2014 William Allan Award Introduction: Stuart Orkin[1]

Haig H. Kazazian, Jr.[2,*]

It is a great pleasure and privilege to introduce Stuart Orkin, the 2014 recipient of the William Allan Award, the highest honor of our society. Orkin's research is unmatched for its cumulative impact on the genetic basis of blood diseases and fundamental aspects of mammalian development. His work is a paradigm for the application of molecular genetics to medicine.

Stu was born to native New Yorkers and grew up in Manhattan. His father was a urologist who made house calls. When Stu was in the fourth grade, the family moved to Riverdale, where he attended a private boys' school. At Riverdale, he had a chemistry teacher who stimulated him to attend the Massachusetts Institute of Technology (MIT). After flirting with physics, he quickly learned that MIT had many physics whizzes, so after Salvador Luria's biology course on early molecular genetics, he decided to pursue biology. Stu then began attending Harvard Medical School in 1967, and around 1970 he took a year of research with John Littlefield to do somatic cell genetics. After the NIH paid for his fourth year in medical school, he did an internship at Boston Children's Hospital. He then went to the NIH to work with Phil Leder, an esteemed molecular geneticist. What a great deal and terrific mentor he had! With Leder, Orkin learned pre-cloning molecular biology. Around this time, I met him for the first time and was quite impressed. After his time at the NIH, he returned to Boston Children's Hospital for a residency year, and David Nathan, the astute director of hematology and oncology at the time, offered Orkin the opportunity to start his own lab while he was still a hematology-oncology fellow. The rest is history!

Personally, he met his wonderful wife, Roz, while both were in college. They've been married 44 years and have one daughter and two grandkids, ages 5 and 3.

I had the pleasure of collaborating with Stu in the early 1980s on the characterization of mutations causing β-thalassemia in various ethnic groups—Mediterraneans, Asian Indians, and Chinese, among others. Stylianos Antonarakis, then a fellow, found in the β-globin gene cluster a number of new restriction-fragment-length polymorphisms that produced nine major haplotypes. Had different β-thalassemia mutations landed on different haplotypes? We picked mutant β-globin genes, which Stu then cloned and sequenced. Indeed, among nine different Mediterranean haplotypes with β-thalassemia, there were eight different mutations affecting transcription, RNA splicing, and translation. What fun this was! Stu would call almost daily with new information! He stayed at our home as we finished the first paper in late 1981. Over the next 5 years, we wrote 25 other papers covering β-thalassemia mutations in other ethnic groups. But this molecular characterization of a Mendelian disease was just the first of many breakthroughs for Orkin.

In 1986, he used the Xp21 deletion found by Uta Francke and help from Tony Monaco and Lou Kunkel to carry out the first positional cloning of any gene: *CYBB*, the gene mutated in chronic granulomatous disease.

In 1989, Orkin found the first transcriptional regulator of hematopoiesis, GATA-1. He followed that with the discovery of many other transcription factors important for blood cell development. Orkin then used gene targeting in mice to determine in vivo roles for many of these transcriptional regulators in blood stem cells or hematopoietic lineages. Because of this work, Orkin is considered the "father" of molecular hematopoiesis.

Recently, Orkin has made another stunning discovery that represents the first inroad into the most important unsolved area in hemoglobin disorders: regulation of the switch from fetal to adult hemoglobin. He found that repressing *Bcl11a* leads to increased fetal hemoglobin and restoration of the normal phenotype in the mouse model

[1]This article is based on the address given by the author at the meeting of The American Society of Human Genetics (ASHG) on October 20, 2014, in San Diego, CA, USA. The audio of the original address can be found at the ASHG website.

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of sickle cell anemia. Orkin might now have a rational approach to curing sickle cell anemia and β-thalassemia by increasing fetal hemoglobin, a holy grail in hematology for many years.

Stu has received many honors, including election to the Institute of Medicine, the American Academy of Arts and Sciences, and the National Academy of Science. Among his many awards is the 2013 Kovalenko Medal of the National Academy of Science, awarded for important contributions to medical science. He has published over 500 scientific papers, including 225 with greater than 100 citations. His mentoring of many graduate students and postdocs is legendary. Among his distinguished mentees are David Ginsburg, Leonard Zon, Nancy Andrews, and my ex-colleagues at Penn Celeste Simon, Gerd Blobel, and Mitch Weiss. A physician-scientist working between fundamental biology and human disease, Orkin has contributed enormously to the development of molecular medicine and genetics. These accomplishments are highly worthy of recognition with the 2014 Allan Award to Stuart Orkin.

2014 William Allan Award: A Hematologist's Pursuit of Hemoglobin Genetics[1]

Stuart H. Orkin[2,*]

I am enormously grateful to The American Society of Human Genetics (ASHG) for selecting me to join the distinguished roster of recipients of the William Allan Award. I especially thank Haig Kazazian, a former recipient and a wonderful collaborator in the early days of my independent career, for the nomination and the gracious introduction today. I am not a conventional awardee for this honor, given that I have never held an appointment in a genetics department or division. Instead, my research career developed in a division of hematology. I have always thought of myself as a geneticist, and I accept the recognition by the ASHG as confirmation of this suspicion.

Reflecting on my career, I realize that I have been privileged to live through successive waves in the revolution of biology over the past three plus decades. My interest in molecular biology and genetics was kindled in my second year at the Massachusetts Institute of Technology (MIT) in an introductory biology course given by Salvador Luria (Figure 1). This was not the biology I knew from high school, which involved cutting up frogs and memorizing phyla. Luria captured the excitement of the early days of molecular genetics and took a special interest in undergraduates. Stimulated by this course, I chose to major in biology, or life sciences as it is called at MIT. In that era, most MIT undergraduates concentrated on engineering or physics, because the true revolution in the biological sciences was still a decade or so into the future. Besides Luria, I encountered many other luminaries as professors, including Boris Magasanik and Vernon Ingram, who introduced us to the single amino acid substitution in sickle hemoglobin.[1] At one meeting of Ingram's course, he invited guest lecturers from the Karolinska Institute in Stockholm to speak, and all I recall is that their presentation might well have been delivered in Swedish. In retrospect, I feel that the Nobel committee sadly overlooked Ingram's monumental contribution to human genetics. My education at MIT was enriched in other ways. Although I wouldn't have predicted it at the time, Bob Weinberg and David Botstein, my teaching assistants in the introductory biology course, would become superstars in the coming years.

Because my father was a devoted old-time surgeon who saw each patient as a potential friend rather than a disease subject, I sought ways to link my early experiences in molecular genetics with medicine. Hence, I crossed the Charles River to attend Harvard Medical School (HMS). Although I might have pursued both the MD and PhD degrees, in those days Harvard wouldn't permit it; they said that one Harvard degree was enough for Kornberg, and therefore it is sufficient for any student! We were encouraged to take a year off from medical school for research if we were so inclined. After my second year, I chose to work with John Littlefield at Massachusetts General Hospital and spent a wonderful year immersing myself in the field of somatic cell genetics. As I re-entered the third year at HMS, I knew I would return to research.

During my junior year at HMS, as US military commitment in Southeast Asia accelerated, the NIH announced a plan by which if one committed to a 2-year tour of duty in the Public Health Service (PHS), Uncle Sam would pay for the final year of medical school. Besides carrying the allure of the NIH, the premier training ground for academic biomedicine at the time, the program provided a generous stipend during the final year of school, making an application a no-brainer. Having heard positive reports from Richard Erbe and David Livingston on cutting-edge

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Figure 1. Three Influential Mentors in My Career

research led by Phil Leder (Figure 1), I applied to and was matched to his laboratory in a small building on the NIH campus. In accord with the PHS program, I spent a year as an intern in pediatrics at Boston Children's Hospital prior to moving to Bethesda. Free of virtually any other responsibilities for 2 years at the NIH, I devoted myself to learning the emerging techniques and concepts of molecular biology. I learned how to purify messenger RNA hand in hand with Leder after bleeding hundreds (yes, hundreds!) of mice, prepare reverse transcriptase from infected chicken blood, and purify restriction enzyme EcoRI—all ancient history to current trainees in molecular biology. I learned how to think about doing clean, direct experiments to answer specific questions without relying on a kit from a biomedical supplier (none of which existed at the time). I cut my teeth on mouse erythroleukemia cells, a convenient model of red cell differentiation, which we still use today for many genomic experiments. What we hoped to learn in my subgroup of the Leder laboratory was how globin messenger was expressed, even though we didn't have a clue that the structure of globin genes (or other mammalian genes) wasn't simple and unbroken, as we imagined then. My experience in the Leder laboratory set me on my lifelong quest to understand blood cell development and apply this knowledge where possible to human disease.

As I was exiting the Leder laboratory, we were just beginning to use restriction enzymes to fractionate genomic DNA in the hopes of purifying globin genes. There was considerable discussion as to whether we might actually be able to purify a gene by standard biochemical purification schemes. I surrendered my laboratory bench to Shirley Tilghman, who accomplished the cloning of the mouse globin genes soon thereafter with the early lambda phage vectors. As I was leaving the NIH, I considered entering either human genetics or hematology as my primary field for a fellowship. Ultimately, I chose to return to Boston Children's Hospital for a second clinical year in pediatrics and join a rather newly formed program in pediatric hematology and oncology led by David Nathan (Figure 1), now recognized as a true giant in academic medicine. As I was midway through my clinical year, Nathan informed me that Bernie Forget, an early pioneer with another HMS student, Ed Benz, in hemoglobin molecular biology, was moving to Yale and that I was to be given his laboratory space. I was instructed to write to the NIH and March of Dimes for grant support to establish an independent lab. Not knowing any better, I did this. Although too naive at the time to understand how risky starting a lab might be without further training, I am grateful to Nathan for giving me an opportunity to be independent at an age when most trainees now are just beginning their postdoctoral training. I chose to focus on the red cell and its major disorders and address the following questions: How are these cells made during development? How are red cells programmed from hematopoietic stem cells? How do red cells or other blood cells go awry in disease? What can we do to alleviate suffering from blood disorders? In this quest, my career transitioned sequentially through the introduction of recombinant DNA methods, prenatal diagnosis of disease by DNA analysis, positional cloning, stem cell biology, the new genetics of genome-wide association studies (GWASs), and now genome engineering. In many respects, my history traces that of modern biomedical science.

With my newly established laboratory, I first set out to understand the mutations that lead to under-expression or no expression of β-globin in β-thalassemia and hoped that this path would provide clues to the mysteries of the disorder but also how red blood cells are made. At the time, it was believed that determining the globin gene molecular defects that lead to thalassemia would provide special insights into cell-specific gene expression. In my growing laboratory, we learned lambda phage cloning of genomic DNA, now a relic of the past, and set out to clone the β-globin gene of patients with β-thalassemia. It was at this juncture that I was fortunate to partner with Haig Kazazian, who had defined haplotypes (or frameworks) of the β-globin gene cluster by restriction enzyme mapping.[2] He hypothesized that thalassemia mutations occurred in the context of these haplotypes and, therefore, that each haplotype would be associated with a specific thalassemia gene mutation. The next several years were devoted to a comprehensive effort to clone β-thalassemia genes successively from different haplotypes in several different geographic or ethnic groups, work that led to validation of the Kazazian hypothesis and to the nearly complete description of β-thalassemia at the molecular level[3,4] (Figure 2). Mutations reside in virtually every part of the gene: promoter, coding region, splice sites, and the poly(A) addition signal. Save for a couple of rather rare promoter mutations, none, however, affected cell-specific aspects of gene expression. It was an exciting era. Without fax machines, computers, or cell phones, Haig and I communicated almost daily by phone to discuss our experiments. Although we took great satisfaction in the comprehensive dissection of mutations in a human disorder for the first time, I had a nagging sense that we didn't quite get to the core of how a red cell is orchestrated at the molecular level or how we could use our new knowledge to treat patients.

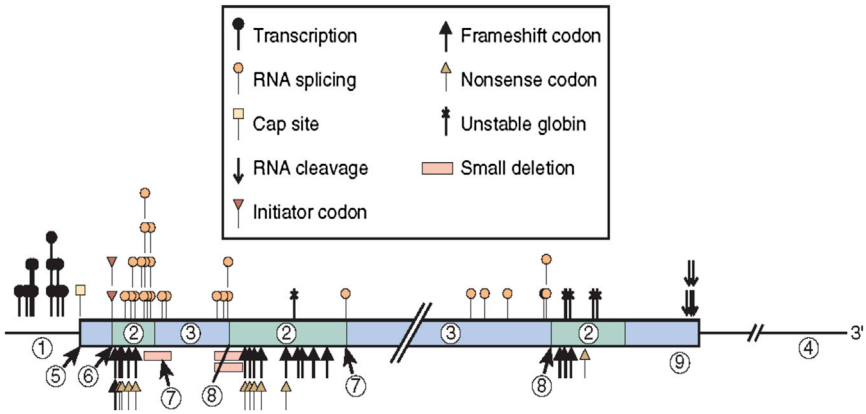

Figure 2. Mutations in the β-Globin Gene in β-Thalassemia

Nonetheless, knowing the specific defects responsible for β-thalassemia had a major practical benefit in that it enabled prenatal diagnosis of disease by DNA analysis. In the early days, this was accomplished by Southern blot analysis (where possible) or with oligonucleotide hybridization. Of course, newer methods, such as PCR, rapidly replaced older technology. Where applied in endemic regions, such as the island of Sardinia, prenatal diagnosis of thalassemia has been enormously successful and virtually eradicated new births of affected individuals.

While thinking about what to do next to address fundamental issues in blood cell development, I took a gratifying detour in collaboration with Lou Kunkel and his MD-PhD student Tony Monaco in the positional cloning of the gene defective in a classic X-linked immune deficiency, chronic granulomatous disease (CGD),[5] a disorder that Nathan and colleagues at Boston Children's Hospital had studied for many years. Although the task of cloning the gene might seem simple by current standards, at the time we could not take advantage of PCR (which had not come on the scene yet) or large-scale DNA sequencing. Instead, we relied on brute force and lots of luck. As often happens after the cloning of a new gene, identification of the gene mutated in CGD led to important insights into the phagocyte oxidase system, a major host defense system of white blood cells.

With my heart in trying to understand how blood cells are programmed, hoping that this path might lead to insights into therapy of the major hemoglobin disorders (Figure 4), I then turned to the nucleus in search of the critical transcriptional regulators that determine blood cell fate. Because we already recognized at the time that having more fetal hemoglobin (HbF) was beneficial to those with the major hemoglobinopathies, we first focused on a region of one of the γ-(fetal) globin promoters where single-base changes were known to greatly increase expression of the γ-gene in *cis*. Such hereditary persistence of fetal hemoglobin (HPFH) mutations was a mystery then and is largely still a mystery in terms of how the mutations lead to a 10- to 50-fold increase in gene expression. Nonetheless, we found by conventional gel shift assay a nuclear factor that bound over one of these HPFH regions.[6] We ultimately cloned its cDNA, which encodes the protein GATA-1, the founding member of the GATA family of transcription factors.[7] GATA-1 is a master regulator of erythroid gene expression. Over the past 25 years, studies of its roles in different contexts have provided a window into extraordinarily diverse biology, including lineage differentiation, lineage choice, lineage reprogramming, rare diseases of red cells and megakaryocytes, and leukemogenesis (Figure 3). Also, GATA-1 led to the discovery of the related GATA factors (GATAs 2–6), which in aggregate are involved in nearly all organ systems and many disease entities.

Using the emerging technology of homologous recombination in embryonic stem cells and the generation of knockout mice,[8] we then proceeded to identify and inactivate numerous nuclear factors that determine blood stem cell or lineage-restricted development[9] (Figure 4). The knowledge gained from this approach led to a deeper understanding of the factors involved in specification of hematopoietic stem cells, differentiation of blood lineages, and the close relationship between normal and malignant hematopoiesis.[10] Most notably, virtually all of the essential transcription factors that drive hematopoiesis have been corrupted by translocations or mutations to engender leukemia. An underappreciated aspect of the studies of hematopoietic transcription factors in the 1990s and early 2000s was the finding that introduction of individual factors into committed blood lineages could reprogram one lineage to another. In my view, experiments of this type, largely pioneered by Thomas Graf,[11,12] set the stage

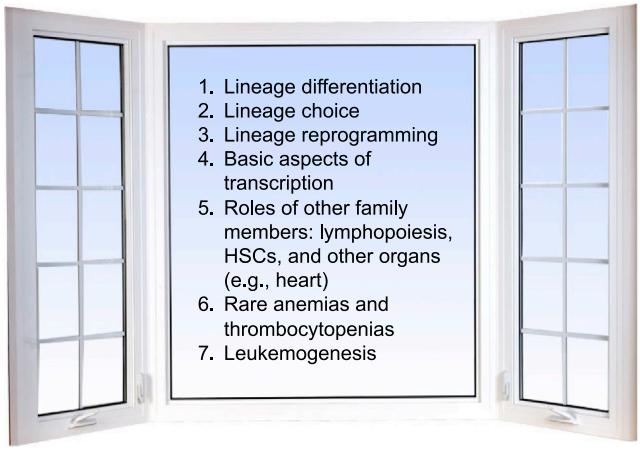

Figure 3. Areas in which Study of GATA-1 Provided Insights into Biology or Disease

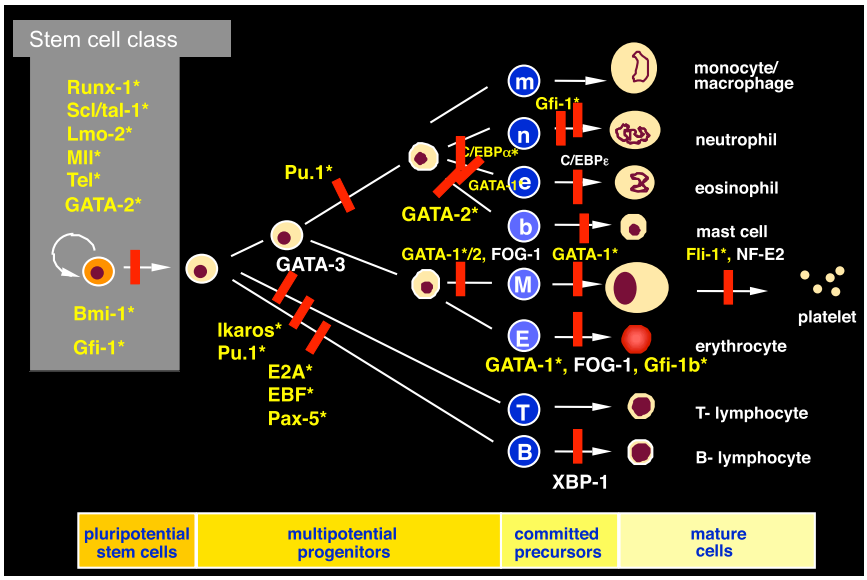

Figure 4. Transcription Factor Requirements in Hematopoietic Development
Asterisks indicate those factors associated with hematopoietic malignancies through overexpression, mutation, or translocation.

for the revolution of somatic cell reprogramming by Yamanaka.[13]

A central problem remained unsettled. How might we employ what we learn in molecular biology to the major hemoglobin disorders? Although sickle cell disease (SCD) was described more than 100 years ago and has served as a "poster child" for a molecular disease caused by a point mutation for more than 50 years,[1] none of the current management of patients has benefited from our genetic understanding. All therapy is directed at the primary mutation's secondary and tertiary consequences, including vascular and organ damage and anemia resulting from polymerization of the altered hemoglobin. Although SCD has not merited the attention it deserves, in my opinion, the problem is global in that it is estimated that there will be more than five million children with SCD on the African continent in the next decade. Taken together with SCD in other regions and β-thalassemia in many parts of the world, the hemoglobinopathies are a global public-health burden, growing rather than decreasing in impact.

Apart from repairing the sickle or thalassemia mutations by gene editing, which is not yet possible because of the low frequency of homologous recombination in hematopoietic stem cells, perhaps the most promising strategy that has been pursued is reactivation of HbF in adults, given that HbF can entirely replace β-containing adult hemoglobin with no ill effects and HbF is an outstanding inhibitor of the sickling process. The switch from fetal to adult hemoglobin is a paradigm of developmental, cell-specific gene expression—one that is taught in introductory biochemistry and genetic courses (Figure 5). Natural history studies of an NIH cohort demonstrated the benefit of higher HbF for survival.[14] Although the DNA sequence of the human β-gene cluster has been on hand for more than 30 years, the sequence alone provided no insights into the critical switch. What was learned about GATA-1 and erythroid gene expression pertains equally well to fetal and adult gene expression. The study of HbF has a long history, starting in the 1950s and 1960s with family studies documenting that the level of HbF is genetically determined and that increased HbF relieves the consequences of SCD or β-thalassemia. Despite intensive efforts by many laboratories to find stage-specific transcription factors that might account for the switch, the basis remained a mystery until quite recently.

The breakthrough for the field came with the application of GWASs to the analysis of HbF levels in populations. Two GWASs were performed: one in England by Thein and the other in Sardinia by the late A. Cao and his colleagues, with whom we were fortunate to collaborate.[15,16] The "we" here includes Joel Hirschhorn and Guillaume Lettre—true geneticists, not hematologists like me—and a gifted MD-PhD student in my group, Vijay Sanakran, who doggedly pursued re-activating my own interest in HbF control. The results of these GWASs were stunning in their simplicity: we observed only three major "hits," including one within the β-locus and one near *c-MYB*, which was previously suggested as a candidate regulator (Figure 6). The remaining new hit resided on the short arm of chromosome 2 at the locus encoding BCL11A, a previously identified transcriptional repressor essential for B-lymphoid cell development but totally

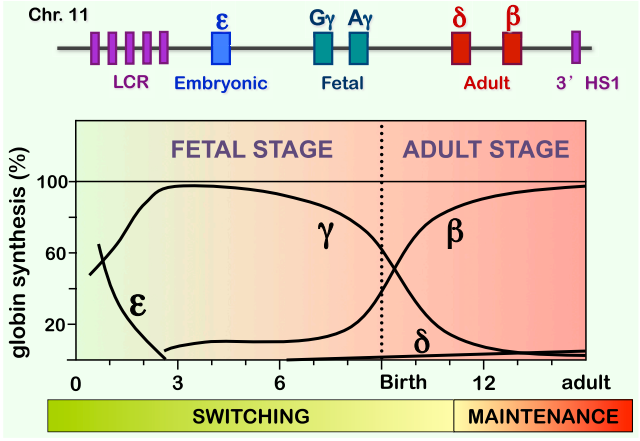

Figure 5. Pattern of Hemoglobin Gene Expression during Ontogeny

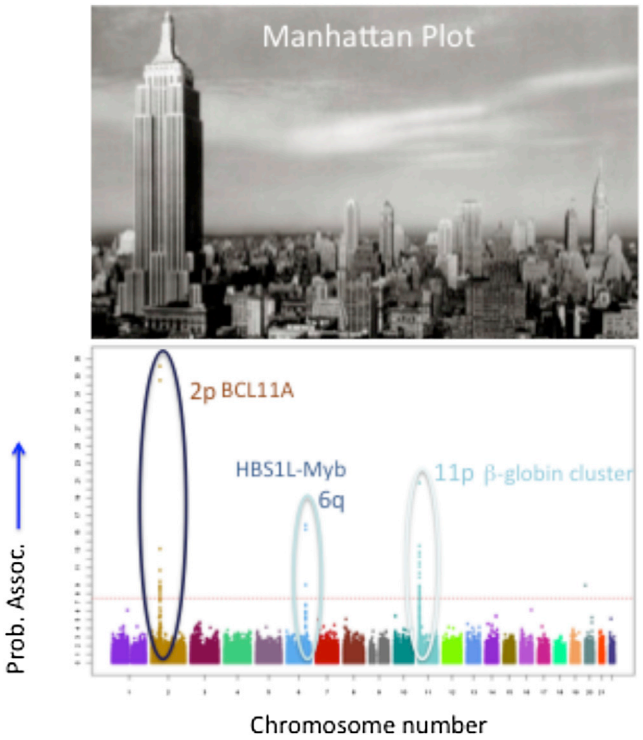

Figure 6. Manhattan Plot of GWAS for HbF Levels in NIH Cohort with SCD

unsuspected as a regulator in erythroid cells. Remarkably, the genetic contribution of the three hits accounts for ~50% of the total genetic variation in HbF.[17] In the US SCD population, high HbF status at BCL11A leads to a modest increase in HbF from about 3% to 11%, a measureable increase but well below the level at which symptoms of disease are regularly ameliorated. Through a series of studies in cells and mice, my group has validated BCL11A as a central regulator of Hb switching in mice and HbF silencing in humans.[18,19] Dial BCL11A up, and HbF is reduced; dial down BCL11A, and HbF is increased (Figure 7). Indeed, erythroid-specific inactivation of BCL11A in humanized SCD mice completely ablates phenotypic consequences and restores hematology to normal through reactivation of HbF.[18] Remarkably, and an exception among transcription factors with which I am aware, loss of BCL11A has no effect on red cell production or differentiation—it has an effect only on the type of hemoglobin within the mature cells. This curious happenstance, I believe, might play an important role in translating findings to patients.

Having established that BCL11A is a major and perhaps the central regulator of the hemoglobin switch and HbF silencing, we were left to account for what GWAS-identified genetic variation at the BCL11A locus signified. This is often the point at which studies of GWAS-identified loci meet insurmountable obstacles. In this instance, the pieces fell into place readily with the tools available to us in terms of prior knowledge of globin gene expression and red cell biology. The critical SNPs associated with

Figure 7. BCL11A as a Quantitative Regulator of HbF Levels

HbF levels are clustered within a large intron of *BCL11A*, suggesting that genetic variation would affect expression rather than the structure of the protein.[20] Indeed, no genetic variation within the coding portion of *BCL11A* has been observed. The region in which the SNPs cluster exhibits the epigenetic hallmarks of a putative regulatory element, marked by appropriate histone modifications and DNase hypersensitive sites. Transient transgenic analysis of a 10-kb segment encompassing the major SNPs demonstrated erythroid-specific and adult-stage-specific enhancer function in mice. Further analysis of SNPs within the region, coupled with additional DNA sequencing of SCD patients, identified a SNP that accounts for the majority but not the entirety of the HbF allele status. We believe that this is a causal SNP because it alters a cognate binding site for the two major erythroid transcription factors, GATA-1 and Tal-1, and in heterozygous individuals for high and low HbF alleles leads to a reduction in allele-specific transcription factor binding and RNA transcript expression.[20] The overall decrease in expression of *BCL11A* transcript from the HbF allele is estimated to be ~40%. Therefore, a modest reduction in BLC11A expression, on the order of 40%, is associated with a rise in HbF from about 3% to 11% on average in the SCD population.

Although natural genetic variation leads to a modest change in HbF, how critical is the enhancer to overall gene expression? To address this, we turned to the last major technology, genome editing. In this instance, we used modified zinc fingers, TALENS, to remove the enhancer from mouse erythroid cells. Remarkably, expression of *BCL11A* was reduced by >99% in enhancer-deleted erythroid cells.[20] In contrast, deletion in B cells, where *BCL11A* is highly expressed, failed to affect expression. Hence, the enhancer is an essential, erythroid regulatory element for *BCL11A* expression. We believe that this finding is important in considering genes identified through GWASs. Although the effect size of a particular SNP to a particular trait or disease might be small, this does not necessarily indicate that the contribution of the gene to the biology or disease under study is also small. Natural genetic variation can only go so far,

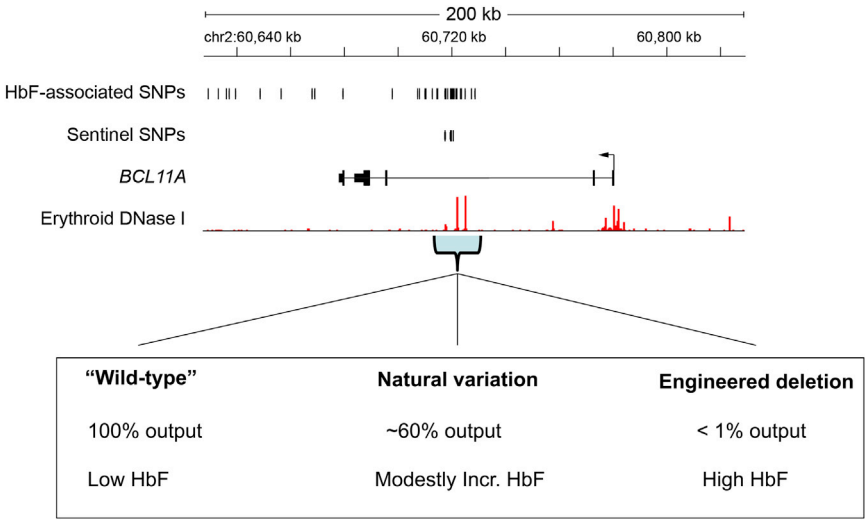

Figure 8. Erythroid Enhancer Element within *BCL11A* Controls the Output of *BCL11A* and, Therefore, the Level of HbF

particularly if a gene is expressed in multiple cellular contexts. The consequences of natural variation and deletion within the erythroid enhancer of *BCL11A* are depicted in Figure 8.

Our finding that the *BCL11A* erythroid enhancer is critical to expression only in the red cell lineage forms the basis for entertaining the possibility of using genetic engineering to inactivate the enhancer in hematopoietic stem cells as an improved strategy of gene therapy (Figure 9).[20] In this manner, the effects of gene modification would be cell restricted and allow for maximal HbF expression. Recent studies of rare microdeletion syndrome have shed light on the quantitative relationship between BCL11A levels and HbF reactivation. Patients with 2p15–p16.1 deletions display an autism spectrum phenotype and developmental delay. Remarkably, these individuals, who are haploinsufficient for *BCL11A*, have normal hematologic and immunologic function and exhibit steady-state HbF levels in the 15%–30% range, equivalent to that of the most potent, classical HPFH mutations (unpublished data). Therefore, we anticipate that editing of the *BCL11A* enhancer by current and emerging gene-editing tools, which often tend to favor homozygous deletions, will lead to expression of HbF at a level that would most likely be curative in SCD or thalassemia. Preclinical studies to bring gene-editing technology to patients with hemoglobin disorders are underway.

No matter how successful genetic engineering might become, it can never meet the global health challenges of hemoglobin disorders. Indeed, in order to reduce the impact of these diseases on a global scale, it will be necessary to develop small molecules, drugs that can achieve a sufficient functional reduction in BCL11A (or some other critical component in HbF regulation) to induce curative levels of HbF. This is a major challenge but is one that modern protein chemistry might be able to address.

I have been blessed to live through the revolution in the biomedical sciences over the past three decades. When I entered the field, we did not know the structure of genes, nor could we express or sequence a gene if it were presented to us. We can now see how the deep knowledge gained can be applied to effect real phenotypic changes in patients to their benefit. Obviously, we all stand on the giants who came before, and so today I feel thankful that I arrived on the scene at a propitious time. None of the work I described was done alone. I have had great collaborators at each step along the way, and remarkably talented trainees, many of whom have moved on to establish their own independent careers of note. I am indebted to the NIH and Howard Hughes Medical Institute for their support and to my colleagues at Boston Children's Hospital and the Dana Farber Cancer Institute, who have provided a wonderful and enriching environment. Finally, I would not be here today if it were not for unwavering support from my family, especially my wife, Roslyn, who has been my steadfast support for 45 years.

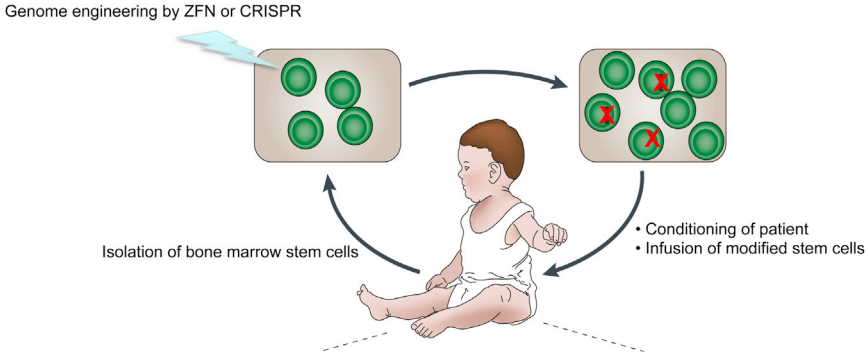

Figure 9. Gene Therapy by Genome Engineering
As in a standard bone marrow transplant, hematopoietic stem cells are obtained (either from the bone marrow or mobilized into the peripheral blood). Genome engineering using a zinc finger nuclease (ZFN) or CRISPR/cas9 is then employed to modify the cells ex vivo. These cells are then reinfused into the recipient to reconstitute the hematopoietic system.

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