6-22-92
3 d 2xp
4-45

probe (10^4 cpm)	35/36	91/92	USF	35/36	91/92	USF
Carrier DNA	← 0.1 μg Cal. Thy. DNA →			← 0.033 μg Sal. Sp. DNA →		
5 μg Nucl. Extr.						

13	14	15	16	17	18	19	20	21	22	23	24
1	2	3	4								

HIF-1

Binding Buf: 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris pH 7.5, 1 mM DTT, 12 alg.
N2 → Carrier DNA → probes.
Incubated @ RT for 15'.
Run @ 4°C, 185 V
6% PA / 0.3 x TBE

HI9 = 1% O_2 x 4 hr.

Cell

Serendipity, Generosity, and Inspiration

Gregg L. Semenza

"Gregg, we won't let you down." This was the remarkable pledge Haig Kazazian made when offering me a post-doc position at Johns Hopkins. With that promise, in the summer of 1986, I packed my things and drove a U-Haul from North Carolina to Baltimore.

I had been working as a resident at Duke Hospital but decided to forgo my third year of pediatrics training and fast track to a medical genetics fellowship. Hopkins had been at the top of my list because it was home to Victor McKusick, a founding father of medical genetics, as well as Haig and Stylianos Antonarakis, who together had done groundbreaking research with Stuart Orkin at Harvard on the molecular genetics of β-thalassemia, an inherited red blood cell disorder that was the topic of my doctoral thesis. Stylianos accepted the task of supervising my day-to-day antics in the lab.

"Gregg, we won't let you down."

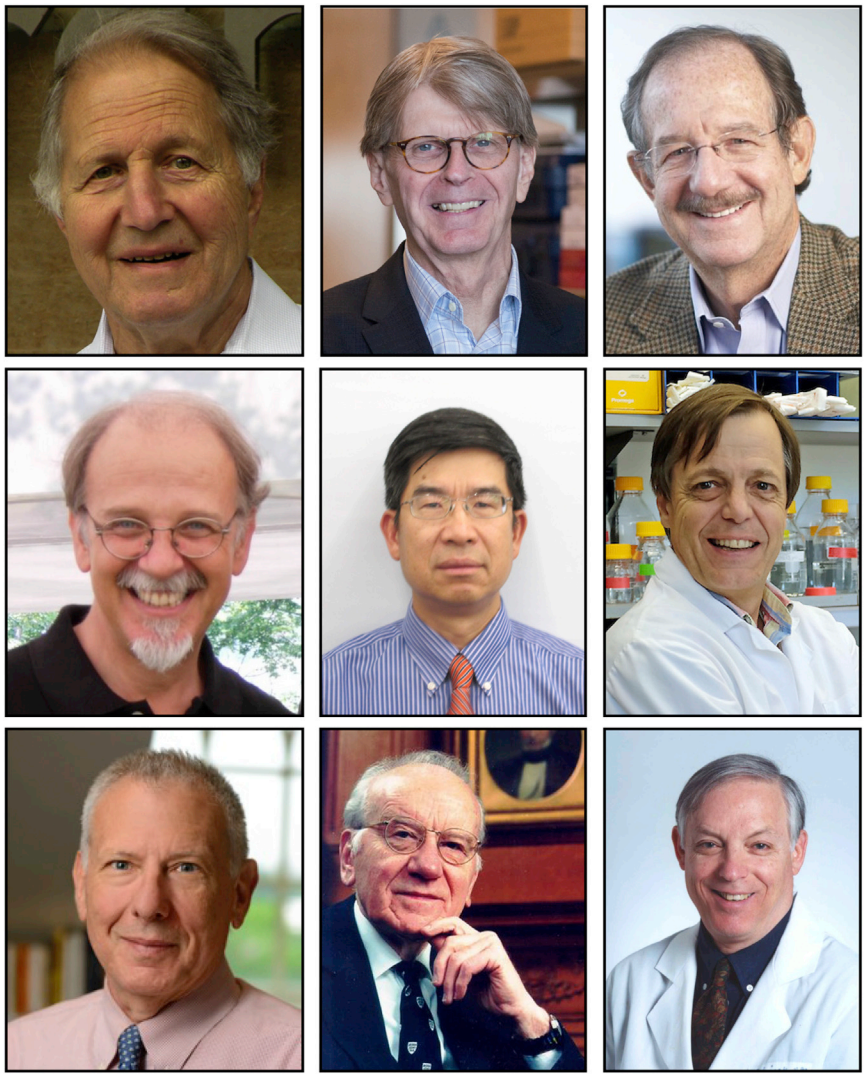
At that time, transgenic mice had just recently been developed, and I was interested in using this new approach to study the regulation of gene expression. Haig and Stylianos had shifted their focus to studying mutations in the *Factor VIII* gene that caused hemophilia A, and so I called Chuck Shoemaker at Genetics Institute, who had isolated the *Factor VIII* gene, to ask if he could send me genomic clones. He said that he could. However, he added that I might want to consider another gene he had cloned. It was called *EPO*.

Erythropoietin (EPO) is made in the fetal liver and adult kidney and is secreted into the plasma, where it controls red blood cell production by binding to erythroid progenitor cells in the bone marrow. Patients with kidney failure can't make EPO and develop anemia, which was treated with blood transfusions that placed them at risk of developing blood-borne infections such as hepatitis. Cloning of the *EPO* gene led to the production of recombinant human EPO protein, which eliminated the need for transfusions.

My initial interest in *EPO* stemmed from the fact that its expression was developmentally regulated with a switch from the fetal liver to the adult kidney. I suspected that there were different enhancers in the gene responsible for expression in each of these tissues. But *EPO* gene expression was also physiologically regulated, making it doubly challenging to study. I had no expertise in generating transgenic mice, but John Gearhart, a faculty member in Physiology, agreed to collaborate on the project. The *EPO* transgenic mice we made had a phenotype—they produced too many red blood cells as a result of expressing both human and mouse EPO. By generating mice with varying amounts of flanking DNA, I was able to confirm my hypothesis: liver and kidney expression were controlled by sequences in the 3′- and 5′-flanking region of the gene, respectively. Having addressed the gene's developmental regulation, our next challenge was to understand its physiological regulation.

The function of red blood cells is to deliver O_2, and exposing animals to a low O_2 environment stimulates EPO production without altering the number of red cells. I set out to identify *cis*-acting elements controlling *EPO* expression using DNase hypersensitivity assays, which Harold Weintraub had pioneered a few years earlier. Using liver nuclei from *EPO* transgenic mice, I found a hypersensitive site that mapped to the 3′-flanking region of the human *EPO* transgene. The DNA sequences that encompassed the hypersensitive site functioned as a

Autoradiograph of Guang Wang's 45th gel shift assay, June 22, 1992. The arrow points to a DNA binding activity present in a nuclear extract prepared from Hep3B cells that were exposed to 1% O_2 for 4 hr (lanes 1 and 3) and absent in a nuclear extract from cells maintained at 20% O_2 (lanes 2 and 4). The DNA binding activity was detected by the wild-type oligonucleotide probe (lanes 1 and 2), but not by a probe with nucleotide substitutions that eliminate hypoxia response element function in the reporter assay (lanes 3 and 4).



Friends and mentors. (Top to bottom, left column: Haig Kazazian, Stylianos Antonarakis, and Charles Wiener; middle column: John Gearhart, Guang Wang, and Victor McKusick [1921–2008]; right column: Thomas Kelly, Charles Shoemaker, and Jerry Spivak.)

hypoxia response element (HRE): its presence enabled transcriptional activation of a heterologous reporter gene.

We hypothesized that a *trans*-acting factor bound to the HRE to activate *EPO* transcription, so we started performing electrophoretic mobility shift ("gel shift") assays, in which we used a radioactively labeled double-stranded oligonucleotide containing part of the HRE hoping to detect a protein that bound to the element in response to hypoxia. These were tricky experiments since non-specific protein-DNA interactions must be blocked, and the optimal salt concentration for binding of different proteins to DNA is idiosyncratic. Or perhaps (I contemplated at the darkest moments), the factor was bound all of the time but for some reason only activated transcription in hypoxic cells.

"I was thumbing through the stack one day … until my eye caught the faintest wisp of a band on one of the autorads. I exclaimed, 'Did you see this? Did you see this?'"

By this time, I had been brought on as a faculty member in the department and had recruited my first post-doc, Guang Wang. Every day, Guang would perform a gel shift assay using different conditions of salt and carrier DNA, and every day he obtained a negative result: no difference in binding between nuclear extracts from hypoxic versus control cells. After weeks of

experiments, Guang had produced a rather impressive stack of autoradiographs on his desk, each one representing a beautifully executed gel shift assay—with a negative result.

I was thumbing through the stack one day, holding each blue plastic sheet up to the fluorescent lights, one after another, looking for a difference in the binding pattern of hypoxic versus control extracts to no avail until my eye caught the faintest wisp of a band on one of the autorads. I exclaimed, "Did you see this? Did you see this?"—a rhetorical question, considering that the experimental result in question was buried deep in the huge pile. Fueled by hope and a prayer, Guang went back to the experimental conditions that had generated the faint band, tweaked the conditions, and within a week produced beautiful gel shift bands that were only observed when nuclear extracts from hypoxic cells were used. Even better, nucleotide substitutions that blocked the sequence from acting as an HRE in the reporter assay also blocked binding in the gel shift assay. This was exactly what we'd been looking for, and we called the DNA binding activity hypoxia-inducible factor 1 (HIF-1).

Next came the formidable task of identifying the protein responsible for the DNA binding activity. Steve McKnight, who was working across town at the Carnegie Institute, had developed a technique in which recombinant bacteriophage expressing mammalian cDNA sequences were screened with a radioactive oligonucleotide to identify proteins capable of binding to the probe. So we embarked on screening millions of bacteriophage—none of which, unfortunately, bound to the gel shift probe. I realized that we had reached a critical decision point with three alternatives: we could continue to screen more bacteriophage (and likely continue to obtain negative results); we could attempt biochemical purification of HIF-1; or we could give up our efforts to determine the identity of HIF-1 and wait for someone else to accomplish the task. Neither the first nor the third choice was appealing, leaving the second option, which involved entering uncharted territory, as our small molecular genetics lab was not even equipped with a fraction collector at the time. Talk about a long shot!

"We could attempt biochemical purification of HIF-1 … which involved entering unchartered territory, as our small molecular genetics lab was not even equipped with a fraction collector at the time."

I had been fortunate to receive funding from the Markey Charitable Trust, the best part of which was the annual meeting of the grantees. At this time, Joe Goldstein gave a particularly inspirational after-dinner speech in which he championed the importance of what he called "technical courage" in pursuing answers to research questions wherever they led, even when this involved using experimental approaches that were outside of one's prior training and comfort zone. His talk made a great impression on me.

I was also very fortunate that, at the time of my dilemma, Tom Kelly was a member of the Department of Molecular Biology and Genetics at Hopkins. Tom was one of the first molecular biologists to purify a protein based on its binding to DNA, and he and his lab members were very generous in sharing their expertise and equipment with us. We had performed a pilot purification from nuclear extracts and performed DNA affinity chromatography using wild-type and mutated versions of the oligonucleotide we'd identified in our gel shift experiments. Two polypeptides—we dubbed them HIF-1α and HIF-1β—co-purified with the DNA binding activity. Thus, the protein was, in fact, a heterodimer and never would have been identified by bacteriophage screening, which relies on expression of individual polypeptides in each clone.

By this time, we had found that HIF-1 was induced in all mammalian cells, so we scaled up and recovered enough purified HIF-1α and HIF-1β to obtain limited protein sequence data from David Speicher's lab at the Wistar Institute. Now this was 1995, when protein sequence databases were still very limited. Nonetheless, Charlie Wiener, a colleague in Pulmonary and Critical Care Medicine, who was one of the first clinician-scientists to grasp the broad medical significance of HIF-1, performed a database search as soon as we received the results from Philadelphia. He came into the lab waving a dot matrix printout, asking, "Have you heard of something called ARNT?" The protein we called HIF-1β appeared to be identical to the aryl hydrocarbon receptor nuclear translocator (originally identified by Oliver Hankinson), which turned out to heterodimerize with either the aryl hydrocarbon receptor or HIF-1α.

The peptide sequences of HIF-1α, on the other hand, were not found in the database, so we designed degenerate oligonucleotides to screen a cDNA library that we had prepared using mRNA

from hypoxic cells. Peter Agre's lab had gone through this process while cloning aquaporin 1 and provided invaluable advice, and within several months, we successfully isolated HIF-1α cDNA.

We sent the cDNAs to dozens of labs, and the roles of HIFs subsequently expanded to encompass development, physiology, medicine, and even evolution. Now, the wheel has come full circle, with ongoing clinical trials of oral agents that stimulate erythropoiesis by inducing HIF activity, which may eliminate the need for injections of recombinant EPO in patients with renal failure. I am now in my 30th year at Hopkins, where I have the great pleasure to mentor students, post-docs, and junior faculty, often with Haig's words in mind: we won't let you down.