widespread activity of the oxygen-regulated erythropoietin enhancer in both erythropoietin and non-erythropoietin producing human cells. (A) RNAse protection assays of the human erythropoietin-producing hepatoma cell line Hep3B, transfected with the indicated sequences from the mouse erythropoietin gene; the work identifies a transcriptional enhancer lying 3' to the mouse erythropoietin gene. (B) RNA protection assays demonstrating that the erythropoietin 3' enhancer confers oxygen regulated activity on an α-globin reporter gene (α) not only in erythropoietin-producing Hep3B cells (right panel) but also in a range of non-erythropoietin-producing cells; U937 monocyte/macrophage; MRC5, lung fibroblast; IBR3, skin fibroblast.; α, α-globin; FGH, transfection control without the erythropoietin enhancer sequence. Reproduced from references.[13,16]

DISCOVERY OF A WIDESPREAD SYSTEM OF OXYGEN SENSING IN HUMAN AND ANIMAL CELLS

We immediately recognised that identification of these oxygen-sensitive enhancer sequences would enable another very important question to be addressed. What would be their activity if transfected into cells that did not produce erythropoietin and were not known to be oxygen-sensitive? Initial experiments gave an unclear result; certainly, the sequence did not appear to be as active as in the erythropoietin-producing hepatoma cells. This initially reinforced the prevalent view that the oxygen-sensing mechanism was a specific property of erythropoietin producing cells, and I set about what I thought was an innovative (though at best high risk) experiment attempting to 'expression clone' components of the 'oxygen sensing' pathway by transfer of genes from the 'oxygen sensing' hepatoma cells, to a line that would be predicted to be oxygen insensitive (according to the then existing prejudice, every other cell type). For the latter, I chose Cos 7 cells, for ease of retrieval of plasmid DNA. Cos 7 cells grow rapidly and when I came to check a rather dense culture of these cells transfected with the erythropoietin enhancer alone, to my surprise I observed very clear oxygen-regulated activity. Of course, this disqualified the planned experimental approach, but fundamentally changed our thinking.

Patrick Maxwell joined the laboratory and set about testing other cells. He saw that responses depended on the culture conditions, but that when rapidly dividing, relatively dense cultures were tested, essentially all cultured cells supported oxygen-regulated activity of the erythropoietin enhancer. Though we never measured it directly, the initial variation almost certainly related to dependence of the actual oxygen concentration at the monolayer on the oxygen consumption rate of the cells, and hence on their growth rate and density. Once this was appreciated, the responses across a wide range of cells were very robust (Figure 2, lower panels) and we were very excited indeed by the implications. If essentially all mammalian cells could support oxygen-regulated activity of this erythropoietin enhancer sequence, irrespective of whether they produced erythropoietin, then the oxygen sensing system was clearly widespread and there must be other targets. Moreover, they might also be predicted to respond to hypoxia with high sensitivity, similar to erythropoietin. Though we had no idea at the time of the extent of the field that was to unfold, I had no doubt about the significance of the findings and confidently drove to the London offices of *Nature*, anticipating a dialogue with the editor that would lead to rapid acceptance for publication.

Needless to say, the final response, four months later, was disappointing. Of course, there will be many who have such an experience and the story itself is not particularly remarkable. There are two reasons for telling it. First, as the editor explained, part of the reason for

the delay was 'difficulty in finding reviewers.' For any early career readers who have the same experience, this is important evidence that you are entering a new field. Be encouraged by that. Second (from the current vantage point) it is easy to have some sympathy with the editor. Essentially it was only one new fact, and we hadn't then followed through by addressing the obvious next question; what were those additional oxygen-sensitive targets? As a young nephrologist, I had little experience of research practice and it didn't occur to me that deriving follow-on conclusions, or a 'more complete story' might be important before publication. And, actually, I still think this often-sought ambition is a problem. As far as I was concerned, the laboratory was stone cold certain of the actual result and we believed the implications to be profound. Once the findings were published, I argued, we and others could follow up. In the end, with a little help from David Weatherall, we did publish, but in another journal,[16] and there was indeed some follow-up. I met with Gregg, at a meeting organised by Christian Bauer at the Lake Lucerne Palace Hotel. He presented his (then unpublished) work defining the protein binding those control sequences in erythropoietin-producing hepatoma cells (hypoxia inducible factor, HIF-1) and I presented the sadly still unpublished findings described above. We left the meeting with considerable enthusiasm for what lay ahead, meeting regularly at conferences now devoted to the expanding field of hypoxia research. (Figure 3).
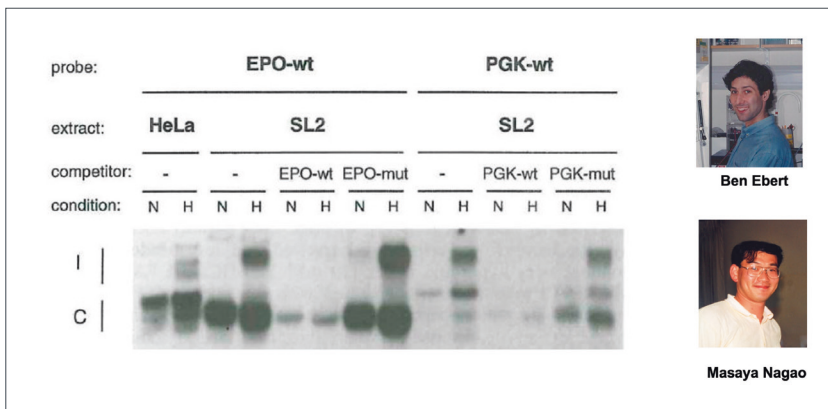
Figure 3. Electrophoretic mobility shift assays demonstrating the presence of hypoxia inducible factor (HIF) in invertebrates. Nuclear extract from normoxic (N) or hypoxic (H) Hela or *Drosophila* (fruit fly) SL2 cells. SL2 cells are shown to contain hypoxia inducible nuclear proteins that bind hypoxia response elements from the human erythropoietin enhancer (EPO-wt) and phosphoglycerate kinase promoter (PGK-wt). Unlabelled oligonucleotides (EPO-wt and PGK-wt), but not mutant version that ablate HIF-binding (EPO-mut and PGK-mut) compete these interactions. Reproduced from[24].

The efforts in my laboratory initially focused on genes encoding glycolytic enzymes, with other clinical trainees, Ben Ebert, John Firth, and Jonathan Gleadle joining the laboratory and contributing much to the work. Dissection of regulatory sequences in the genes encoding the glycolytic enzymes phosphoglycerate kinase and lactate dehydrogenase revealed that their oxygen regulated expression (despite lower amplitude of regulation) was dependent on sequences resembling those in the erythropoietin enhancer.[17] These sequences had a similar core motif 'ACGTG', which bound HIF, and they cross-competed with the erythropoietin enhancer in electrophoretic mobility shift assays of HIF binding.[17,18] These results then clearly confirmed that other genes respond to the same oxygen-regulated signals and we rapidly found many other examples. Angiogenic growth factors such as vascular endothelial growth factor and platelet-derived growth factor,[19] glucose transporters and glycolytic enzymes[18,20] all manifest the classical features associated with erythropoietin regulation; the response to hypoxia was mimicked by cobaltous ions, and (as had more recently been established by Gregg[21]) by iron chelators.[19] All were transcriptional targets of HIF.

Apart from corroborating the evidence for a widespread mechanism of oxygen sensing in mammalian cells, this early work took us in two new directions. I read the work of researchers following up on Warburg's findings on enhanced glycolysis in apparently well oxygenated malignant cells.[22,23] These studies described isoform specificity in the upregulation of genes encoding glycolytic enzymes in cancer cells. It was quickly apparent that the up-regulation of glycolytic enzymes and glucose transporters by HIF manifests a closely similar isoform specific pattern, immediately suggesting a link between HIF activation and cancer.[20] Second, as glycolysis and the genes encoding glycolytic enzymes are more conserved than erythropoiesis and the erythropoietin gene, the new findings raised an important question as to the phylogeny of the pathway, and whether we could apply the powerful genetic technologies available in model organisms to dissect the mechanism of oxygen sensing. Substantial unrewarded effort went into attempting to define an orthologous system in yeast. However, we did obtain clear evidence for the existence of a HIF-like protein that bound to the human erythropoietin control sequences in insect cell extracts derived from *Drosophila melanogaster* SL2 cells (Figure 4).[24] Quite clearly the system ante-dated the appearance of specialised blood vascular systems that have evolved for oxygen delivery in higher animals. Importantly, the findings opened new opportunities for analysis in lower 'model' animal organisms, which we were able to capitalise on later.

Figure 4. The author discussing the future of hypoxia research with Gregg Semenza (Woods Hole, Massachusetts, 1993).

Our own findings on different HIF target genes and transcriptional pathways downstream of HIF were amplified by a great deal work from Gregg and many other groups, with the field undergoing an explosion of interest during the later years of the 1990s. It became clear that the HIF system was involved in many processes beyond those with well-established connections to oxygen homeostasis. In addition to erythropoiesis, angiogenesis and energy metabolism, further studies identified hypoxia and HIF-regulated genes with central roles in iron metabolism, pH regulation, matrix metabolism, cell differentiation, cell motility, immune regulation and stem cell behaviour. Quite quickly, the list (now encompassing hundreds or thousands of genes) extended beyond even our most optimistic perspective.

REGULATORY DOMAINS IN HYPOXIA INDUCIBLE FACTORS

Our major efforts now focussed on defining the oxygen sensing and signalling pathways that lay upstream of HIF. Gregg had identified the HIF DNA binding complex as an α/β heterodimer of basic-helix-loop-helix PAS proteins; HIF-1β being a constitutively expression protein that also dimerises with the aryl hydrocarbon receptor and HIF-1α being a novel protein, specifically transducing the response to hypoxia.[25] Reasoning that definition of isolated sequences from HIF-α sub-units that could confer oxygen sensitivity on heterologous proteins would direct a search for the upstream signal pathways, we set about fusing HIF-α sequences to heterologous transcription factors and assaying for oxygen sensitive responses. Overall, it was possible to define three distinct domains in HIF-α, each of which could convey oxygen sensitivity in heterologous transcriptional reporter systems.[26,27] These experiments demonstrated

that two of these sequences operated, at least in part, through oxygen-regulated proteolysis, whereas a third, overlapping the C-terminal activation of HIF-α operated through changes in transcriptional activity that did not reflect altered protein stability. All of them displayed responses to hypoxia, cobaltous ions and iron chelators, consistent with the characteristics of both erythropoietin and HIF regulation. In one way these experiments (together with similar studies from others[28,29]) were very successful in directing the search for upstream oxygen signalling pathways, as each of these discrete domains must interact in some way with those pathways. In other ways, we were disappointed. We had confidently expected to identify a common protein phosphorylation motif that would surely direct us to the signal transduction pathway. But surprisingly, we were initially unable to define a common motif at all, despite the apparently identical regulatory characteristics. Chris Pugh went as far as to mutate every phospho-acceptor amino acid in one of these domains, the more C-terminal of the oxygen dependent degradation domains.[26] None of the mutations made any difference to the oxygen sensitivity. Although initially disappointing, these experiments were critical in excluding what had been believed to be the likely mechanism of signal transduction (a protein phosphorylation cascade linking an 'upstream' oxygen sensor to HIF), and hence were important in directing attention elsewhere.

CONDITIONAL DEGRADATION OF HIF BY THE VON HIPPEL-LINDAU TUMOUR SUPPRESSOR

Our next major step was the recognition that the von Hippel-Lindau tumour suppressor protein was absolutely necessary for the oxygen sensitive proteolysis of HIF-α. We found that pVHL physically bound to HIF-α sequences, that binding was prevented by exposure of cells to the 'hypoxia-mimetics' (cobaltous ions and iron chelators) and that this process governed the stability of HIF-α and the expression of HIF target genes.[30] This work interfaces with contributions from my other co-laureate Bill Kaelin. The interplay between the different contributions illustrates how knowledge gained from different perspectives interacts to build a picture. Bill and others had examined some of the recently-identified hypoxia-inducible transcripts described above (encoding vascular endothelial growth, glucose transporter-1) in renal cancer cells that are defective for the von Hippel-Lindau tumour suppressor.[31,32] They reported constitutive upregulation irrespective of oxygen, but didn't identify the mechanism. We heard about these findings, observed the upregulation of hypoxia inducible transcripts, but initially were also puzzled as to the mechanism as we could not detect HIF-1α at all in the renal cancer cell lines that were being studied.

How then was the matter resolved? In a different line of work, sequencing of the human genome was revealing new opportunities for gene identification; Steve McKnight was hunting for basic-helix-loop-helix PAS proteins. He identified what he proposed to be a HIF-α paralogue strongly expressed in endothelial cells. He termed the gene product EPAS1 (endothelial PAS protein-1).[33] But the regulation of EPAS1 by oxygen was not proven. To study this, it was necessary to measure expression at the protein level. To that end, we made an antibody, PM (Patrick Maxwell) 9, which clearly established that EPAS1 (now more commonly known as HIF-2α) was indeed regulated by oxygen, like HIF-1α.[34] We had, by then, identified more HIF-target transcripts, encoding other glycolytic enzymes and found them also to be constitutively upregulated in VHL deficient cells. So, we went back to HIF, this time also using the new anti-EPAS1 antibody. The result was striking. The renal carcinoma cells that we and others had used (786-0) expressed EPAS1/HIF-2α alone (and not HIF-1α), but the HIF-2α protein was constitutively stabilised and upregulated irrespective of oxygen levels. When we examined other renal carcinoma cell lines, we found some of these expressed HIF-1α as well as HIF-2α; importantly whenever HIF-1α or HIF-2α was expressed, the proteins were constitutively stabilised (Figure 5).[30] We were rapidly able to build on this as described above, with Matthew Cockman and Norma Masson going on to demonstrate directly that pVHL (as predicted by its structure)[35] was a ubiquitin E3 ligase that conditionally targeted HIF-α sub-units for destruction by the ubiquitin-proteasome pathway.[36]

Renal cancer-associated mutations affecting a specific region of pVHL abrogated the interaction with HIF-α, directly linking dysregulation of HIF to the function of a tumour suppressor protein, and hence to can-
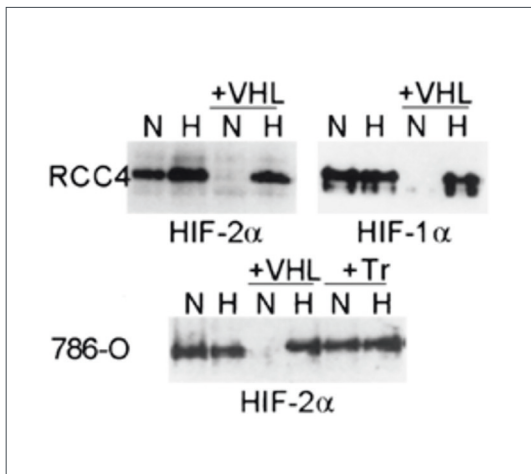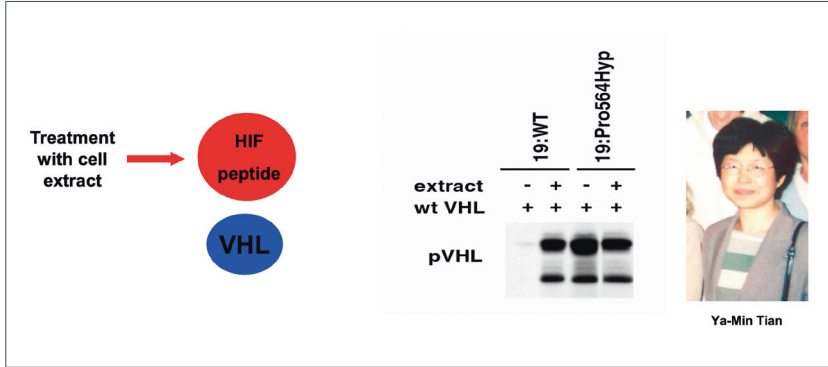

Figure 5. Regulation of HIF by the von Hippel-Lindau protein (VHL). Panels show HIF-1α or HIF-2α immunoblots of extracts from normoxic (N) and hypoxic (H) VHL-defective kidney cancer cell lines, RCC4 and 786-0. HIF-α proteins are stabilised in VHL defective cells irrespective of oxygen levels. Transfection with a wildtype (+VHL) but not a truncated (+Tr) *VHL* gene restored regulation of HIF-α by oxygen. 786-0 cells do not express HIF-1α. Reproduced from[30].

cer.[37] But it was puzzling to find that this unphysiological upregulation of HIF was associated with a very unusual pattern of HIF expression, a very strong bias to HIF-2. By examining kidneys from patients with VHL disease (the familial form of clear cell renal cell carcinoma, ccRCC), we observed that this bias appeared to evolve during progression from morphologically normal renal tubular cells (expressing HIF-1) to dysplastic and overtly malignant cells (dominantly expressing HIF-2).[38] Consistent with this, we (together with Bill and others) subsequently found that, in this setting, HIF-2 appears to be supporting oncogenic drive, with HIF-1 even restraining growth of renal carcinoma cells as experimental tumours.[39] We have since become very interested in the implications of the 'unphysiological switching' of massively interconnected pathways, such as HIF, during oncogenesis, and in the actions of selective pressures that apparently operate during cancer evolution to 'tune' the pathway output (such as by modulating the HIF-1 to HIF-2 bias).[40,41] However, at the time our major quest was to move on to understand the process of oxygen sensing.

A MECHANISM FOR OXYGEN SENSING

Identification of the HIF-α/pVHL connection opened the way to defining the mode of regulation of HIF. We narrowed down sequences in HIF-α that were necessary for interaction with pVHL, initially focusing on the C-terminal of the two regions that showed oxygen dependent proteolysis, the one in which we had mutated every phospho-acceptor amino acid without impairing its function.[26] We found that peptides representing this sequence required treatment with cell extract to promote interaction with pVHL, implying some form of modification (that was presumably not protein phosphorylation). But one result was very puzzling. By whatever means we used to assess the pVHL-HIF association, we always found that the association was blocked when extracts were made from cells treated with hypoxia mimetics (cobaltous ions and iron chelators), but apparently not when they were made from hypoxic cells. Could it be that the oxygen dependent reaction was continuing in the cell extracts post-lysis? This was shown to be the case by Panu Jaakkola and David Mole, who worked with a large oxygen-controlled work-station, a chance purchase from Baker-Ruskinn that I had made with an (unusual) surplus on my grants that year. This enabled extracts to be made, and immuno-precipitations performed, entirely within a hypoxic atmosphere, and using buffers pre-equilibrated to the same level of hypoxia. These and other experiments clearly revealed that the process was dependent on oxygen, but not ATP, and that it was inhibited by cobaltous ions and iron chela-

Figure 6. Definition of prolyl hydroxylation as the regulatory modification of HIF-α proteins governing binding to the von Hippel-Lindau protein (VHL). Binding of VHL to synthetic peptides derived from HIF-1α sequences (556−574) was assayed by pull-down. The unmodified HIF peptide (19:WT) requires treatment with cell extract to promote binding of VHL. In contrast a HIF peptide bearing a trans-4-hydroxyprolyl substitution at residue 564 (19:Pro564Hyp) binds VHL without such treatment. Adapted from[42].

tion. Other experiments narrowed the target HIF-α sequences, mutational analyses implicating a specific proline residue (Pro 564 in human HIF-1α). Mass spectrometry revealed oxidation compatible with hydroxylation of that proline, but the eureka moment came with the synthesis of a HIF-1α peptide with a substituted residue (*trans*-4-hydroxyproline) at the suspected site, Pro 564. It was one of those rare moments where things come together. I remember well the moment Ya-Min Tian phoned excitedly to tell me the result; untreated hydroxyproline HIF peptide bound VHL very tightly, just like the extract-treated HIF peptides containing proline (Figure 6).

All findings were therefore consistent with the action of a prolyl hydroxylase in the cell extract. Nevertheless, the known human prolyl hydroxylases, the collagen modifying enzymes, did not seem good candidates for an oxygen sensor. Moreover, the target sequence did not fit with the collagen consensus and indeed Johanna Myllyharju in Oulu, Finland, tested the HIF-α peptides with recombinant pro-collagen prolyl hydroxylases and found no activity. Nevertheless, the procollagen prolyl hydroxylases belong to the family of 2-oxoglutarate dependent dioxygenases and we found that a 2-oxoglutarate analogue, dimethyloxalylglycine, caused striking up-regulation of HIF when applied to cells, strongly suggesting the operation of a similar type of enzyme.

In the catalytic cycle of 2-oxoglutarate dependent dioxygenases, molecular oxygen is split and oxidation of the prime substrate (in this case the target prolyl residue) is coupled to the oxidative decarboxylation of 2-oxoglutarate, yielding succinate and carbon dioxide. The co-ordination

of the catalytic Fe (II) ions by the 2-histidine-1-carboxylate motif is suffi-
ciently labile to allow inhibition by iron chelating agents or substitution
by cobaltous ions or other transition metals that cannot support or are
very ineffective in supporting catalysis. Thus their properties fitted with
the regulatory characteristics of the HIF system. Furthermore, the abso-
lute dependence of catalysis on molecular oxygen clearly implied a mech-
anism of 'oxygen sensing'. In hypoxia, the reaction would slow, enabling
HIF to escape destruction and assemble into a transcriptional complex.

The work therefore conceptualised the mechanism of sensing and was
published simultaneously with independent work from Bill Kaelin.[42,43]
That story may itself be of some interest. Shortly after we first identified
the HIF-α/pVHL connection, I had suggested that Patrick Maxwell attend
the international VHL meeting to find out current thinking. He reasonably
asked 'what should I say we are doing ourselves, given that the work is
unpublished.' The answer, we agreed, was straightforward. Since we had
been working for some years on HIF, and now attending a meeting on
VHL, then it might reasonably be deduced we were working on HIF and
VHL and that's what should be said. Patrick returned and indicated that
he had indeed revealed our position in conversation with Bill; it was
agreed (since we were clearly competing) to keep in touch regarding
future publication intentions. Bill was as good as his word, and in due
course, shortly after we had done the work I've just described identifying
prolyl hydroxylation as the regulatory modification of HIF, there was a
call from Boston saying they 'had something very interesting' and were
ready to publish. There was a rapid set of exchanges when it was ascer-
tained that we did indeed have very similar findings. Then things pro-
ceeded very quickly to publication, perhaps aided by the fact that two
groups were reporting the same key findings, derived by somewhat differ-
ent experimental approaches.

Further work soon completed other parts of the picture. As I indicated
earlier, we had defined not one, but two, distinct sequences that could
confer oxygen dependent degradation on heterologous proteins. Although
sequence similarities between the two domains are not striking, Norma
Masson, working in my laboratory, showed that the more N-terminal of
the two sites in HIF-α also contained a site of prolyl hydroxylation (Pro
402 in human HIF-1α), which promotes binding to pVHL, and hence pro-
teolytic destruction in the presence of oxygen.[44] Though the two domains
can function independently, they ordinarily operate in a co-ordinated
manner; C-terminal hydroxylation precedes N-terminal hydroxylation,
hydroxylation of both sites co-operating to promote efficient destruction
of the HIF-α protein. Again, binding experiments using synthetic peptides
revealed remarkable discrimination of the VHL complex for the
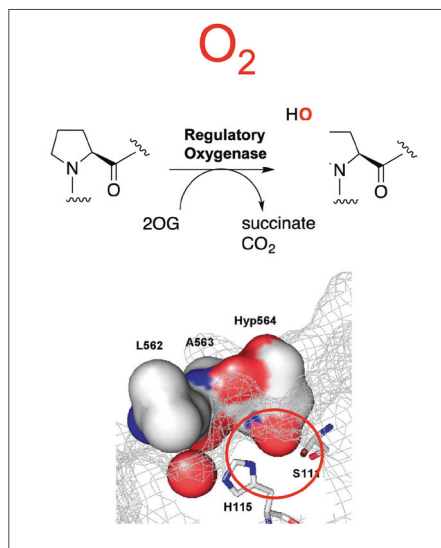*trans*-4-hydroxylated versions. In subsequent work, both my laboratory

Figure 7. Diagram depicting the molecular 'switch' at the core of the oxygen sensing mechanism. The regulatory prolyl hydroxylase a 2-oxoglutarate dependent dioxygenase, splits molecular oxygen, incorporating one oxygen atom into its prime substrate (the HIF-α polypetide) and uses the other in the coupled oxidative decarboxylation of 2-oxoglutarate yielding succinate and carbon dioxide. The alcohol group of the trans-4-hydroxyprolyl residue in the HIF-α product is able to form an optimised hydrogen bonding network with residues serine 111 and histidine 115 in the floor of a hydroxyprolyl- binding pocket in VHL, increasing affinity *circa* 1000-fold. Adapted from[45.]

working with Yvonne Jones and David Stuart[45] and Bill's laboratory, working with Nicola Pavletich,[46] independently showed that the exquisite discrimination (>1000 fold increase in affinity) of the VHL-E3 ligase complex for *trans*-4-hydroxylated over non-hydroxylated HIF was dependent on optimised hydrogen-bonding between the oxygen atom of the alcohol group in *trans*-4-hydroxyproline and each of two specific residues (Ser 111 and His 115) in the floor of the hydroxyproline binding pocket in human pVHL (Figure 7).

IDENTIFICATION OF THE HIF PROLYL HYDROXYLASES AS ENZYMATIC OXYGEN SENSORS

Whilst the demonstration of oxygen sensitive enzymatic prolyl hydroxylation governing the HIF-α/pVHL association, and hence degradation of HIF, clearly suggested a mechanism of oxygen sensing, it left open a key question: the identity of the actual enzyme(s). By this stage we had linked with a colleague who was to become an excellent and much valued collaborator for many years, Christopher Schofield, Professor of Organic Chemistry at Oxford. The story well exemplifies one of the most important aspects of scientific discovery, one often overlooked in the quest for findings of immediate value. Knowledge builds on knowledge in unexpected ways.

Chris had worked on the structure and enzymology of prokaryotic enzymes related to the 2-oxoglutarate dioxygenases, including an enzyme catalysing the key step in penicillin synthesis, isopenicillin *N* synthase.

This work revealed a core jelly-roll structure at the catalytic domain. Together with sequence alignments for a number of 2-oxo-acid-dependent dioxygenases, this led Chris and colleagues to propose that many such enzymes use a 2-histidine-1-carboxylate triad of residues, typically positioned on the 2nd and 7th stands of the jelly-roll, to co-ordinate the catalytic iron atom.[47] As genome sequencing programmes progressed, this work enabled the informatic prediction of other 2-oxoglutarate dioxygenases, including many members encoded by the human genome, in addition to the known procollagen modifying enzymes.

In a wholly different line of work, Bob Horvitz (Nobel Laureate, Physiology or Medicine 2002) and colleagues had been identifying mutations in the nematode worm *Caenorhabditis elegans*, carefully identifying large numbers of distinct mutants whose phenotypes corresponded to broad categories of developmental abnormality. Many different mutants were characterised that were associated with the retention of eggs within a swollen body, 'egg-laying defective', the mutant genes being termed *EGL*.[48]

In my laboratory we had been following up on our earlier work defining a HIF-like DNA binding activity in *Drosophila* SL2 cells. We identified the HIF-α orthologue in *Drosophila*, again using a fusion gene strategy to assay candidate *Drosophila* proteins that could confer oxygen regulated activity on the transcription factor Gal4, when transfected into human cells.[49] We used this information to predict a HIF-α orthologue in *C. elegans*. John O'Rourke, who was doing this work, then made an antibody to this protein. Slightly to our surprise (since we had no idea what level of ambient air would induce the putative HIF system in nematode worms) when we exposed worms to 1% oxygen using a simple bell jar, we saw a dramatic upregulation of the predicted HIF-α protein. We used this system to test mutants affecting genes encoding molecules that had been previously proposed as candidate oxygen sensors, including a range of mitochondrial mutants, but saw no clear alteration in the regulation of HIF.

But the system now enabled us to test candidate prolyl hydroxylases as predicted by Chris Schofield, in particular one group that was highly conserved across human and invertebrate animal genomes and represented in worms by the mutant *egl-9*. The result was striking: I have a vivid memory of Andrew Epstein, who had taken on the laboratory's *C. elegans* work, bursting through the door with the result. Worms bearing different *egl-9* mutations all showed clear upregulation of HIF irrespective of oxygen (Figure 8).[50] We rapidly proceeded to demonstrate that the EGL-9 gene product had HIF prolyl hydroxylase activity. Jonathan Gleadle identified the three human orthologues on the basis of their highly conserved catalytic domains, naming them PHD (prolyl hydroxylase domain) 1, 2 and 3.[50] Each of the PHDs catalysed the prolyl hydroxylation of peptides
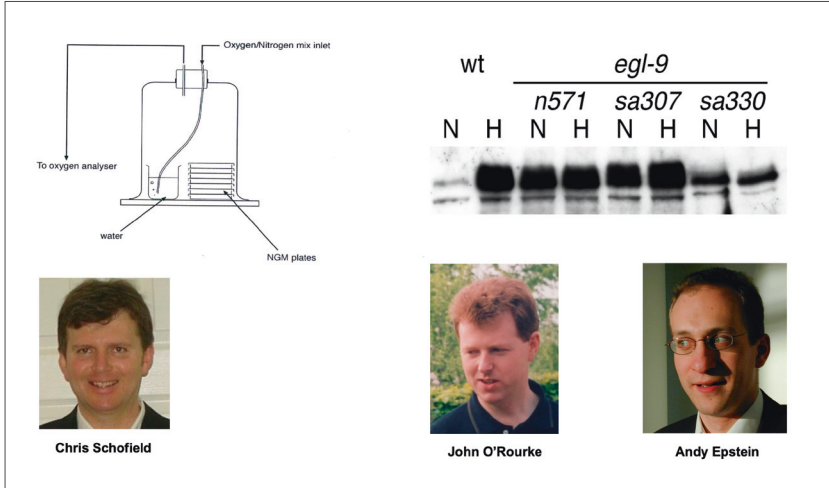
Figure 8. Identification of the 'oxygen sensing' HIF prolyl hydroxylase in the nematode *worm C.* elegans. (A) simple bell jar apparatus that was used to expose worms to a hypoxic atmosphere (B) Immunoblot of *C. elegans* HIF-α protein using an antibody produced 'in house' to test responses of wild-type and mutant worms to hypoxia; whereas HIF-α is strongly induced by hypoxia in wild-type worms (wt), HIF-α protein is stabilised and upregulated irrespective of oxygen in worms bearing different mutant alleles of the gene encoding the HIF prolyl hydrolylase *egl-9*. Adapted from[50].

and polypeptides corresponding to sites of prolyl hydroxylation identified within the oxygen dependent degradation domains of HIF-α proteins, though some differences in site selectivity were observed.[50] We were rapidly able to demonstrate the striking sensitivity of the PHDs to even modest reductions in the ambient oxygen concentration using capture of pVHL by HIF-α peptides as a measure of HIF hydroxylation.[50] More formal kinetic measurements by Johanna Myllyharju and colleagues confirmed these findings, revealing high Michaelis-Menton K_mO_2 values in the range 200−250 μM.[51]

Elucidation of the HIF-α/PHD/pVHL pathway clearly explained the function of two of the three domains in HIF-α that we had identified as being able to independently confer regulation by oxygen on heterologous proteins. But it left open a question as to the mode of regulation of the third oxygen-regulated domain at the C-terminal of HIF-α, which encompasses the C-terminal activation domain. This isolated domain was stable and did not appear to be regulated by proteolysis. Otherwise, it manifested closely similar behaviour to the other two regulatory domains, being activated by cobaltous ions and iron chelators as well as hypoxia.[26] Somewhat to our surprise, despite an apparent consensus (LxxLAP), revealed by identification of the first two prolyl hydroxylated domains, we could see no similar sequence in the C-terminal domain. As we were puz-

zling over this, two papers appeared; one from Gregg identifying a molecule he termed FIH (factor inhibiting HIF) as a protein associating with HIF-1α,[52] and one from Murray Whitelaw's laboratory describing a new modification, hydroxylation of an asparaginyl residue, within the third domain of HIF-α.[53] Based on a predicted jelly roll motif suggestive of a 2-oxoglurarate dioxygenase, Chris Schofield suggested that FIH might be the implied HIF asparaginyl hydroxylase. Working together we were rapidly able to confirm that and show that hydroxylation prevented association of the C-terminus of HIF-α with the p300 transcriptional co-activator,54 thus explaining, at least in part, the activity of this third regulatory domain.

Thus, HIF is subjected to dual regulation through stability (prolyl hydroxylation) and activity (asparaginyl hydroxylation), both the oxygen sensitive signals being generated by the catalytic activity of 2-OG dioxygenases. Studies of the genes encoding HIF and these HIF hydroxylases across multiple animal genomes identified a basic HIF-α/PHD/pVHL triad in all genomes examined. FIH is also represented in most animal species, generally as a single gene. However, multiple *PHD* and *HIF-α* genes appear to have arisen by gene duplication events at the base of vertebrate evolution.[55] This raised a question as to whether these additional isoforms have specialised roles in the regulation of specialised oxygen delivery systems observed in higher animals.

When expressed together in cells, HIF isoforms have partial overlapping functions.56 However, some key homeostatic responses observed in higher animals appear largely dependent on the cell-specific expression of one isoform. Thus, the induction of erythropoietin in the renal interstitial cells is exclusively driven by HIF-2.[57] In more recent work, Tammie Bishop and others in my laboratory have observed that the increase in respiratory sensitivity to hypoxia observed after acclimatisation is again specifically dependent on HIF-2.[58,59] In keeping with these findings, both the interstitial fibroblasts that make erythropoietin,[60] and the type 1 cells of the carotid body, which mediate hypoxic ventilatory control, specifically express very high levels of the HIF-2α isoform.[59] Thus, remarkably, both the classical features of acclimatisation, as studied by FitzGerald, Haldane and colleagues more than 100 years ago, appear critically dependent on a specific component of this system; HIF-2α.

To summarise at this point, the work achieved my original goal as a young nephrologist, of connecting erythropoietin to oxygen. But so much more was found along the way: a panorama of entirely unforeseen responses to hypoxia, an unanticipated mode of cell signalling that might be used in other types of biological circuitry, and new opportunities for the therapeutic manipulation of the human oxygen sensing system. In the final part of the lecture I will briefly consider some of these extensions.

SIGNALLING OXYGEN BY ENZYMATIC PROTEIN OXIDATION COUPLED TO PROTEOSTASIS IS A COMMON PARADIGM

We were initially surprised that the HIF system operated in all animal cells (and was not restricted to erythropoietin producing cells). However, once it became clear that HIF also regulated highly conserved cellular functions, such as glycolysis, we were *then* surprised not to be able to trace it further across non-animal organisms. Nevertheless, following elucidation of the mode of HIF regulation by oxygenase-catalysed post-translational hydroxylation, it has now become clear that many other life forms use oxygenase-catalysed protein oxidation coupled to proteostasis to signal oxygen levels in their cells, but deploy different types of enzyme-substrate couple.

Work by Christopher West on the slime-mould *Dictyostelium discoideum* has characterised a similar prolyl-4-hydroxylase that catalyses hydroxylation of Skp1, a sub-unit of E3^{SCF} ubiquitin ligases.61 In this system, post-translational prolyl hydroxylation of Skp-1 is followed by glycosylation of the hydroxyproline residue to signal oxygen levels to pathways involved in culmination. In the fission yeast *Schizosaccharomyces pombe*, another prolyl hydroxylase, Ofd1, has been demonstrated by Peter Espenshade to transduce the oxygen regulated stability of the sterol regulatory element-binding protein Sre1.[62] In this case it is not the transcription factor itself that is hydroxylated; rather, the prolyl hydroxylation reaction regulates stability of Sre1 indirectly. Ofd1 is represented in human cells by the enzyme OGFOD1. Unexpectedly we found that OGFOD1 and its orthologues are ribosomal hydroxylases that catalyse hydroxylation of a prolyl residue in the small subunit protein, Rps23.[63] In fission yeast it is proposed that Rps23 hydroxylation imparts oxygen sensitivity on Ofd1's nuclear functions through competitive association;[64] whether an analogous process occurs in human cells is not yet clear. In plants, a group of plant transcription factors belonging to the ERF (ethylene response factor) VII family are subject to control by oxygen dependent instability via the N-degron pathway.[65,66] In this system an N-terminal cysteine is generated by the action of methionine aminopeptidases. Oxidation of this N-cysteine residue in turn generates a substrate for arginyl transferases; addition of arginine then creating an N-terminal destabilising residue that is targeted by ubiquitin E3 ligases.[67] In plants, Francesco Licausi showed that this N-cysteine oxidation is enzymatically catalysed by a set of closely related plant cysteine oxidases and that this is the step conferring oxygen sensitivity on the degradation pathway.[68] Like the animal PHDs, the plant cysteine oxidases are dioxygenases (i.e. they incorporate oxygen atoms derived from molecular oxygen directly into their substrates). Thus, this work identified a system signalling oxygen levels in plants using a quite differ-

ent type of enzyme, but the same general process of oxygenase-cata-lysed protein oxidation coupled to proteostasis.

A chance meeting with Francesco led us into a collaborative project, which resulted in the discovery of a human orthologue of the plant cysteine oxidases. To our surprise, introduction into human cells of a fusion protein reporter in which sequences from the *Arabidopsis thaliana* ERF-VII transcription factor RAP2.12 were fused to green fluorescent protein, showed proteolytic regulation by oxygen even without co-trans-fection of genes encoding the plant cysteine oxidases. We identified the human enzyme orthologue of the plant enzymes, which was implied by this work, as ADO (2-aminoethane thiol dioxygenase, otherwise known as cysteamine dioxygenase) and showed that this enzyme catalyses the dioxygenation of N-cysteine residues on a range of human polypeptides.[69] Like the PHDs, ADO manifested an unusually low affinity (high KmO2) for oxygen. We found that ADO catalysed what had previously been pro-posed as non-enzymatic oxidation of an N-terminal cysteine,[70] on the R4 family of RGS (regulator of G-protein signalling) proteins to target these proteins for oxygen-dependent degradation, thus modulating G-protein signalling.[69] Potentially, direct proteolytic regulation of these signalling molecules in human cells would allow for more rapid responses to oxygen than those transduced via transcriptional regulation. Overall, what is now clear is that all four eukaryotic kingdoms use enzymatic protein oxidation coupled to proteostasis to signal oxygen levels, and that at least some of these systems contribute, alongside the HIF hydroxylase pathway, to oxy-gen homeostasis in human cells.

PHYSIOLOGY AND THERAPEUTICS

Before finishing, I'd like to discuss briefly a general question regarding the precision with which a reductionist biochemical understanding, such as has been gained by the work I have described, can be used to predict inte-grated physiological responses and therapeutic outputs. This question is common to many fields of biomedical research, but here brought into sharp focus by the precision demanded in physiological oxygen homeo-stasis, the enormous complexity of the pathways defined to date, and the development of drugs with the potential to modulate these pathways therapeutically.

The widespread operation of the HIF hydroxylase oxygen signalling pathways across animal cells and the existence in those cells of thou-sands of transcriptional targets of HIF, clearly qualifies this simple molec-ular pathway for a homeostatic function on countless cellular processes. These must operate with great precision across highly heterogeneous tis-sue oxygen concentrations. To exemplify from my nephrology perspec-

tive, within the mammalian kidney, oxygen tension varies by an order of magnitude between the renal cortex and the physiologically hypoxic renal papilla.71 Yet at each site, levels of HIF proteins are low under physiological conditions, but raised by a systemic hypoxic challenge.[60] Presumably this reflects an ability of cells in their native environment to respond to changes in intra-renal tissue pO_2 over entirely different ranges. This 'range finding' problem is just one example of many in which the outputs specified by different types of homeostatic response appears to require an adjustment to the operation of the basic oxygen signalling pathway.

Multiple feedback systems have been described by which activity in the HIF-α/PHD/pVHL system is modulated.[72] Such systems might alter the *in cellulo* oxygen kinetics by altering substrate to enzyme ratios. Equally co-factor and co-substrate availability may tune the activity of the PHD enzymes to adjust their regulatory range with respect to oxygen. These and other molecular interactions *might, in principle*, contribute the tuning necessary for organismal homeostasis. The outstanding question is: *do they?*

This physiological challenge of oxygen homeostasis is compounded in therapeutics, where the aim is usually to modulate one component of a complex matrix but not another, again with considerable precision. Conceivably, massive computational modelling might ultimately assist our ability to understand or even predict such outputs. But at present there are limitations in our ability to study in *cellulo* biochemistry at the required resolution, and the complex interconnected signalling networks in the necessary detail, to accurately predict integrated physiological outputs. The nineteenth and early twentieth century physiologists who first considered the problem of oxygen homeostasis were woefully ill-equipped to link their beautifully precise observations on integrated physiology to molecular mechanisms. That came about decades later, based on progress in countless areas of biochemistry and biology that underpinned the studies described in this lecture. But excitement in our power to deliver reductionist biology should not make us over-confident in our ability to go back and precisely predict actions in those integrated systems.

Nevertheless, studies of several classes of drug that modulate the output of the HIF pathway are showing promising results: two in the clinical setting. One class of agent aims to inhibit HIF itself, the most advanced of these being agents that bind a large pocket in the PAS domain of HIF-2α to block dimerisation and activity.[73] Such HIF-2 antagonists are showing efficacy in the treatment of clear cell renal carcinoma,[74–76] which manifests the unusual bias to HIF-2α expression that I described earlier; Bill Kaelin will be speaking more about the efficacy of these agents in cancer. But the data raises a number of interesting questions. Structural work has also

revealed pockets in the PAS domains and dimerisation interfaces of other HIF-α iso-forms;[77] although they are smaller, might these also be targeted? Will it be possible to manipulate the HIF-2 transcriptional responses productively in non-malignant disease? As predicted by genetic studies on Hif-2α in the mouse,[58] our recent work reveals effects of pharmacological antagonism of HIF-2 on the control of respiration,[78] indicating that the benefits and risks of systemic inhibition of HIF-2 will need to be considered as clinical development progresses.

The development of PHD inhibitors that activate HIF offers a different strategy for a different set of conditions. These drugs are 2-oxoglutarate analogues that (like hypoxia) reduce the rate of prolyl hydroxylation. Following the principle that augmentation of an endogenous physiological response might be predicted to be an effective therapeutic strategy, a large number of pre-clinical studies support the potential of these molecules to ameliorate ischaemic and hypoxic diseases.[79] So far however, clinical studies have largely been confined to correcting the erythropoietin deficient anaemia that complicates chronic kidney disease. Here, the ability to mimic the natural erythropoietic response to hypoxia, likely entrains other actions (such as direct effects on iron metabolism and on the bone marrow itself), which complement the erythropoietin-stimulating action. As might be predicted from investigations of the erythropoietin gene at the inception of this field, PHD inhibitors have been shown to be very effective in inducing erythropoietin and erythropoiesis. What is less clear is whether effects on the many HIF targets unrelated to erythropoietin and erythropoiesis can be largely avoided by careful adjustment of dose, whether such effects are generally beneficial, or whether they may be harmful in some circumstances. As outlined above, with current (and foreseeable) analytical methods we cannot eliminate these uncertainties. The complexity and inter-connectivity that Darwinian evolution has built, runs counter to precise predictions of therapeutic action, however desirable that might be. Drug development entrains a substantial element of empiricism.

But I will conclude with the prescient words of Sir Michael Jagger and Mr Keith Richards; *'you can't always have what you want, but if you try, sometimes you get what you need'*. As we enjoy this special time in Stockholm, the results of clinical use of PHD inhibitors and HIF-2 antagonists are looking promising.[76,80–83] And as clinician scientists, Gregg, Bill and I look forward to further exploring the therapeutic potential of these agents.

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