Hypoxia-Inducible Factors in Physiology and Medicine

Nobel Lecture, December 7, 2019 by
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OXYGEN HOMEOSTASIS: A BALANCING ACT

In 1865, the French physiologist Claude Bernard famously stated: *"La fixité du milieu intérieur est la condition de la vie libre* (the constancy of the internal environment is necessary for independent life)." In 1932, the American physiologist Walter B. Cannon published his influential book *The Wisdom of the Body*, in which he captured Bernard's concept of a stable equilibrium in a single word: homeostasis, a modern Latin term that is derived from the ancient Greek words *homoios* 'similar' and *histemi* 'standing still'. Although this term has been applied to virtually every physiological system, there is no steady state that is more fundamental, more essential to survival of the organism, than oxygen homeostasis. Indeed, each of the hundred trillion cells in the adult human body requires a constant supply of O_2, principally for mitochondrial respiration. If the delivery of O_2 is disrupted for mere minutes, cells will die, as is most dramatically demonstrated by the catastrophic pathological processes of myocardial infarction (heart attack) and cerebrovascular accident (stroke).

Before considering the mechanisms by which O_2 homeostasis is maintained, let us first consider how this concept also serves an organizing principle for understanding the evolution of life on Earth. We will focus on two extraordinary biological milestones. The first of these occurred approximately 2.5 billion years ago, with the evolution of primitive single-celled organisms capable of transducing solar energy into the chemical energy of carbon bonds through the process of photosynthesis, which utilizes light, carbon dioxide and water as substrates to generate glucose and, as a side product, O_2. These primitive organisms were capable of breaking one of the carbon bonds in glucose, through the process of glycolysis, to generate a small amount of adenosine triphosphate (ATP), which was used to power other biochemical reactions. Initially, the O_2 released from these cells was consumed by reaction with the abundant iron in the Earth's crust, but after much of the iron had been oxidized, the concentration of O_2 in the atmosphere began to rise.

The second extraordinary milestone occurred about 1.5 billion years ago when (slightly less) primitive single-celled organisms evolved the ability to catalyze a complex series of chemical reactions in which glucose and O_2 were utilized to generate ATP, with carbon dioxide and water as side products. Compared to the fermentation of glucose to lactate, the complete oxidation of glucose yielded approximately 18-fold more ATP per mol of glucose. Next, some of these primitive respiring cells established a symbiotic relationship within other cells and became the mitochondria of present day eukaryotic organisms.

Just as the evolution of photosynthetic organisms and the resulting increase in atmospheric O_2 levels was a necessary prerequisite for the evolution of organisms capable of respiration, so the evolution of multicellular organisms was dependent upon the efficient generation of energy, which was needed in much greater amounts to generate and maintain complex collections of different cell types acting in a coordinated manner over space and time. Finally, because small animals tend to be eaten by large animals, metazoan evolution over a period of several hundred million years made a quantum leap with the increased body mass of vertebrates, which in turn required more complex systems for O_2 delivery. Thus, the roundworm *Caenorhabditis elegans* consists of a thousand cells, all of which obtain O_2 by direct diffusion from the atmosphere. By contrast, the fruit fly *Drosophila melanogaster* consists of hundreds of thousands of cells and developed tracheal tubes to conduct air to the interior of the organism, whereas *Homo sapiens* has evolved complex respiratory and circulatory systems to capture and deliver O_2 to trillions of cells.

O_2 homeostasis is a simple matter of supply and demand – matching O_2 consumption with O_2 delivery – but achieving this goal is not so simple when multiplied by trillions of cells integrated over the millions of

minutes of a human life. Worse yet, there is little room for error at the cellular level, as either too little or too much O_2 for too long can be lethal to cells. The basis for this conundrum is the mechanism by which ATP is synthesized in the mitochondria. During the oxidation of glucose, reducing equivalents are captured in the molecules NADH and $FADH_2$ and passed to complex I and II, respectively, of the electron transport chain, which in turn transfer the electrons to complex III and then to complex IV. This process of electron transport generates a proton gradient that is used to drive the synthesis of ATP. In the final step, complex IV (cytochrome c oxidase) catalyzes the transfer of electrons to O_2 to form H_2O. However, the process of electron transfer is not completely efficient, and some electrons escape prior to complex IV and react with O_2 to form superoxide anion, which is converted to hydrogen peroxide. These reactive oxygen species (ROS) oxidize cellular macromolecules and, if present in large quantities for prolonged periods of time, result in cell dysfunction and death. The electron transport chain is optimized to function most efficiently at physiological O_2 levels, such that any decrease or increase in O_2 availability results in less efficient electron transport and greater production of ROS. Hence, the need to precisely maintain O_2 levels within a narrow physiological range. How is this formidable task accomplished?

ERYTHROPOIETIN AND THE CONTROL OF RED BLOOD CELL PRODUCTION

I arrived at Johns Hopkins University School of Medicine to start my postdoctoral fellowship in Medical Genetics in 1986. I had earned an M.D. degree and a Ph.D. in genetics at the University of Pennsylvania in 1984 by performing thesis research in the laboratory of Elias Schwartz and Saul Surrey in the Hematology Division at Children's Hospital of Philadelphia. We studied a family with a variant form of β-thalassemia, which was the first disease in which many different mutations affecting the expression of a gene (in this case, the gene encoding β-globin) were identified in a comprehensive manner. The leading research team in the field was a collaboration involving Stuart Orkin at Harvard and Haig Kazazian and Stylianos Antonarakis at Johns Hopkins. After completing internship and residency training in pediatrics at Duke University Medical Center, I accepted a postdoctoral fellowship at Hopkins because of my familiarity with the genetics research being performed there and the opportunity to train in clinical genetics with the renowned Victor McKusick.

As a pediatric geneticist, my interest at the time was in studying the regulation of gene expression during development. I thought that this could best be accomplished by utilizing a newly developed technique in which cloned DNA fragments were microinjected into fertilized mouse

eggs to create transgenic mice. At the time, Haig Kazazian and Stylianos Antonarakis had transitioned from identifying mutations in the β-globin gene that caused β-thalassemia to analyzing mutations in the factor VIII gene that caused hemophilia A. Haig advised me to contact Chuck Shoemaker at Genetics Institute to ask whether he could provide me with cloned genomic DNA from the factor VIII gene. Chuck said that he could, but that there was another gene I might want to consider studying called erythropoietin (EPO). Chuck and his colleagues had previously cloned the cDNA and genomic DNA encoding erythropoietin, which is the hormone that controls red blood cell production.

I learned that EPO was produced in the liver during fetal life and in the kidney after birth. EPO is secreted into the bloodstream and binds to receptors on the surface of erythroid progenitor cells in the bone marrow, stimulating their survival, proliferation, and differentiation. In addition to this interesting developmental regulation, expression of the gene was induced by hypoxia; i.e. EPO producing cells were able to sense a decrease in local O_2 levels and respond with increased *EPO* gene transcription, leading to increased red blood cell production and increased blood O_2-carrying capacity, thereby correcting the tissue hypoxia – a beautiful homeostatic system.

However, patients with chronic kidney disease (CKD) have a progressive loss of EPO production as their disease progresses, resulting in anemia of progressively greater severity. The molecular cloning of EPO was a landmark event in the biotech industry, as it enabled commercial production of recombinant human EPO for the treatment of CKD patients with anemia, which had previously required transfusions and the accompanying risk of transfusion-associated diseases, such as hepatitis and AIDS.

I was fortunate that John Gearhart's lab in the Department of Physiology was accomplished at making transgenic mice. I went to John and asked him whether he would be willing to inject the human *EPO* gene into fertilized mouse eggs. He said yes.

Like the renowned physiologist Walter B. Cannon, I had obtained my undergraduate education at Harvard College. During my junior and senior years, I had worked in Park Gerald's laboratory on the Pediatric Genetics Unit at Children's Hospital in Boston for my honors thesis, using somatic cell hybrids for human gene mapping. I had also spent six years at the University of Pennsylvania and Children's Hospital of Philadelphia for my M.D.-Ph.D. training, so I had already worked in several major biomedical research institutions. When I arrived at Johns Hopkins, I was impressed by the culture of collegiality that is interwoven into the fabric of the institution. I learned that whatever scientific technique I wanted to apply to my research, there was someone who knew how to do it – and was willing to help me.

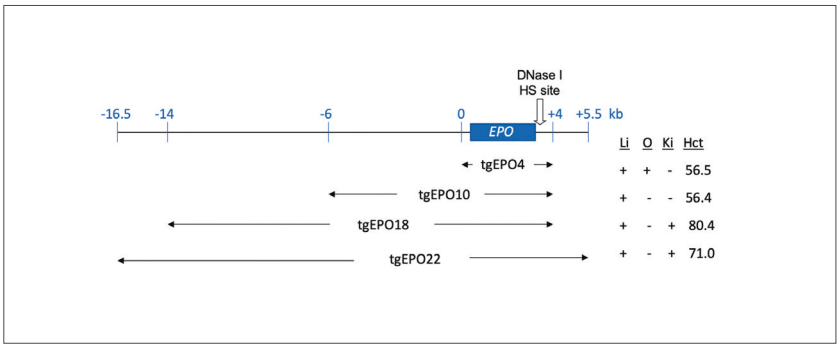

Figure 1. Human EPO transgenic mice. Nucleotide coordinates are given relative to the transcription start site (drawing is not to scale). Expression in liver (Li), kidney (Ki), or other tissues (O) and mean hematocrit (%) are indicated.

The Gearhart lab injected into mouse eggs a 4-kilobase (kb) DNA fragment containing the human *EPO* gene that I had purified from a plasmid vector after amplification in *E. coli*. We obtained mice in which the DNA had stably incorporated into the mouse genome such that the *EPO* transgene was passed on to half of each transgenic mouse's offspring at each mating. These transgenic mice were polycythemic – their hematocrit (i.e. the proportion of blood volume that is occupied by red blood cells), which is normally around 45%, was increased to over 55% (Semenza *et al.*, 1989). When adult mice were subjected to bleeding or were treated with phenylhydrazine to induce hemolysis, the expression of the human EPO transgene was dramatically induced in the liver, but not in the kidney. In addition, the transgene was expressed at high levels in organs where the endogenous gene was not expressed.

We concluded that the 4-kb *EPO* transgene: (i) contained regulatory sequences that mediated inducible human EPO expression in the liver; (ii) lacked a positive regulatory element required for inducible expression in the kidney; and (iii) lacked a negative regulatory element required to silence the gene in other tissues. To test this hypothesis, we next injected a 10-kb transgene that contained an additional 6 kb of 5'-flanking DNA beyond the 0.4-kb present in the first transgene (Figure 1). Mice transgenic for the 10-kb transgene were also polycythemic, with a mean hematocrit of 56%, and expressed human EPO in an inducible manner in the liver, but not in the kidney, and did not express human EPO mRNA in other tissues (Semenza *et al.*, 1990). We concluded that the putative negative regulatory element was present within the additional 6-kb of 5-flanking DNA.

Next we generated mice with an additional 8 kb of 5'-flanking DNA. These mice had a mean hematocrit of 80%, expressed the 18-kb human *EPO* transgene in an inducible manner in both the liver and kidney, and

renal expression was localized by *in situ* hybridization (in collaboration with Stephen Koury at Vanderbilt) to the same rare interstitial/peritubular fibroblast-like cell type that expressed the endogenous mouse *Epo* gene (Semenza *et al.*, 1991a). Mice with a 22-kb transgene with additional 5' and 3' flanking sequences showed similar expression, suggesting that there no additional regulatory elements in proximity to the gene. Thus, we had successfully obtained regulated, tissue-specific expression of the human *EPO* gene in mice. I subsequently performed a deoxyribonuclease I (DNase) assay, using nuclei purified from the livers of mice carrying the 10-kb *EPO* transgene, and this experiment suggested the presence of an important transcriptional regulatory element in the 0.7-kb of 3'-flanking DNA, which was present in all three of the transgenes (Semenza *et al.*, 1991b).

Our next task was to establish the basis for hypoxia-induced *EPO* gene transcription. EPO was discovered by Alan Erslev, a hematologist at Thomas Jefferson University in Philadelphia (Erslev, 1957). Dr. Erslev's colleague Jaime Caro first reported that mice subjected to anemia or hypoxia responded with increased renal expression of EPO mRNA (Schuster et al., 1987). Exposure of the Hep3B human hepatoma cell line to hypoxia was shown to induce increased EPO expression (Goldberg *et al.,* 1987) and the Caro lab demonstrated that this was due to increased transcription (Costa-Giomi et al., 1990). I decided that we would utilize Hep3B cells to localize the *EPO* gene sequences required for hypoxia-inducible transcription and Jaime Caro generously sent us a flask of Hep3B cells to start our work.

My understanding of transcriptional regulation was greatly influenced by the work of Harold Weintraub, who had also received his M.D. and Ph.D. degrees from the University of Pennsylvania School of Medicine. He pioneered use of the DNase sensitivity assay as a means of identifying transcriptional regulatory elements (Weisbrod and Weintraub, 1979). In order for transcription factors to bind to DNA, the chromatin must be unwound from its nucleosomal structure, in which the DNA is tightly associated with histone proteins. Such unwound DNA, because it is not protected by histones, is exposed and accessible for binding of transcription factors, but also for degradation by DNase. Our finding of nuclease sensitivity of the *EPO* gene 3'-flanking region (Semenza *et al.*, 1991b) pointed to this area of the gene as the potential location of a hypoxia response element (HRE).

To extend this analysis to single nucleotide resolution, I performed a DNase footprint assay, in which a purified 256-bp DNA fragment from the *EPO* 3'-flanking region was incubated with nuclear lysate prepared from the livers of *EPO* transgenic mice that were either untreated or subjected to phlebotomy to cause anemia. The assay revealed a region of the 256-bp fragment that when incubated with nuclear extract from livers of control

mice was hypersensitive to DNase I digestion, indicating absence of protein binding, and when incubated with liver nuclear extract from anemic mice was protected from DNase I digestion, suggesting that a transcription factor had been induced to bind to the DNA in response to anemia (Semenza *et al.*, 1991b). Furthermore, we inserted the 256-bp fragment into a reporter gene, in which expression of bacterial chloramphenicol acetyltransferase (CAT) coding sequences was driven by a basal SV40 promoter, and then transfected the reporter into Hep3B cells, which were incubated for 24 hours at 20% O_2 (standard tissue culture conditions of 95% air and 5% CO_2) or in a chamber flushed with a hypoxic gas mixture consisting of 1% O_2, 5% CO_2 and 94% N2. Insertion of the 256-bp fragment led to 2-fold increased CAT expression in cells subjected to hypoxia. Taken together, these experiments suggested that we had localized a cis-acting HRE to the 3'-flanking region of the *EPO* gene and there appeared to be a nuclear factor capable of binding to this sequence that was present specifically in the nuclei of liver cells from anemic mice. The requirement for 3'-flanking region sequences for hypoxia-inducible human *EPO* gene expression was also reported by the Caro lab (Beck *et al.*, 1991) and Peter Ratcliffe's lab at Oxford identified a candidate HRE in the 3'-flanking region of the mouse *Epo* gene (Pugh *et al.*, 1991).

We tested various fragments of the 256-bp region for their ability to function as an HRE and identified a 50-bp fragment that mediated a 7-fold increased reporter gene expression in hypoxic Hep3B cells (Semenza and Wang, 1992). We focused on the 5' end of this fragment (Figure 2), which had the sequence 5'-GCC.CTA.CGT.GCT.GTC.TCA-3'. Scanning mutagenesis performed in six 3-bp intervals revealed that the mutation CTA→TAT, CGT→AAA, or GCT →ATG (underscored in the sequence above) resulted in a complete loss of HRE activity (Semenza and Wang, 1992).

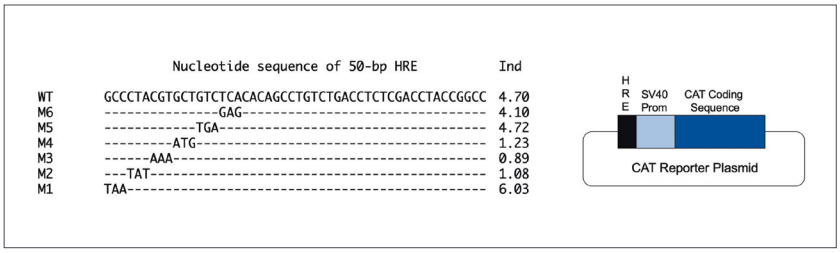

Figure 2. Functional analysis of human *EPO* gene hypoxia response element (HRE). Scanning mutagenesis of the first 18 bp of the HRE was performed. Wild type (WT) and mutant (M) nucleotide sequences were inserted into the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into Hep3B cells, which were exposed to 20% or 1% O_2 for 24 hours. The fold hypoxic induction (Ind; ratio of CAT activity at 1%: 20% O_2) was determined.

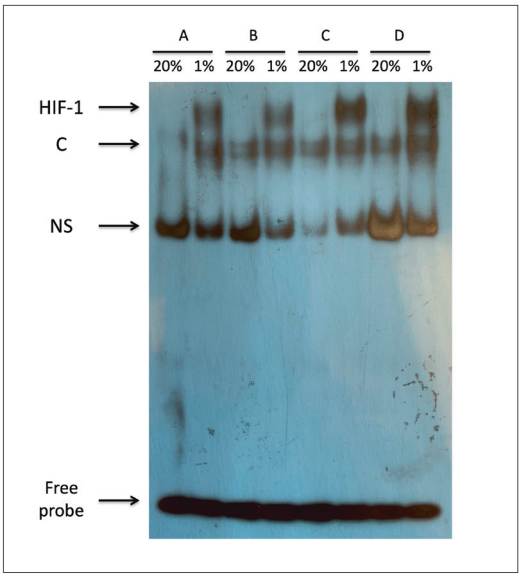

Figure 3. Identification of HIF-1 by electrophoretic mobility shift assay. A ^{32}P-labeled 18-bp oligonucleotide probe W18 was incubated (in four different binding buffers labeled A–D) with aliquots of nuclear extract prepared from Hep3B cells that were exposed to 20% or 1% O_2 for 4 hours. After incubation, polyacrylamide gel electrophoresis was performed to separate the free probe from probe that was bound by nuclear proteins. One of these bound proteins (HIF-1) is only present in nuclear extracts from hypoxic cells. The binding of a protein that is constitutively expressed (C) and a binding activity that is non-specific with respect to the nucleotide sequence of the probe (NS) are also indicated. The identification of HIF-1 as a DNA binding activity in 1992 led to its purification and the isolation of DNA encoding the HIF-1α and HIF-1β subunits in 1995, which opened up the field of oxygen biology to molecular analysis. The autoradiograph shown in the figure was donated to the Nobel Prize Museum in Stockholm, where it is currently on display.

An electrophoretic mobility shift assay (EMSA) was performed using a ^{32}P-labelled double-stranded oligonucleotide probe containing the 18-bp sequence shown above. This probe, designated W18, was incubated with nuclear extracts, which were prepared from Hep3B cells that were exposed to either 20% or 1% O2 for 4 hours (Figure 3). The binding of a nuclear factor that was only present in hypoxic cells was identified. Furthermore, the factor did not bind to an oligonucleotide, designated M18, which contained the same CGT→AAA mutation that abrogated hypoxia-inducible reporter gene expression, thereby linking the hypoxia-induced binding of the factor to hypoxia-induced transcriptional activation (Semenza and Wang, 1992). We designated this DNA-binding activity hypoxia-inducible factor 1 (HIF-1).

My postdoctoral fellow, Guang Wang, performed methylation interference assays, which revealed that HIF-1 directly contacted four guanine

residues in the W18 oligonucleotide, two each on the sense and antisense strands in the sequence 5'-CGTGC-3' (Wang and Semenza, 1993a), which was consistent with the mutagenesis studies demonstrating that these residues were required for HRE activity and for HIF-1 DNA-binding activity (Semenza and Wang, 1992), as described above. Remarkably, the induction of HIF-1 DNA-binding activity was detected within 30 minutes and was maximal after 4 hours of hypoxic exposure; when hypoxic cells, were reoxygenated, HIF-1 DNA-binding activity was markedly reduced within 5 minutes (Wang and Semenza, 1993a). These were quite impressive kinetics because this was essentially the amount of time required for O_2 to diffuse out of or into the tissue culture media. Furthermore, we found that both *EPO* gene expression and HIF-1 DNA-binding activity were induced by exposure of Hep3B cells to cobalt chloride (Wang and Semenza, 1993c) or the iron chelator desferrioxamine (Wang and Semenza, 1993b). These findings tightly linked HIF-1 activity to *EPO* gene transcription.

OXYGEN SENSING BEYOND EPO

Hypoxia-induced *EPO* gene expression was demonstrated in Hep3B and HepG2 cells (Goldberg et al., 1989) but was not a general property of mammalian cell lines, which was consistent with its restricted expression *in vivo*. However, we found that HIF-1 DNA-binding activity was induced by hypoxia in all mammalian cell lines that we tested (Wang and Semenza, 1993c). These results were consistent with studies from the Ratcliffe lab indicating that the expression of a reporter gene containing the *Epo* gene HRE was induced by hypoxia after transfection into several different mammalian cell lines (Maxwell et al., 1993). These results indicated that the ability to sense decreased O_2 availability and to respond by induction of HIF-1 activity was a general property of mammalian cells.

Based on our finding that HIF-1 was ubiquitously induced by hypoxia, we hypothesized that HIF-1 regulated the expression of genes in addition to *EPO* and that these genes were likely to also contain HREs. To test this hypothesis, we focused on genes encoding glycolytic enzymes, since the switch from oxidative to glycolytic metabolism is a fundamental and perhaps universal response of animal cells to hypoxia, as described above, and Keith Webster had reported induction of glycolytic gene expression in response to hypoxia (Webster, 1987). We identified the core HIF-1 binding-site sequence 5'-ACGTG-3' in the human genes encoding aldolase A (*ALDOA*), enolase 1 (*ENO*1), and phosphoglycerate kinase 1 (PGK1) and in the mouse genes encoding lactate dehydrogenase A (*Ldha*) and phosphofructokinase L (*Pfkl*) (Semenza *et al*., 1994). Using Hep3B cells, which express EPO, and HeLa cells, which do not, we demonstrated hypoxia-in-

duced binding of HIF-1 to these glycolytic gene sequences by EMSA; HRE activity of DNA fragments encompassing these HIF-1 binding sites; and induction of endogenous glycolytic gene expression by exposure to hypoxia, cobalt chloride or desferrioxamine (Semenza *et al.*, 1994). The Ratcliffe lab reported similar results for PGK1 and *Ldha* (Firth *et al.*, 1994).

PURIFICATION OF HIF-1

To thoroughly investigate the molecular basis for hypoxia-induced gene expression, we needed to isolate the DNA sequences encoding HIF-1. We performed an experiment in which we fractionated Hep3B nuclear proteins by polyacrylamide gel electrophoresis (PAGE), transferred the proteins to a nitrocellulose membrane, and incubated it with the W18 probe. We reasoned that if HIF-1 was a monomer and was successfully renatured after transfer it might bind to the probe, whereas if HIF-1 consisted of multiple subunits, the binding activity would not be reconstituted. We observed a single band on the autoradiograph, suggesting that the protein was monomeric. Unfortunately, excitement trumped scientific rigor and we did not attempt to replicate this result, but instead employed an expression cloning strategy that Steven McKnight's lab at the Carnegie Institution (which like Johns Hopkins is located in Baltimore) had used to identify cDNA encoding the transcription factor C/EBP (Vinson *et al.,* 1988). We generated a recombinant bacteriophage library expressing cDNA sequences prepared from mRNA that we had isolated from hypoxic Hep3B cells. Several million bacteriophage plaques were screened using the W18 probe with no positive results.

I went to Haig Kazazian for advice. He suggested that I speak with Tom Kelly, the chairman of the Department of Genetics and Molecular Biology, whose lab was one of the first to purify a protein by DNA affinity chromatography (Rosenfeld and Kelly, 1986). Just as when I went to see John Gearhart in 1987, my entire research plan was dependent on obtaining essential assistance from a senior Hopkins faculty member. I met with Tom Kelly, told him what I wanted to do, and asked if he could help. Tom said yes. Two postdoctoral fellows in the Kelly lab, Dan Herendeen and George Brush, provided invaluable advice and instruction to my postdoctoral fellow Guang Wang, who performed the purification.

The purification strategy that I designed had two important components. First, it was based on our critical observation that the 3-bp mutation CGT→AAA abrogated the ability of the 50-bp HRE to mediate hypoxia-inducible transcription and abrogated the ability of HIF-1 to bind to the oligonucleotide probe. Second, it relied on a pilot analytical purification, which involved thirty 15-cm tissue-culture dishes of Hep3B cells that were exposed to 1% O_2 for 4 hours, and a large-scale purification, which involved

120 liters of HeLa S3 cells grown in suspension culture in the presence of 125 μM $CoCl_2$ for 4 hours. [HeLa, the first and still the most commonly-used human cell line, was established by Dr. George Gey from a cervical cancer biopsy obtained from Mrs. Henrietta Lacks at the Johns Hopkins Hospital in 1951 (Scherer *et al.,* 1953).] The HIF-1 DNA-binding activity present in hypoxic Hep3B cells was our gold standard, but it would be very difficult to isolate sufficient HIF-1 from Hep3B cells to perform even limited amino acid sequence analysis, which would require at least 50 picomoles of purified protein. The use of HeLa cells in spinner cultures provided a means for the necessary scale-up, but we needed to be certain that the HIF-1 DNA-binding activity that was cobalt-induced in HeLa cells was in all respects identical to the hypoxia-induced HIF-1 from Hep3B cells, so we analyzed the two preparations in parallel.

Guang first performed anion exchange chromatography using DEAE-Sepharose, and tested aliquots from each fraction by EMSA using the W18 probe. The positive fractions were pooled and DNA affinity chromatography was performed, using the W18 oligonucleotide coupled to Sepharose beads; the bound protein was eluted in high salt and applied to a column containing the M18 oligonucleotide coupled to Sepharose beads; the flow-through from this column was then applied to another W18 column and the bound protein was eluted and fractions were tested by EMSA using the W18 probe. In the purification from HeLa cells, we started with 3,040 mg of nuclear extract containing 608,000 units of HIF-1 DNA-binding activity (we defined one unit as equivalent to the DNA binding activity present in 5 μg of crude nuclear extract) with a specific activity of 0.2 units per μg, and we recovered 0.06 mg of purified protein with a specific activity of 2,250 units per μg, which represented a purification of 11,250-fold and a yield of 22% (Wang and Semenza, 1995).

HIF-1 purified from HeLa and Hep3B cells was fractionated by SDS-PAGE and the gel was silver-stained to visualize the protein, which revealed a polypeptide with an apparent molecular mass of 120 kDa, which was designated HIF-1α, and a series of polypeptides with an apparent molecular mass of 91–94 kDa, which were designated HIF-1β (Wang and Semenza, 1995). Glycerol gradient sedimentation, UV crosslinking to the W18 oligonucleotide, and methylation interference studies suggested that HIF-1 was a heterodimer of HIF-1α and HIF-1β, and that both subunits contacted DNA in the major groove (Wang and Semenza, 1995).

HIF-1 purified from HeLa cells was fractionated by SDS-PAGE and the polypeptides were transferred to a membrane, individually digested with trypsin, and fractionated by reverse-phase HPLC. Protein microsequence analysis was performed in the laboratory of David Speicher at the Wistar Institute in Philadelphia. The sequences of four peptides from HIF-1α were obtained and degenerate oligonucleotides were synthesized, based

on the sequences of two of the peptides, and used as primers for PCR with cDNA prepared from mRNA purified from hypoxic Hep3B cells. The PCR product was sequenced and shown to encode the predicted amino acids, demonstrating that the two polypeptides were contiguous. I received advice regarding primer design and PCR strategy from my faculty colleague (and future Nobel laureate) Peter Agre, who had used a similar approach to isolate cDNA that encoded aquaporin 1 several years earlier (Preston and Agre, 1991).

My postdoctoral fellow Bing-Hua Jiang isolated a 3.4-kb cDNA from the Hep3B library that encoded an 826-amino-acid HIF-1α protein, which contained a basic helix-loop-helix (bHLH) domain and a PAS homology domain in its amino-terminal half (Wang *et al.*, 1995). The bHLH domain is a DNA-binding domain that defines a large superfamily of transcription factors. The PAS domain was first identified in the PER, ARNT, and SIM proteins (Hoffman et al., 1991). Analysis of two tryptic peptides from the 94-kDa HIF-1β polypeptide yielded partial amino acid sequences that were used to design degenerate oligonucleotide primers that amplified a PCR product of the expected size from Hep3B cDNA. Nucleotide and amino acid sequence analysis revealed that the HIF-1β sequence was identical to the aryl hydrocarbon nuclear translocator (ARNT), which is a bHLH-PAS protein that dimerizes with the aryl hydrocarbon receptor, another bHLH-PAS protein (Reyes et al., 1992). Two isoforms of ARNT that differ by the presence or absence of a 15-amino-acid sequence encoded by an alternative exon had been described (Hoffman et al., 1991), providing a basis for the purification of 91- and 93/94-kDa HIF-1β polypeptides. Further studies revealed that HIF-1β/ARNT was the common subunit for multiple bHLH-PAS proteins (Wu and Rastinejad, 2017).

We raised antibodies against HIF-1α and HIF-1β and showed that these antibodies supershifted the HIF-1: W18 complex in the EMSA. We analyzed the time course of induction of HIF-1α and HIF-1β in nuclear extracts of Hep3B cells exposed to 1% O_2. Immunoblot assays revealed that the levels of both proteins were increased after 30 min and peak levels were reached after 4 hours (Wang *et al.*, 1995), very similar to the induction of HIF-1 DNA-binding activity as detected by EMSA (Wang and Semenza, 1993a). When hypoxic cells were reoxygenated, HIF-1α and HIF-β protein levels in nuclear extracts were decreased by more than 50% within 5 min and completely lost by 15 min (Wang *et al.*, 1995). Our paper reporting the cloning of HIF-1 concluded with the following statement: "The cDNA and antisera described here will permit a detailed analysis of the molecular mechanisms by which HIF-1 activity is regulated and the role of HIF-1 in mammalian O_2 homeostasis" (Wang *et al.*, 1995). We shared these reagents with all of the other investigators in the field as soon as the work was published.

IDENTIFICATION OF HIF-2α AND HIF-3α

Once we had published the nucleotide and amino acid sequence of HIF-1α, it was possible to perform database queries for related sequences, which led to the discovery by four labs of a protein that they named endothelial PAS domain protein 1 (Tian *et al*., 1997), HIF-like factor (Ema et al., 1997), HIF-related factor (Flamme *et al.,* 1997) and member of PAS domain family 2 (Hogenesch *et al*., 1997), respectively, but which is now known as HIF-2α. Another ortholog, HIF-3α, was subsequently identified (Gu et al., 1998). Each of these proteins heterodimerizes with HIF-1β under hypoxic conditions and is subject to O_2-dependent degradation. Unlike HIF-1α and HIF-1β, which are found in almost all metazoan species, HIF-2α and HIF-3α are only found in vertebrates (Duan, 2016; Graham, 2017).

CHARACTERIZATION OF HIF-1α PROTEIN STRUCTURE AND FUNCTION

We demonstrated that efficient dimerization with HIF-1β and binding to the *EPO* gene HRE required the amino terminal half of HIF-1α (residues 1-390), which encompassed the bHLH and PAS domains (Figure 4), whereas transcriptional activation of the HRE-reporter gene required the carboxyl terminal half of HIF-1α (residues 531–826) (Jiang *et al.*, 1996a). When these C-terminal residues were fused to the GAL4 DNA-binding domain, they mediated hypoxia-inducible transactivation of a reporter gene in which firefly luciferase coding sequences were downstream of a basal promoter containing five GAL4 binding sites. Remarkably, expression of the GAL4-HIF-1α fusion protein was not regulated by hypoxia, indicating that hypoxia directly regulated the transactivation domain (TAD) function (Jiang *et al.*, 1997). TAD function was also inducible by treatment of cells with cobalt chloride or desferrioxamine, similar to the induction of endogenous HIF-1α protein levels by these agents (Wang et al., 1993b), but these agents did not affect the levels of the fusion protein, only its TAD function. These results suggested that similar mechanisms might underlie the O_2-dependent regulation of HIF-1α protein stability and TAD function.

A detailed analysis revealed two different regions that functioned as TADs when fused to the GAL4 DNA-binding domain: residues 531–575 and 786–826 (Jiang *et al*., 1997). The residues between these two TADs, amino acids 576–785, had a negative effect on TAD function, and were designated the inhibitory domain (Jiang *et al.*, 1997). In order to identify proteins interacting with HIF-1α to regulate TAD function, we performed a yeast two-hybrid assay and postdoc Connor Mahon identified a protein

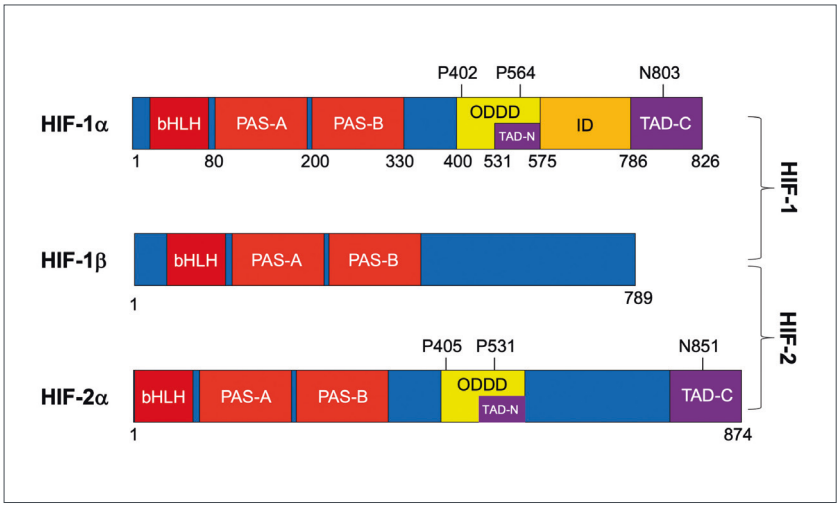

Figure 4. Structure of HIF-1 and HIF-2. The well-defined structural/functional domains of HIF-1a are the basic helix-loop-helix (bHLH) domain, PER-ARNT-SIM homology (PAS) domains, oxygen-dependent degradation domain (ODDD), amino-terminal transactivation domain (TAD-N), inhibitory domain (ID), and carboxy-terminal transactivation domain (TAD-C). Sites of O_2-dependent prolyl (P402, P564) and asparaginyl (N803) hydroxylation are shown. Each of the subunits has multiple isoforms as a result of alternative transcription initiation and splicing; the longest isoform is shown (drawing is not to scale).

that bound to residues 757–826, but not to 786–826, and negatively regulated TAD function; we named this protein factor inhibiting HIF-1 (FIH-1) (Mahon *et al.*, 2001). Remarkably, residues 757–826 functioned as a hypoxia-inducible TAD, whereas residues 786–826 functioned as a constitutively active TAD (Jiang *et al.*, 1997).

O2-DEPENDENT REGULATION OF HIF-1α PROTEIN STABILITY AND TAD FUNCTION

In order to investigate the expression of HIF-1α in cells exposed to a range of O_2 concentrations, we collaborated with Christian Bauer's lab at the Institute of Physiology in Zurich. They exposed HeLa cells to precisely defined O2 levels, ranging from 20% to 0%, for 4 hours in an instrument known as a tonometer. The cells were flash-frozen and sent to my lab in Baltimore where Bing-Hua Jiang prepared nuclear extracts and performed immunoblot assays to quantify the levels of HIF-1α and HIF-1β. He found that HIF-1α levels increased gradually as O_2 levels decreased from 20% to 6% O_2, then rose rapidly at lower O_2 levels, with a half-maximal response at 1.5–2% and maximal response at 0.5% O_2 (Jiang *et al.*, 1996b). It was known that there was considerable variation in the O_2 lev-

els in various organs and even in different areas of the same organ. Other than in the lungs, most cells are exposed to O_2 levels between 3% and 6% O_2, which correspond to a PO_2 of 20 and 40 mm Hg, respectively. This meant that if the induction of HIF-1α *in vivo* was similar to that demonstrated in HeLa cells then, for most cells in the body, any decrease in O_2 levels would occur on the steep portion of the stimulus-response curve, allowing a graded transcriptional response to hypoxia: the greater the decrease in O_2 levels, the greater the increase in HIF-1α protein levels and in the expression of HIF target genes. We subsequently showed a similar graded response in vivo, using an isolated and perfused lung preparation (Yu *et al.*, 1998).

Our initial studies had demonstrated the remarkably rapid degradation of HIF-1α in post-hypoxic cells (Wang *et al.*, 1995) and the Caro lab demonstrated that this degradation was dependent upon ubiquitination and proteasomal degradation (Salceda and Caro, 1997). HIF-1α residues 401–603 were shown to be necessary and sufficient for O2-dependent protein degradation (Huang *et al.,* 1998). The Ratcliffe lab reported that in renal carcinoma cell lines that lacked expression of the wild-type von Hippel-Lindau tumor suppressor protein (VHL), HIF-1α and HIF-2α protein were constitutively expressed regardless of O2 availability (Maxwell *et al.*, 1999). When an expression vector encoding wild-type VHL was transfected into VHL-deficient cells, O_2-dependent degradation of HIF-1α and HIF-2α was restored. VHL directly bound to the HIF-α subunits and this binding was disrupted when cells were treated with cobalt chloride or iron chelator (Maxwell *et al.*, 1999). Structural data indicated that VHL, in a complex with Elongin B and C, likely functioned as a ubiquitin-protein ligase (Stebbins et al., 1999). The trigger for VHL binding and subsequent HIF-1α ubiquitination and proteasomal degradation was the hydroxylation of proline residue 402 and/or 564 in a reaction, which utilized O_2 and α-ketoglutarate as substrates and Fe^{2+} as a co-factor, catalyzed by prolyl hydroxylase domain (PHD) proteins (Epstein *et al.*, 2001; Jaakkola *et al.,* 2001; Ivan *et al.*, 2001; Masson *et al.,* 2001; Yu *et al.*, 2001). Three genes encoding PHD1-3, respectively, were identified in mammalian species (Bruick and McKnight, 2001; Epstein *et al.*, 2001).

Whereas O_2-dependent hydroxylation of Pro-402 and/or Pro-564 was required for binding of VHL, O_2-dependent hydroxylation of asparagine residue 803 abrogated the binding of the coactivators p300 and CBP to the carboxyl-terminal TAD of HIF-α subunits in a reaction that was catalyzed by FIH-1 (Lando *et al.*, 2002a,b). Taken together, these studies indicated that both the half-life and activity of HIF-α subunits were regulated by O_2-dependent post-translational modifications that transduce changes in O_2 availability to the nucleus as changes in HIF–mediated gene transcription. Thus, the fundamental transcriptional mechanism that under-

lies oxygen homeostasis, composed of cis-acting HREs and trans-acting HIFs regulated by PHDs, VHL, and FIH-1 was delineated by a small number of laboratories over the course of a dozen years from 1991 to 2002.

VASCULAR ENDOTHELIAL GROWTH FACTOR AND THE CONTROL OF ANGIOGENESIS

Systemic hypoxia due to anemia or ascent to high altitude induces a systemic response, including the induction of EPO expression to induce red blood cell production and thereby increase the blood O_2 carrying capacity. In contrast, localized hypoxia due to reduced tissue perfusion in the context of vascular disease results in a local response, the production of new blood vessels, which is known as angiogenesis. In 1992, Eli Keshet's laboratory reported that exposure of cells to hypoxia induced the expression of mRNA encoding vascular endothelial growth factor (VEGF) (Shweiki *et al.*, 1992). Analysis of glioblastoma tumor sections revealed VEGF expression in cells surrounding areas of necrosis – which represent tumor cells that have died because they are too far from a blood vessel to receive adequate O_2 for survival, suggesting that intratumoral hypoxia was a key stimulus for tumor vascularization (Shweiki *et al.*, 1992). Werner Risau's lab also reported a role for VEGF in tumor angiogenesis (Plate *et al.*, 1992).

We identified a 47-bp sequence located approximately 1 kb 5' to the transcription start site of the *VEGF* gene, which mediated hypoxia-inducible reporter gene expression and contained a HIF-1 binding site; furthermore, the same 3-bp mutation (TACGTG to TAAAAG) that inactivated the *EPO* gene HRE and abrogated HIF-1 binding also inactivated the *VEGF* HRE and abrogated HIF-1 binding to an oligonucleotide from the *VEGF* gene (Forsythe *et al.,* 1996). We obtained from Oliver Hankinson's lab wild-type Hepa1 cells; an ARNT/HIF-1β-deficient subclone that they had identified and designated c4; and a subclone of c4 cells (designated VT2) that had been transfected with an ARNT expression vector (Hoffman *et al.*, 1991). We found that in the absence of ARNT/HIF-1β, there was very little induction of HIF-1α protein and no HIF-1 DNA-binding activity under hypoxic conditions in c4 cells, whereas in VT2 cells HIF-1β protein expression and hypoxia-induced HIF-1α protein expression as well as HIF-1 DNA-binding were rescued. Analysis of VEGF mRNA expression revealed hypoxic induction in wild type and VT2 cells but not in c4 cells (Forsythe et al., 1996). In addition, co-transfection of the VEGF HRE reporter gene with expression vectors encoding HIF-1α and HIF-1β dramatically increased reporter gene expression under both hypoxic and non-hypoxic conditions (Forsythe *et al.*, 1996).

Taken together, these studies demonstrated that HIF-1 was necessary and sufficient for hypoxia-induced *VEGF* transcription. Judah Folkman

had drawn attention to the critical role of angiogenesis in tumor progression (Folkman, 1971) and had characterized a "tumor angiogenesis factor" (Folkman *et al.*, 1971). Napoleone Ferrara subsequently purified the protein, cloned the gene, and named it VEGF (Leung *et al.*, 1989). The discovery of hypoxia-induced and HIF-mediated *VEGF* gene transcription provided a molecular mechanism underlying the observation of increased VEGF expression in human tumors.

GENERATION OF HIF-1α DEFICIENT EMBRYONIC STEM CELLS AND KNOCKOUT MICE

In order to further investigate the role of HIF-1 in mammalian development, physiology, and disease pathogenesis, my postdoctoral fellow Narayan Iyer engineered (in collaboration with John Gearhart's lab) mouse embryonic stem (ES) cells in which the HIF-1α coding sequence was disrupted by homologous recombination (Iyer *et al.,* 1998). Loss of HIF-1α expression was associated with decreased expression of mRNAs encoding the glucose transporters GLUT1 and GLUT3 and the glycolytic enzymes hexokinase (HK1, HK2), glucose phosphate isomerase (GPI), PFKL, ALDOA, ALDOC, triose phosphate isomerase (TPI), PGK1, ENO1, pyruvate kinase (PKM), and LDHA. Thus, HIF-1 coordinately regulated the entire glycolytic pathway in hypoxic ES cells (Iyer *et al.*, 1998).

Hypoxia-induced VEGF mRNA expression was completely eliminated in the HIF-1α-deficient ES cells. The ES cells were injected into mouse blastocysts and *Hif1a*$^{+/-}$ mice, which were heterozygous for the HIF-1α knockout allele, were obtained. However, when these mice were interbred, the *Hif1a*$^{-/-}$ embryos arrested in their development at embryonic day 8.5 and died by day 10.5. I was fortunate to recruit as a postdoctoral fellow Lori Kotch, who had considerable expertise in mouse embryology. She discovered that complete HIF-1α deficiency resulted in impaired erythropoiesis, cardiac malformations, and vascular defects, demonstrating that all three components of the circulatory system are dependent on HIF-1α expression for their proper development (Iyer *et al.*, 1998).

ADAPTATION OF CELLULAR METABOLISM TO HYPOXIA: A PARADIGM SHIFT

We established mouse embryo fibroblast (MEF) lines from wild type and *Hif1a*−/− embryos. Remarkably, exposure of the HIF-1α-null MEFs to 1% O2 resulted in death of virtually all of the cells over 72−96 hours (Kim et al., 2007; Zhang *et al.*, 2008). The conventional wisdom held that cells switch from oxidative to glycolytic metabolism to maintain ATP production. Yet ATP levels in HIF-1α-null MEFs cultured at 1% O_2 were higher

than in wild-type MEFs cultured at 20% O_2. The cells were not dying from lack of ATP, but rather due to excess reactive oxygen species (ROS) production (Kim *et al.,* 2007; Zhang *et al.*, 2008). The reason that both ATP and ROS levels were high in HIF-1α-null MEFs was that they could not switch from oxidative to glycolytic metabolism without HIF-1α. In collaboration with Jay Kim and Chi Dang we found several HIF-1 target genes that were required to prevent excess ROS production under hypoxic conditions. The first of these was pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates the catalytic subunit of pyruvate dehydrogenase, the enzyme that converts pyruvate to acetyl coenzyme A (Acetyl CoA) for entry into the tricarboxylic cycle. Transfection of HIF-1α-null MEFs with an expression vector encoding PDK1 prevented excess ROS production and cell death under conditions of chronic hypoxia (Kim et al., 2006).

Acetyl CoA can also be generated from fatty acid oxidation. The second critical HIF-1 target gene required to prevent excess ROS production under hypoxic conditions was BNIP3, which my postdoc Huafeng Zhang showed mediated mitochondrial-selective autophagy: after 48 hours at 1% O_2 wild-type MEFs reduced their mitochondrial mass by 50%. Forced expression of BNIP3 was sufficient to reduce mitochondrial mass and significantly reduced hypoxia-induced death of *Hif1a*$^{-/-}$ MEFs (Zhang *et al.*, 2008). Thus, by reducing mitochondrial mass, wild-type MEFs reduced respiration and thereby reduced ROS generation.

A third mechanism of adaptation was more subtle and involved cytochrome c oxidase (complex IV of the electron transport chain), which catalyzes the terminal transfer of electrons to O_2 to form H_2O. In response to hypoxia, HIF-1 induces a subunit switch in complex IV by inducing expression of COX4-2 and LON, which is a mitochondrial protease that is required for the degradation of COX4-1 (Fukuda *et al.*, 2007). This COX4 subunit switch optimizes the efficiency of respiration under hypoxic conditions and may represent an initial response (perhaps under conditions of mild hypoxia) that allows cells to maintain oxidative metabolism with its much more efficient energy production. When hypoxia is severe and/or prolonged, the more draconian measures mediated by PDK1 and BNIP3 may be required. Thus, cells switch from oxidative to glycolytic metabolism, long before O_2 is limiting for ATP production, in order to prevent ROS-mediated cell death.

Hif1a$^{+/-}$ MICE ARE PROTECTED FROM HYPOXIA-INDUCED PULMONARY HYPERTENSION

The embryonic lethality of *Hif1a*$^{-/-}$ mice precluded the possibility of studying the effect of complete HIF-1α deficiency on physiological responses to hypoxia in adult mice. Fortunately, the *Hif1a*$^{+/-}$ heterozygous knockout

mice developed normally but had impaired responses to hypoxia. It was known that unlike arterioles in the systemic circulation, which dilate in response to hypoxia in order to increase blood flow (and O_2 delivery) to the affected tissue, pulmonary arterioles constrict in response to hypoxia so that blood flow is shunted away from areas of the lung that are not ventilated. This is an adaptive response in the context of a lobar pneumonia; however, during ascent to high altitude, the entire lung is exposed to hypoxia and the generalized pulmonary hypertension (PH) that develops in sensitive individuals can be a life-threatening component of acute mountain sickness. Individuals with chronic obstructive pulmonary disease are also at risk of PH, which leads to increased right ventricular pressure (RVP), right ventricular hypertrophy, and progressive heart failure.

In collaboration with my colleague Larissa Shimoda of the Pulmonary division, wild-type (*Hif1a*$^{+/+}$) and heterozygous knockout (*Hif1a*$^{+/-}$) mice were placed in a chamber in which the O_2 concentration was maintained at 10%, which is comparable to the summit of Mt. Kilimanjaro (19,000 feet), for three weeks. In wild-type mice, the mean RVP increased from 7.3 to 18 mm Hg, whereas in the heterozygous mice, RVP only increased from 6.8 to 12 mm Hg, demonstrating a role for HIF-1α in the pathogenesis of PH (Yu *et al.,* 1999). The Shimoda lab showed that treatment of mice with either of two different HIF inhibitors (digoxin and acriflavine), which we originally identified in order to investigate the role of HIFs in cancer progession (as described below), blocked the development of PH induced by chronic hypoxia and prevented further progression in animals in which PH had already developed (Abud *et al.*, 2012).

ESTABLISHING THE ROLE OF THE HIF PATHWAY IN REGULATING ERYTHROPOIESIS IN HUMANS

George Dover, who was then the director of Pediatric Hematology at Johns Hopkins, referred to me a family affected by a condition known as congenital polycythemia or hereditary erythrocytosis, in which the body makes too many red blood cells – the same phenotype displayed by our *EPO* transgenic mice. However, the cause of the disease in humans had not been established. I contacted the family and explained that I was a geneticist interested in identifying the molecular basis for the condition, which affected a man, his mother, and his two children. This immediately told us that the disease was inherited in an autosomal dominant manner, meaning that affected individuals had one wild type and one mutant allele, and that unaffected family members did not have the mutant allele in their DNA, which we extracted from routine blood samples obtained from family members.

I contacted Josef Prchal, a hematologist who was an expert on the

diagnosis of congenital polycythemia, and we began a scientific collaboration, which is now in its third decade. A frameshift mutation, the insertion of an additional guanine residue at codon 430, was identified in one copy of the EPOR gene, which encodes the EPO receptor, in affected family members (Sokol et al., 1995). *EPOR* is expressed on erythroid progenitor cells in the bone marrow and, when activated by EPO binding, stimulates their survival, proliferation and differentiation. The mutation resulted in the synthesis of a receptor that lacked the last 64 amino acids of the protein. This mutant EPOR had increased sensitivity to EPO, providing a molecular basis for the disease in this family (Sokol *et al.,* 1995). Albert de la Chapelle's group studied another family with polycythemia and identified a nonsense mutation in EPOR that led to synthesis of a protein lacking the last 70 amino acids (de la Chapelle *et al.,* 1993). This mutant protein also endowed erythroid progenitors with increased sensitivity to EPO (Sokol *et al.*, 1995).

Joe Prchal became involved in a collaboration to study congenital polycythemia that was endemic in the Chuvash region of the Russian Federation (Sergeyeva *et al.,* 1997). Many affected individuals experienced headaches, which were thought to be due to increased blood viscosity, leading to decreased blood flow in cerebral capillaries. Some patients received relief after partial exchange transfusion, in which blood is removed and only the plasma is returned to the patient's circulation in order to reduce the hematocrit. Joe observed that reduction of the hematocrit from 66% to 48% (upper normal range) was associated with an increase in serum EPO levels from 51 to 121 U/ml (Sergeyeva *et al.*, 1997). In other words, the patients were behaving as if their "normal range" had been shifted upward. Based on these results, Joe hypothesized that these patients had a disorder of oxygen sensing. He queried me regarding candidate genes that he should analyze for the presence of a mutation.

At the top of my list of candidates were the genes encoding HIF-1α, HIF-2α, PHD2, and VHL. Unlike the family from Baltimore, the Chuvash polycythemia pedigrees indicated autosomal recessive inheritance. Affected individuals were found to be homozygous for a missense mutation in the VHL gene that changed the amino acid at position 200 from arginine to tryptophan, which resulted in a mutant VHL protein that bound to hydroxylated HIF-1α or HIF-2α with decreased avidity, resulting in impaired degradation of the HIF-α subunits (Ang *et al.,* 2002). Thus, at any given O2 concentration, there were higher levels of HIF-α proteins and higher expression of HIF target genes, including EPO. Chuvash polycythemia was indeed, as Joe had predicted, a disorder of O_2 sensing.

The *VHL* gene was mutated in every cell of affected individuals and the HIFs regulated many genes in addition to *EPO*, which raised the question as to whether other physiological responses to hypoxia were altered.

Compared to control subjects, affected individuals were shown by Peter Robbins at Oxford to have greater increases in heart rate, respiratory rate, and pulmonary artery pressure in response to a brief decrease in inspired O2 levels (Smith *et al.,* 2006). Other missense mutations in the *VHL* gene were subsequently identified in patients with congenital polycythemia; in one case, severe pulmonary hypertension resulted in death of the patient at 2 years of age (Sarangi *et al.,* 2014).

Subsequent studies by Frank Lee's lab identified heterozygous missense mutations in the EGLN1 gene, which encodes PHD2, in several families with polycythemia; a proline-to-arginine change at residue 317 impaired binding of PHD2 to HIF-1α, whereas a histidine-to-arginine change at residue 374 affected Fe^{2+} binding and catalytic activity, which may also be affected by an arginine-to-histidine substitution at residue 371 (Percy et al., 2006, 2007; Ladroue *et al.*, 2008). Finally, heterozygous missense mutations in the *EPAS1* gene, which encodes HIF-2α, also cause congenital polycythemia (Percy *et al.*, 2008, 2012). Thus, mutations that cause congenital polycythemia have been identified at each level of the HIF-PHD-VHL pathway, demonstrating the key role of these proteins in the regulation of erythropoiesis in humans.

THE PROTECTIVE ROLE OF HIF-1 IN ISCHEMIC CARDIOVASCULAR DISEASE

Atherosclerotic narrowing (stenosis) of major arteries in the brain, heart and leg result in cerebral, myocardial and limb ischemia, respectively. These disorders have a progressive (i.e., age-related) course that leads to stroke, heart attack, or gangrene requiring limb amputation, respectively. Limb ischemia has been modeled in mice by femoral artery ligation (FAL); however, the vast majority of studies were performed in young, healthy mice whereas patients with critical limb ischemia (CLI), meaning that perfusion is not sufficient to maintain tissue viability, are usually of advanced age and suffer from chronic diseases, most notably, diabetes.

To investigate the role of HIF-1 in limb ischemia, my postdoc Marta Bosch-Marcé performed unilateral FAL on *Hif1a*$^{+/-}$ and *Hif1a*$^{+/+}$ littermates and used laser Doppler perfusion imaging for serial and non-invasive analysis of blood flow. Mice are sexually mature (adult) by 2 months and have a lifespan of approximately 2 years. In young (2-month-old) wild-type (*Hif1a*$^{+/+}$) mice, blood flow (compared to the contralateral limb) was reduced to 20% immediately following surgery and gradually recovered to > 70% by 5 weeks after ligation; by contrast, middle-aged (8-month-old) mice recovered < 55% and old (20-month-old) mice recovered only 30% of blood flow (Bosch-Marcé *et al.*, 2007). At each age, *Hif1a*$^{+/-}$ mice recovered less well than their wild-type littermates.

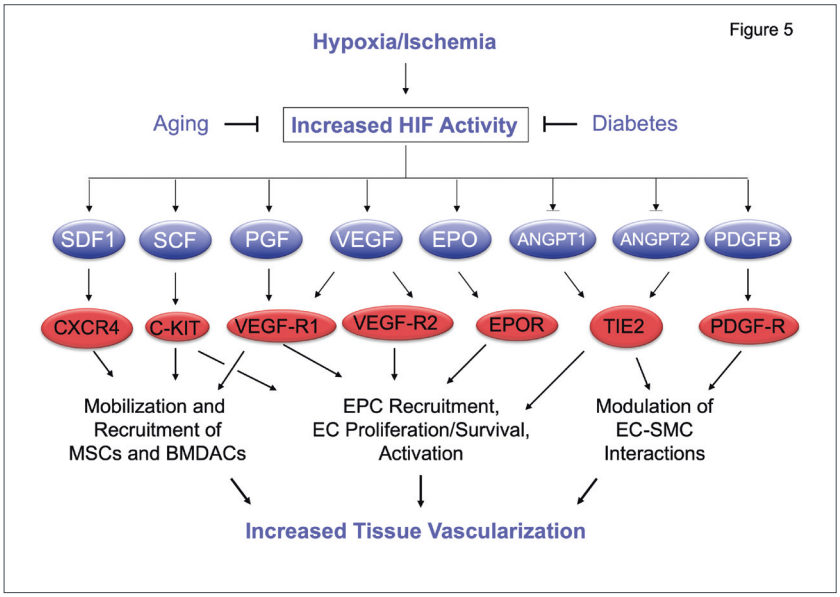

Figure 5. Regulation of tissue vascularization by hypoxia-inducible factors. HIFs activate transcription of genes encoding the angiogenic factors stromal-derived factor 1 (SDF1), stem cell factor (SCF; also known as kit ligand), placental growth factor (PGF), vascular endothelial growth factor (VEGF), erythropoietin (EPO), angiopoietins 1 and 2 (ANGPT1, ANGPT2), and platelet-derived growth factor B (PDGFB), which bind to cognate receptors (CXCR4, CKIT, VEGFR1, VEGFR2, EPOR, TIE2, PDGFR) to recruit and activate vascular endothelial cells, vascular pericytes/smooth muscle cells, and various bone marrow-derived angiogenic cells (BMDACs) to promote angiogenesis and vascular remodeling to increase tissue perfusion. Aging and diabetes impair the induction of HIFs in response to hypoxia and thereby impair ischemia-induced vascular remodeling.

These differences in the recovery of blood flow were correlated with the clinical outcome: all of the young wild-type mice recovered without any permanent tissue damage (limb salvage), whereas 40% of middle-aged mice developed soft tissue necrosis or spontaneous amputation of one or more toes and 15% of old mice lost their entire foot because the recovery of blood flow was insufficient to maintain tissue viability. Again, at every age, the clinical phenotype of *Hif1a*$^{+/-}$ mice was more severe than their wild-type littermates (Bosch-Marcé *et al.*, 2007).

Next, postdoc Ye Liu analyzed the expression of HIF-1α protein in the limb three days after FAL. In young wild-type mice, strong induction of HIF-1α protein expression was observed in skeletal muscle from the ipsilateral compared to contralateral limb. In middle-aged mice, the induction of HIF-1α protein was less robust and in old mice very little HIF-1α protein was detected in the ischemic limb (Bosch-Marcé *et al.*, 2007). Analysis of mRNA expression revealed induction of HIF-1α and mRNAs encoding multiple angiogenic growth factors, including VEGF, stromal-derived

factor 1 (SDF1), stem cell factor, angiopoietin 1, angiopoietin 2, placental growth factor, and platelet-derived growth factor B (Bosch-Marcé *et al.*, 2007) (Figure 5). Ischemia-induced expression of all of these mRNAs was impaired by aging or HIF-1α deficiency. These mRNAs encode angiogenic factors that are all secreted proteins, which bind to receptors on vascular endothelial and smooth muscle cells as well as mesenchymal stem cells and other bone marrow-derived angiogenic cells (BMDACs) that are recruited to sites of ischemia and promote vascular remodeling to increase tissue perfusion. Indeed, analysis of circulating $CD34^{+}/VEGFR^{2+}$ or $Sca1^{+}/CXCR4^{+}$ BMDACs, which express a stem cell marker (CD34 or Sca1) and the cognate receptor for VEGF or SDF1, respectively, revealed a two-fold increase in the peripheral blood of wild type mice, but not in *Hif1a*$^{+/-}$ mice, on day 3 after FAL (Bosch-Marcé *et al.,* 2007).

We hypothesized that because (i) HIF-1α was required for expression of multiple angiogenic factors that controlled the homing and activation of multiple cell types contributing to vascular remodeling; and (ii) aging was associated with impaired induction of HIF-1α and all downstream events, that HIF-1α gene therapy might represent a novel therapeutic approach to CLI. Kiichi Hirota, a postdoc in the lab, constructed a replication-defective recombinant adenovirus (designated AdCA5) encoding a modified form of HIF-1α containing a deletion and several missense mutations that rendered the protein resistant to O_2-dependent degradation and, thus, constitutively active. Infection of AdCA5 into various cell types was sufficient to induce cell-type-specific changes in angiogenic gene expression that were identical to those induced by hypoxia (Kelly *et al.,* 2003).

Marta Bosch-Marcé showed that the injection of AdCA5 into the limb of a healthy mouse was sufficient to increase the number of circulating BMDACs in peripheral blood by three-fold within three days, which was similar to the response elicited by FAL (Bosch-Marcé *et al.*, 2007). When injected into the limb muscle of young wild-type mice immediately following FAL, AdCA5 significantly increased the recovery of blood flow to > 90% at three weeks after ligation (Bosch-Marcé *et al.,* 2007). However, young wild-type mice recovered without any therapy and were not a model of CLI. When middle-aged mice were treated with AdCA5 injected into the ischemic limb after FAL, their recovery was comparable to control young mice. Thus, HIF-1α gene therapy could overcome the age-dependent impairment of vascular remodeling. Postdoc Kakali Sarkar showed that injection of AdCA5 in the ischemic limb of *db/db* diabetic mice also dramatically improved recovery of perfusion and limb salvage (Sarkar *et al.*, 2009).

Remarkably, when postdoc Sergio Rey injected AdCA5 into the ischemic limb immediately after FAL of 13-month-old mice, the beneficial

effect on the recovery of blood flow observed in 8-month-old mice was completely absent (Rey *et al.*, 2009). We reasoned that in addition to the failure to induce HIF-1 and HIF-dependent angiogenic factors, there was an additional impairment in old mice, which was a failure of BMDACs to respond to angiogenic signals induced by AdCA5, and the failure to respond was due to a failure to induce HIF-1 in BMDACs. To test this hypothesis, Sergio harvested bone marrow mononuclear cells from a donor mouse and cultured these cells for 4 days in the presence of angiogenic growth factors and dimethyloxalylglycine (DMOG), an α-ketoglutarate analog that the Ratcliffe lab had shown inhibits PHD activity, leading to induction of HIF-α protein expression even under aerobic conditions (Epstein *et al.*, 2001). Sergio performed FAL and injected AdCA5 into the ischemic limb muscle and then waited 24 hours before injecting the DMOG-treated bone marrow cells intravenously. We waited in order to allow AdCA5 to induce production of angiogenic factors, which would serve as a homing signal to attract BMDACs to the ischemic tissue, and we performed intravenous injection so that BMDACs bearing cognate receptors for the angiogenic factors induced by AdCA5 would be selectively recruited to the ischemic tissue. This two-stage, combined gene-and-cell therapy resulted in complete limb salvage: old mice had no permanent tissue damage or motor defects after FAL (Rey *et al.*, 2009). Remarkably, like AdCA5 alone, DMOG-treated bone marrow cells alone had no beneficial effect in old mice, because there was no homing signal to attract BMDACs to the ischemic tissue.

In the experiments described above, the recipient of the cell therapy was an old mouse, but the bone marrow donor was a young mouse. Sergio next repeated the experiments using 17-month-old mice as both donors and recipients, in order to model autologous bone marrow donation, which would be used if this approach were to be translated to a clinical trial involving elderly patients with CLI. Again, a dramatic recovery of perfusion was observed over a four-week period after FAL, with a marked reduction in tissue damage and motor impairment (Rey *et al.,* 2011). These results provide a potential novel strategy for treatment of CLI. Remarkably, a Phase I clinical trial, involving injection of a recombinant adenovirus encoding a HIF-1α/VP16 fusion protein into the limbs of elderly CLI patients did not improve perfusion or clinical measures (Rajagopalan *et al.*, 2007), as predicted by the mouse model.

HIF-1 PROMOTES WOUND VASCULARIZATION AND HEALING

Patients with CLI develop non-healing foot ulcers, which precede 85% of limb amputations. As in the case of peripheral arterial disease, wound healing is impaired by both aging and diabetes, and the pathogenic effects

of these two conditions are synergistic (Brem *et al.,* 2007). In collaboration with the lab of surgeon John Harmon, we demonstrated that correction of the age-dependent impairment of HIF-1α expression in diabetic mice by gene therapy increased wound healing, angiogenesis, and recruitment of BMDACs (Liu *et al.*, 2008). Subsequent studies revealed important roles for HIF-1 in the vascularization and healing of burn wounds as well (Zhang *et al.,* 2010, 2011; Sarkar *et al.*, 2012).

HIF-1 PLAYS A PROTECTIVE ROLE IN LUNG TRANSPLANTATION

Lung transplant patients have a 5-year survival of 50%, which is the poorest outcome of all solid organ transplants. The lung is the only allograft for which the major blood supply (the bronchial arteries) is not re-established after transplantation. Autopsy data indicate that chronic rejection is preceded by a loss of airway microvasculature. Mark Nicolls, the director of the Pulmonary division at Stanford University developed a mouse model of chronic rejection using orthotopic tracheal transplantation into MHC-mismatched mice. Mark's postdoc Xinguo Jiang showed HIF-1 dependent recruitment of recipient $Tie2^+$ BMDACs into the donor airway was required for repair of the airway microvasculature and maintenance of graft survival (Jiang *et al.*, 2011). Treatment of the allografts with AdCA5 prior to transplantation increased graft perfusion and survival. Mark and Xinguo demonstrated that treatment of allografts with desferrioxamine nanoparticles also improved perfusion and survival in the mouse model of chronic rejection (Jiang *et al.*, 2011). Most recently, they have demonstrated that conditional knockout of HIF-2α in endothelial cells of the allograft accelerates, whereas HIF-2α overexpression protects against, microvascular loss after airway transplantation (Jiang *et al.*, 2019). Taken together, these elegant studies suggest a potential role for HIF inducers in the prevention or treatment of chronic rejection of lung allografts.

HIFS PLAY KEY ROLES IN THE PATHOGENESIS OF SLEEP APNEA-ASSOCIATED HYPERTENSION

Hypoxemia (reduced blood O_2 content) is sensed by glomus cells in the carotid bodies (CBs), which depolarize, triggering adaptive reflex arcs in the sympathetic nervous system that lead to increased breathing, heart rate, and blood pressure, thereby increasing O_2 capture and delivery. These homeostatic responses of the CB are subverted in the context of obstructive sleep apnea (OSA), which is characterized by airway obstruction leading to 15–30 seconds of hypoxemia that eventually awakens the patient, who clears his airway, leading to reoxygenation; this process is

repeated dozens of times per night. The major sequela of OSA is hypertension, which places the patient at increased risk for heart failure, heart attack and stroke. Approximately 1 in 5 men and 1 in 15 women in the U.S. have OSA, which is the major cause of treatment-resistant hypertension. Although OSA causes both chronic intermittent hypoxemia (CIH) and chronic intermittent hypercarbia, only exposure of rodents to CIH leads to hypertension and only if the animal's CBs are intact (Fletcher *et al.,* 1992).

Nanduri Prabhakar's lab at the University of Chicago has utilized an OSA model in which rodents are placed in a chamber and the O_2 concentration is rapidly decreased from 21% to 5% O_2 for 15 seconds followed by rapid reoxygenation, which results in CIH similar to what is observed in OSA patients. These cycles of hypoxia and reoxygenation are repeated for 8 hours per day during the sleep cycle, leading to hypertension within 10 days. Nanduri's lab showed that CIH leads to increased ROS in CB glomus cells, which increases HIF-1α levels and transcriptional activity as a result of increased intracellular Ca^{2+} levels, which activate protein kinase C and Ca^{2+}/calmodulin-dependent kinase (Yuan *et al.*, 2008). CBs isolated from *Hif1a*$^{+/-}$ mice have dramatically impaired depolarization in response to hypoxia (Kline *et al.,* 2002) and are completely protected from CIH-induced ROS in the CB and CIH-induced hypertension (Peng *et al.,* 2006). Expression of NADPH oxidase 2 (NOX2) in the CB is induced by CIH in a HIF-1α-dependent manner (Yuan *et al.*, 2011). Remarkably, whereas CIH induces HIF-1α synthesis in the CB, it induces HIF-2α degradation by calpains, which are Ca^{2+}-dependent proteases. Whereas HIF-1α is required for expression of the pro-oxidant enzyme NOX2 and superoxide production in CBs, HIF-2α is required for expression of superoxide dismutase 2 (SOD2), the antioxidant enzyme that converts superoxide to hydrogen peroxide, which can then be converted to water by the action of glutathione peroxidase or catalase. Whereas *Hif1a*$^{+/-}$ mice are completely protected from CIH-induced ROS production in the CB and CIH-induced hypertension, *Hif2a*$^{+/-}$ mice have: increased ROS levels in their CBs due to decreased SOD2 and increased NOX2 expression; augmented depolarization of isolated CBs in response to hypoxia; and hypertension under normoxic conditions (Nanduri *et al.*, 2009; Peng *et al.*, 2011). These studies indicated that the balance between HIF-1α and HIF-2α levels determines the redox state of glomus cells in the CB, which in turn determines the set point of the sympathetic nervous system: high HIF-1α:HIF-2α ratio → high ROS → high sympathetic activity. The dramatic confirmation of this hypothesis came from the analysis of *Hif1a*$^{+/-}$ *Hif2a*$^{+/-}$ double heterozygous mice, which have normal ROS levels in their CBs, a normal CB response to hypoxia, and normal blood pressure (Yuan *et al.*, 2013). Most recently, Gabriela Pavlinkova's lab at the Czech Academy of Sciences has

shown that HIF-1α is also required for development of the sympathetic nervous system (Bohuslavova *et al.*, 2019).

HIFS PROMOTE PATHOLOGICAL OCULAR NEOVASCULARIZATION

We collaborated with Peter Campochiaro at the Johns Hopkins Wilmer Eye Institute to study the role of HIF-1 in ocular neovascularization, which occurs in the wet type of age-related macular degeneration (AMD) and in proliferative diabetic retinopathy (PDR). We showed that the induction of HIF-1α protein expression was temporally and spatially correlated with VEGF expression in a mouse model of ischemic retinopathy (Ozaki *et al.*, 1999) and that retinal and choroidal neovascularization could be blocked by administration of a HIF inhibitor (digoxin or acriflavine) by intraocular or intraperitoneal injection (Yoshida *et al.*, 2010; Zeng *et al.,* 2017).

In collaboration with Akrit Sodhi, another retinal surgeon at Hopkins, we found that the two major angiogenic factors induced in the mouse model of ischemic retinopathy were VEGF and angiopoietin-like 4 (ANGPTL4). In diabetic patients, Akrit found that intraocular ANGPTL4 levels were significantly correlated with the presence of PDR, whereas VEGF levels were not (Babapoor-Farokhran *et al.*, 2015). Drugs targeting VEGF are effective in approximately 40% of patients with vision loss due to ocular neovascularization. The Sodhi lab demonstrated that vitreous fluid from the eyes of many patients treated with anti-VEGF therapy still had angiogenic activity, which could be blocked by an anti-ANGPTL4 antibody (Babapoor-Farokhran *et al.,* 2015). In the mouse model of ischemic retinopathy, administration of the HIF inhibitor digoxin blocked the induction of both VEGF and ANGPTL4 mRNA expression (Xin *et al.,* 2013), suggesting that HIF inhibitors may be beneficial in patients who do not respond to anti-VEGF therapy.

HIFS PLAY CRITICAL ROLES IN PROMOTING CANCER PROGRESSION

Most advanced human cancers contain areas of hypoxia as a result of increased O_2 consumption, due to dysregulated cell proliferation, and inadequate O_2 delivery, due to the formation of blood vessels that are structurally and functionally abnormal. In collaboration with oncologist Jonathan Simons, we found high levels of HIF-1α in human primary and metastatic cancers compared to normal tissue by immunohistochemistry (Zhong *et al.*, 1999). Furthermore, high levels of HIF-1α in the diagnostic tumor biopsy was associated with failure to achieve a complete response to radiation therapy in oropharyngeal cancer (Aebersold *et al.*, 2001) and

with patient mortality in lymph node negative breast cancer (Bos *et al.,* 2003). Similar results have been reported in many other types of cancer (Semenza *et al.*, 2010).

In collaboration with Professor Jun Liu in the Department of Pharmacology, my postdoctoral fellows David Qian, Huafeng Zhang, and Kang-Ae Lee performed a cell-based screen for inhibitors of hypoxia-induced HIF reporter activity in Hep3B cells using a library of 3000 drugs that were FDA approved or had been evaluated in a phase I trial. Huafeng discovered that digoxin blocked the accumulation of HIF-1α protein in hypoxic cells, whereas Kang-Ae showed that acriflavine bound to HIF-1α and HIF-2α and blocked their dimerization with HIF-1β; both drugs potently inhibited tumor xenograft growth in mice (Zhang *et al.*, 2008; Lee *et al.,* 2009).

In a mouse model of triple-negative breast cancer (TNBC), in which human MDA-MB-231 cells were implanted in the mammary fat pad of immunodeficient mice, postdocs Huafeng Zhang and Carmen Wong, and graduate student Luana Schito showed that treatment of mice with digoxin or acriflavine blocked both blood vessel and lymphatic metastasis (Wong *et al.,* 2012; Zhang *et al.,* 2012; Schito *et al.*, 2012). Through the work of Huafeng, Carmen and postdocs Daniele Gilkes, Pallavi Chaturvedi, and Ting Wang, we subsequently deconvoluted the process of blood vessel metastasis from the mammary gland to the lungs of mice into a series of discrete steps and identified specific HIF target genes required for each step (Semenza, 2016; Table 1).

As part of the metastatic process, we focused on the specification of breast cancer stem cells, because although many cancer cells may successfully metastasize to the lungs, only a cancer stem cell is capable of giving rise to a secondary (metastatic or recurrent) tumor. Graduate students Lisha Xiang, Chuanzhao Zhang, and Jie Lan identified multiple

Table 1. HIF target genes required for metastasis of MDA-MB-231 human triple-negative breast cancer cells from the mammary fat pad to lungs of immunodeficient mice.

METASTATIC STEP	HIF TARGET GENES REQUIRED
MSC* and macrophage cooptation	*CCR5, CSF1, CXCL16, CXCR3, PGF*
Migration and invasion	*P4HA1, P4HA2, PLOD2, RHOA, ROCK1*
Microvesicle formation	*RAB22A*
Margination and extravasation	*ANGPTL4, L1CAM*
Immune evasion	*CD47, CD73, PDL1*
Premetastatic niche formation	*LOX, LOXL2, LOXL4*
Cancer stem cell specification and maintenance	*ALKBH5, CD47, GCLM, GSTO1, IL6, IL8, MDR*1 *PHGDH, SIAH1, SLC7A11, WWTR1, ZNF217*

*MSC, mesenchymal stromal cell

genes that were induced by hypoxia in a HIF-dependent manner and increased the specification of breast cancer stem cells by increasing the expression of the pluripotency factor Nanog (Xiang and Semenza, 2019; Table 2). Remarkably, postdocs Debangshu Samanta and Haiquan Lu demonstrated that exposure of TNBC cells to cytotoxic chemotherapy (such as paclitaxel, gemcitabine, doxorubicin, or carboplatin), either in vitro or in vivo, induced HIF-dependent expression of genes that increase Nanog expression and breast cancer stem cell specification. Remarkably, whereas chemotherapy treatment markedly increased the percentage of cancer stem cells among the surviving breast cancer cells, this counter-therapeutic effect could be blocked by co-administration of the HIF inhibitor digoxin.

Another critical requirement for cancer cells to form primary and metastatic cancers is that they evade killing by the immune system. Postdoc Debangshu Samanta found that the expression of three genes involved in immune evasion was induced by exposure of breast cancer cells to hypoxia or cytotoxic chemotherapy (Samanta *et al.*, 2018). CD47 encodes a cell surface protein that interacts with a receptor on macrophages called SIRPα, which blocks the ability of macrophages to engulf cancer cells, thereby earning CD47 the nickname of the "don't eat me signal." CD73, also known as ecto-5'-nucleotidase, is a cell surface protein that converts AMP to adenosine, which binds to receptors on T lymphocytes and induces anergy or apoptosis, enabling cancer cells to evade killing. Finally, PDL1 encodes a cell surface protein that interacts with PD1, a cell surface receptor expressed by T lymphocytes, and thereby induces anergy or apoptosis. Remarkably, using flow cytometry we demonstrated coordinate expression of all three genes in individual cancer cells exposed to hypoxia or chemotherapy, thereby rendering the cells resistant to killing by both innate and adaptive immunity (Samanta *et al.,* 2018).

Table 2. HIF target genes that increase Nanog expression and breast cancer stem cell specification in response to hypoxia or cytotoxic chemotherapy.

GENE	STIMULUS TESTED	EFFECT ON NANOG EXPRESSION
A2BR	Hypoxia	Increased Nanog mRNA synthesis mediated by STAT3
ALKBH5	Hypoxia	Increased Nanog mRNA stability
CD73	Hypoxia	Increased Nanog mRNA synthesis mediated by STAT3
DUSP9	Chemotherapy	Increased Nanog mRNA synthesis mediated by FOXO3
GCLM	Chemotherapy	Increased Nanog mRNA synthesis mediated by FOXO3
GSTO1	Chemotherapy	Increased Nanog mRNA synthesis mediated by STAT3
REST	Chemotherapy	Increased Nanog mRNA stability
SLC7A11	Chemotherapy	Increased Nanog mRNA synthesis mediated by FOXO3
ZNF217	Hypoxia	Increased Nanog mRNA stability

Debangshu injected mouse 4T1 mammary carcinoma cells into the mammary fat pad of syngeneic, immunocompetent female BALB/c mice and treated the mice with vehicle, paclitaxel or paclitaxel plus the HIF inhibitor acriflavine. Compared to tumors from vehicle-treated mice, tumors from paclitaxel-treated mice showed a 3-fold increase in the percentage of $PDL1^+/CD47^+/CD73^+$ cancer cells, a 3-fold decrease in $CD8^+/CD44^+/CD69^+$ activated effector T cells, and a 2-fold increase in $CD4^+/CD25^+/FoxP3^+$ regulatory T cells, which promote T cell anergy (Samanta *et al.,* 2018). Thus, treatment with cytotoxic chemotherapy resulted in a profound change in the immune cell microenvironment in the residual breast tumor that favored immune evasion. Remarkably, all of the counter-therapeutic effects of paclitaxel on the immune microenvironment were blocked by co-administration of the HIF inhibitor acriflavine. Similar results were obtained using carboplatin, another cytotoxic chemotherapeutic agent (Samanta *et al.*, 2018).

Taken together, our studies indicate that TNBC cells that survive cytotoxic chemotherapy are more likely that untreated cells to have cancer stem cell and immune evasive phenotypes, i.e., the lethal cancer phenotype, providing a molecular basis for the recurrent and metastatic disease that is responsible for the high mortality of TNBC compared to all other breast cancer subtypes. Our results also suggest that combining a HIF inhibitor with chemotherapy might result in a major improvement in the survival of women with TNBC. Furthermore, HIF inhibitors may improve the response to immune checkpoint blockers such as anti-PD1 or anti-PDL1 antibodies. Although digoxin and acriflavine have been useful HIF inhibitors for animal models, they are not suitable for this use in patients. We are currently devoting considerable efforts towards the identification of novel HIF inhibitors that are safe and effective in cancer patients.

CONCLUSION

Over the last 30 years, our lab with the help of many essential collaborators at Johns Hopkins and other institutions has had the good fortune to discover and characterize HIF-1 at the molecular level as well as to investigate the involvement of HIF-1 in embryonic development, cellular and systemic physiology, and disease pathophysiology. It has been particularly rewarding to witness the translation of our discovery to the clinic with the development of HIF inducers and inhibitors that are now in advanced clinical trials for the treatment of anemia and cancer, respectively. Based on work in animal models described above, we can look forward to many other applications for these two new classes of drugs in the years to come.

ACKNOWLEDGMENTS

I have endeavored to present the work of as many of my lab members and collaborators as possible, and I regret that I was unable to include many others. Likewise, many laboratories have made important contributions to the field and I apologize to those individuals who may be offended by this one-sided accounting of my lab's activities.

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