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Of mice and men: From hematopoiesis in mouse models to curative gene therapy for sickle cell disease

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Through studies in mice and in humans, Stuart Orkin showed that *GATA-1* is a master transcriptional regulator of hematopoiesis. He has highlighted the role of *BCL11A* in the fetal-adult hemoglobin switch. The Gairdner Foundation Award recognizes Orkin's contribution to the development of gene therapy of sickle cell disease.

Pioneers and historians of molecular biology have perceived two strands at the origin of this science: understanding structures, mainly of nucleic acids and proteins, and understanding how genetic information is transmitted and expressed (Stent, 1968). Initially the preferred organisms were bacteria and bacteriophages, but F.H.C. Crick remarked that sometimes the choice was "oddly enough, man himself, though mainly because of a single one of his molecules – hemoglobin." Indeed hemoglobin was the first oligomeric protein the structure of which was solved at high resolution by Max Perutz, over 2 decades of work. mRNA was difficult to investigate in bacteria on account of its heterogeneity, whereas in mammalian reticulocytes, over 90% of mRNA could be expected to encode globin chains. In a series of papers that helped to make this very journal rapidly prominent, the group of Tom Maniatis and others made use of this unique advantage in order to sequence the mRNA itself and then clone and sequence the globin genes. Soon after introns were discovered, it was from the globin genes that we learnt, through work in Phil Leder's lab, that this was a general feature in the mammalian genome. Equally important for us physicians, hemoglobin is at the center of genetic diseases: sickle cell disease (SCD), a major public health problem especially in Africa (Makani et al., 2018), and the thalassaemias, a group of genetic diseases that are a problem in many countries, the molecular genetics of which was spearheaded by David Weatherall and many others. It is the contribution of Stuart Orkin to understanding in depth these problems that the Canada Gairdner Awards has recognized

In 1972, a short paper in *Nature*, published by the distinguished Hungarian hematologist Susan Hollàn, had more than tickled the hemoglobin field. That paper reported the discovery of three hemoglobins co-existing in the same person: normal hemoglobin (Hb A), and two abnormal hemoglobins. The entirely novel intriguing finding was that each of the two abnormal hemoglobins, called J-Buda and G-Pest, had different amino acid replacements, both in the α chain, implying that there must have been at least three α-globin genes in that person. In fact, that the α-globin locus might be duplicated in humans had been suggested already by Hermann Lehmann and Robin Carrell in 1968, and this notion was supported by a painstaking genetic analysis of families in Thailand with different forms of α-thalassemia (see ;Wasi et al., 1972). However, the notion only became a proven fact when Orkin was able to construct a detailed restriction map (Orkin, 1978) of the α-globin gene region, now known to be on chromosome 16p13.3 (Figure 1B). This was one of the first examples where the physical location of two neighboring genes was established within the human genome.

For the older one of us (L.L.), memories go back to the 19th Congress of the International Society of Hematology held in 1982 in Budapest. Susan Hollàn, the president of that congress, confided to L.L. that she had succeeded in advocating for the young Stuart Orkin, rather than a more senior person, to give a keynote lecture. Stuart Orkin had trained as MD at Harvard Medical School and had worked in Phil Leder's lab at the National Institutes of Health (NIH). He was then recruited by David Nathan to the Faculty at Children's Hospital, Harvard Medical School, where he took on the challenge of studying the physiology and pathology of hemoglobins and erythropoiesis. Stuart's lecture was a success, and he was quick to realize that his findings had important medical implications. Until then, prenatal diagnosis of thalassemia was carried out by sampling of fetal blood from the placenta at 18–20 weeks pregnancy: this technique was invasive and not free of risks. When Y.W. Kan and An M. Dozy discovered that a single base change, now called a single nucleotide polymorphism or SNP, was in strong linkage disequilibrium with the β^S mutation they set the ground, long before the human genome project was even started, for what became known as genome wide association studies (GWASs). As a result, prenatal diagnosis of SCD could be done at the DNA level, without the need for fetal blood sampling, Following Orkin's work, this was now possible also for severe forms of α-thalassaemia. This meant that prenatal diagnosis was now feasible at 10–12 weeks by the less invasive technique of sampling chorionic villi. Programs based on this approach have eventually reduced by more than 80%

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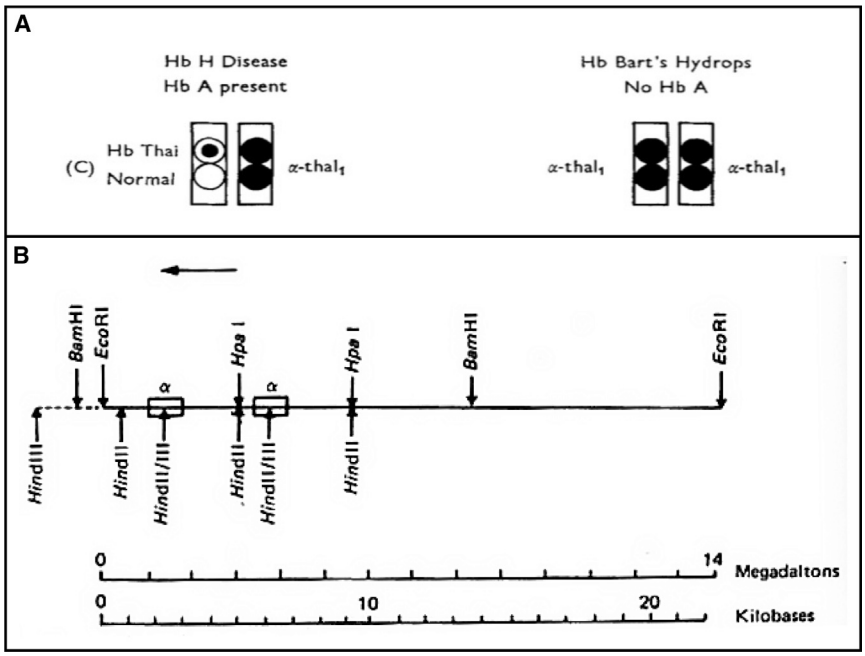

Figure 1. Duplication of the α-globin gene locus in humans
(A) Hypothetical diagram of the duplicated α-globin locus based on genetic analysis of families with hemoglobin variants in Thailand. This panel was reproduced with permission from Wasi et al. (1972).
(B) Physical map of the DNA region containing the α-globin genes, constructed on the basis of the sizes of DNA fragments obtained by using a set of restriction enzymes (*EcoR1*, *BamH1*, *Hin*dIII, *Hpa*I). This panel was reproduced with permission from Orkin (1978).

the births of babies with severe β-thalassemia in Sardinia, thanks to the single-minded efforts of Antonio Cao, and in Cyprus.

Orkin's major contributions to understanding the molecular and genetic basis of hematopoiesis were just getting started. Some of his most seminal contributions were in understanding the genetic basis of erythrocyte differentiation. Erythropoiesis is an extreme example of how a program of cell differentiation is implemented. From a stem cell that is anything but red, the program produces erythrocytes that contain 34% hemoglobin; no other cell in the body has a comparable concentration of a single protein. The differentiation process involves selective expression of heme and globin genes: this is gradual at first, but then the expression of the latter becomes explosive and almost exclusive. In the course of the process, major cellular events take place: the nucleus becomes pycnotic and is eventually extruded, the cytoskeleton is re-arranged, mitochondria are lost, and in the end, even ribosomes are lost. Despite these major losses, the mature red blood cells are endowed with extraordinary pliability and will circulate in the blood for 120 days. This is why we, haematologists, tell students that the erythrocyte is the most altruistic among cells: it has sacrificed aerobic metabolism, it has sacrificed the ability to make protein, and it has even given up reproduction for the sake of the single purpose to convey the maximum amount of oxygen to all other cells in the body.

The differentiation program must involve cell-intrinsic regulatory elements in the genome; growth factors such as erythropoietin, discovered by Eugene Golwasser and studied in depth by Harvey Lodish and others; and physical signals from the environmental niche. An important discovery by Orkin was to identify GATA-1, a zinc-finger protein (independently isolated by Gary Felsenfeld) as a transcriptional regulator of blood cell development that cooperates with another zinc finger protein that they called Friend of GATA-1 (FOG) (Whitelaw et al., 1990). This work was the basis for the subsequent understanding of the role of other transcription factors and of epigenetic events involved in both determination of the erythroid lineage from multipotent progenitors and cell maturation within the erythroid lineage itself. *GATA-1* can be regarded as a "master gene" within hematopoiesis (Figure 2), and its study has unveiled the close relationship between the erythroid and the megakaryocytic lineage. Most of this work was carried out in mice, thanks to increasingly sophisticated technologies, based on targeted homologous recombination pioneered by Mario Capecchi, for generating gene knockouts. Thus, it had become possible not only to model human diseases in the mouse but also to invent new combinations of genetic abnormalities. Added interest by hematologists in GATA-1 arose when it was discovered by Michelle Le Beau, John Crispino, and collaborators that truncating somatic mutations of GATA-1 were responsible, in infants with trisomy 21, for the transient myeloproliferative disorder (TMD) that may evolve to acute megakaryoblastic leukemia. Stuart Orkin, again by using mouse models, then found that truncated GATA-1 leads to hyperproliferation of yolk sac and fetal liver progenitor cells, which may account for the usually transient nature of TMD. In the meantime, Orkin was also involved in pinpointing pathology arising from germline *GATA-1* mutations, including thalassemia and X-linked thrombocytopenia, dyserythropoietic anemia and thrombocytopenia, and Diamond-Blackfan anemia. Interestingly, *GATA-1* maps to the X chromosome just like *ATRX*, the α-globin gene regulator and chromatin remodeller discovered by David Weatherall, Richard Gibbons, and Doug Higgs. This is important from the clinical point of view, because one mutant X-linked gene is enough to give disease in male hemizygotes.

For many years, one of the most intriguing features of the developmental biology of human hematopoiesis has been the transition, around birth, from producing fetal hemoglobin (Hb F) to producing adult hemoglobin (Hb A), known as "the Hb switch." It has been clear for over half a century that, around birth, the fetal genes *HBG1* (formerly $^{G}\gamma$) and *HBG2* (formerly $^{A}\gamma$) are turned off at the transcriptional level, whereas *HBB* (β) and *HBD* (δ) are turned on. However, the

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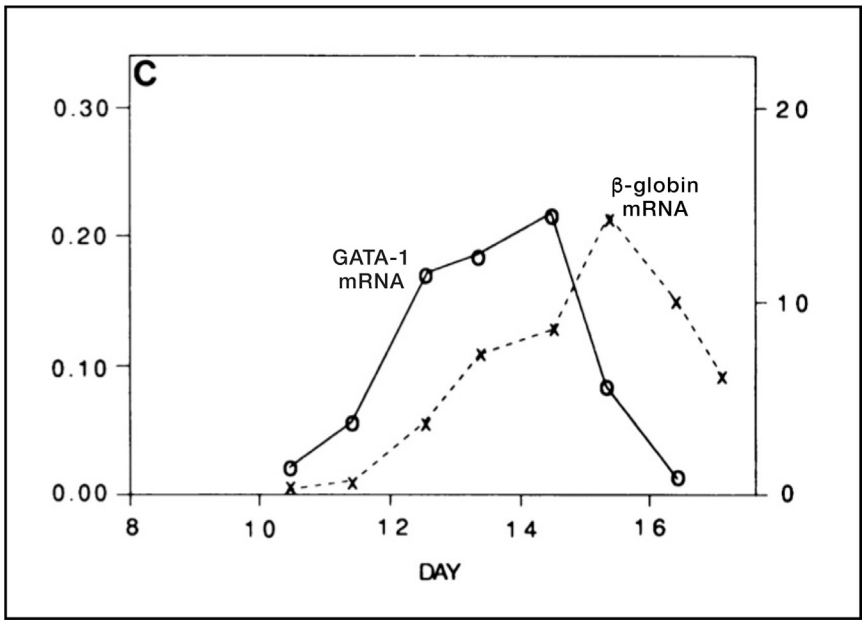

Figure 2. Genetic control of erythropoiesis: Time course of gene expression in hematopoietic cells in the course of embryonic development in the mouse

On the abscissa, days post-coitum; on the ordinate, amount of RNA (arbitrary units) per 2.5 μg of tissue. This time relationship was at the root of GATA-1 (formerly referred to as EryfIINF-EIIGF-1 or as EF-I/EFγa), which came to be known as a *master transcriptional regulator of erythropoiesis.* This figure was reproduced with permission from Whitelaw et al. (1990).

precise mechanism has remained elusive, until GWASs carried out by Swee-Lay Thein and others revealed that at least three loci significantly influence the residual Hb F synthesis that takes place in adults. One of these, *BCL11A*, became a main focus of investigation by Stuart Orkin and his colleague Vijay Sankaran: their work identified an erythroid-specific enhancer within this gene, and they found that *BCL11A* expression and enhancer activity were influenced by GATA-1. They then showed that the BCL11A protein targets specific sites in the γ-globin gene promoters and thus is key to the Hb switch (Liu et al., 2021). It was this work that inspired the younger of us (J.M.) to analyze the variability of Hb F levels and of F cells in a selected population of patients with SCD in Tanzania.

The pathophysiology of β-thalassemias and of SCD is fundamentally different, as the defect is quantitative in the former and qualitative in the latter; however, they are allelic; i.e., they result from mutations at the same locus. Genetic correction of somatic cells aiming to cure human disease has been a coveted objective since the dawn of molecular genetics. Given the hemoglobin genes were the first to be identified and available, hemoglobinopathies were from the outset an obvious target for genetic correction. For some time, retroviral vectors seemed the best candidates to transfer the β-globin gene into hematopoietic stem cells (HSCs), but David Williams and Stuart Orkin, among others, were well aware that, in the case of hemoglobinopathies, a major hurdle would be the very high level of expression needed. Indeed, this is a main reason why gene therapy has been successful in some other genetic diseases before hemogobinopathies. In that respect, a major advance came from Frank Grosveld's group, when they discovered a set of DNase hypersensitive sites upstream of the β-globin gene cluster that act as a crucial enhancer and that they named the locus control region (LCR). When introduced in the mouse genome, along with the human β-globin gene, the LCR caused the latter to be expressed at the same level as the endogenous mouse β-globin gene (Talbot et al., 1989). It became clear that the LCR, or at least some of it, was absolutely required for high expression. By that time, a lentivirus was preferred over a retrovirus, because it could transduce into quiescent stem cells: after testing dozens of constructs, and after fine-tuning regulator and insulator elements without compromising viral titre, Michel Sadelain came up with a suitable vector and succeeded in curing thalassemic mice (May et al., 2000). Subsequently, in Marina Cavazzana-Calvo's unit, this approach succeeded in human patients, both in thalassaemia and in SCD (Ribeil et al., 2017).

SCD is formally listed as a rare disease in many countries, yet it is anything but rare in Africa. In some African countries, up to 2% of infants may be born with this condition, and the heterozygote frequency may reach 25%. SCD is recognized by the WHO as a major public health problem. There are disease-modifying treatments including hydroxyurea, which has been abundantly time tested and is low cost. There are also curative treatments: hematopoietic stem cell transplantation (HSCT) and now gene therapy through the advances described here, both with a high cost. This creates a difficult situation: should curative treatments be made available in Africa (HSCT has been done in Nigeria), when most patients are still not receiving even hydroxyurea? Medicine has evolved in every human culture: it is a distinctive feature of the human species that through medicine we have chosen to oppose Darwinian selection. Therefore, we feel strongly that any kind of medicine/intervention should be without barriers; at the same time, we must be realistic. Developing facilities for curative therapies in Africa is important, and we should not relent on these necessary efforts. In the meantime, to provide hydroxyurea to all patients with SCD in Africa is a must; how this might be achieved and some of the hurdles we have discussed elsewhere (Luzzatto and Makani, 2021).

There is hope that the predicament outlined in the previous paragraph might benefit from progress that again owes to the perseverance of Stuart Orkin (2021) and of many others, who have contributed to the biennial hemoglobin switching conference, established by George Stamatoyannopoulos in 1978, with the expressed aim of curing thalassemia and SCD by reactivating fetal hemoglobin

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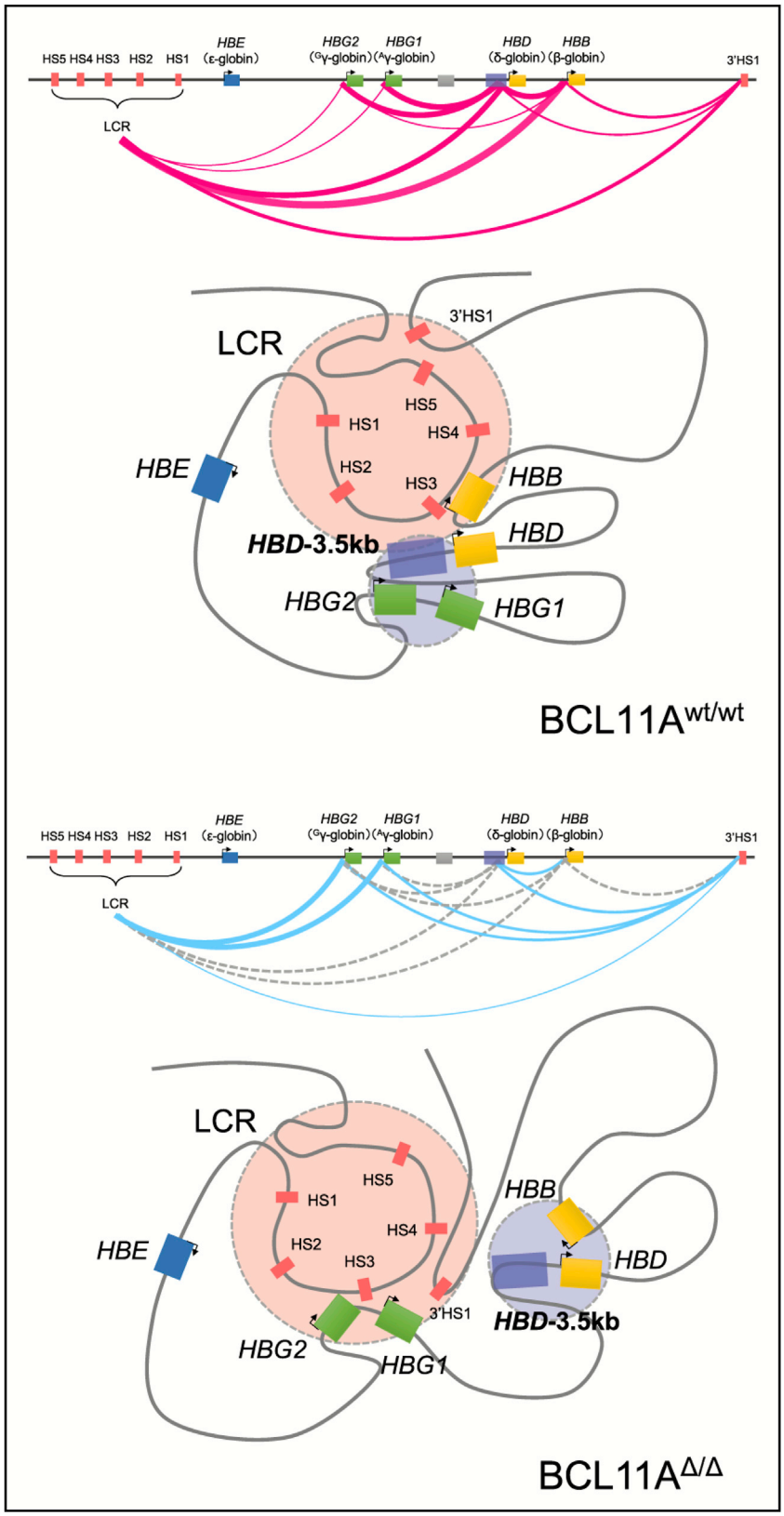

Figure 3. The major role of *BCL11A* in the human hemoglobin fetal to adult switch makes it a good candidate for gene therapy of SCD

(Top) With *BCL11A* active, long-range chromatin interactions (at the top) are such that the β (*HBB*) and δ (*HBD*) genes are actively transcribed (although *HBD* at a very low rate, because it has a weak promoter), whereas the two γ genes (*HBG1* and *HBG2*) have minimal transcription.

(Bottom) With both copies of the *BCL11A* gene inactivated in hematopoietic stem cells, the long-range interactions are changed and the fetal genes are actively transcribed. This figure was reproduced with permission from Shen et al. (2021).

synthesis. It has been known for a long time that the biallelic combination of the Hb S (HBB^{E6V}) gene with a gene responsible for the syndrome of hereditary persistence of fetal hemoglobin (HPFH) is clinically benign, because Hb F interferes potently with the polymerization of deoxy-hemoglobin S that causes sickling. Based on the role of *BCL11A* discussed above, one might expect that silencing *BCL11A* in a patient with SCD will increase Hb F and conversely decrease Hb S. Indeed, by using a short RNA homologous to the *BCL11A* transcript the translation of this gene is inhibited; this has produced a marked increase in Hb F in patients with SCD (Esrick et al., 2021): in this way, SCD is converted to the phenotype of Hb S/HPFH. A similar result has been obtained by CRISPR-Cas-mediated editing of a short sequence within the erythroid-specific enhancer of the *BCL11A* gene itself (Frangoul et al., 2021). In general, inhibiting or disrupting a gene can be regarded as easier than replacing a gene—there may be several ways to target *BCL11A*, whether permanently or transiently, and this opens up therapeutic prospects.

In his spirited article that narrated the romantic phase and the dogmatic phase of molecular biology, Gunther Stent (1968) seemed to lament that the golden era might be finished. Well, if the legacy is gene therapy, we are not complaining. At the same time, the genetic strand and the structural strand at the origin of molecular biology have become vigorously intertwined. The linear (one-dimensional) information in the DNA sequence encodes a developmental program that cannot be understood without taking into account the three-dimensional arrangement of DNA

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in chromatin, and millions of DNA-protein and protein-protein interactions. Some of the more recent work by Stuart Orkin and colleagues bears witness to this: they have used single-cell genome editing assay in conjunction with quantitative modeling and chromatin capture analyses in order to foster a comprehensive understanding of how distinct regulatory mechanisms can synergistically modulate Hb F expression (Shen et al., 2021). These studies have confirmed and refined (Figure 3) Grosveld's model, whereby through DNA looping, the LCR comes in contact in turn with the fetal globin gene promoters and then with the adult globin gene promoters. It remains to be uncovered, perhaps in the next switching conference, what triggers this remarkably timely physiological developmental phenomenon: whether it is a count of cell divisions from the onset of embryogenesis or subtle specific cues from a physical change in the environmental related to bone marrow organogenesis.

It is a natural question to ask, in biology and medicine, to what extent what we learn in a particular system has more general validity. The DNA region embedding the α-globin gene cluster is different in several respects from the region embedding the β-globin gene cluster, and the α-thalassaemias are clinically different from the β-thalassaemia, and they present much earlier (before birth) because there is no fetal-to-adult switch. However, there is a much earlier switch, from ζ to α. Doug Higgs's group has shown recently that in embryonic erythroid cells, the ζ-gene lies within open, acetylated chromatin, where it is able to interact with the α-globin super-enhancer; whereas in adult erythroid cells it is packaged within hypo-acetylated, facultative heterochromatin, where it no longer interacts with its enhancers. The ζ-gene can be partially re-activated by inhibition of histone de-acetylases, suggesting possible therapies for severe α-thalassemia (King et al., 2021).

It is clear that Stuart Orkin's contributions, spanning 5 decades, have been prompted by scientific curiosity, by the challenge of tackling difficult problems, and by his judgment of what questions are important. Like in many other cases, discoveries in basic biology have led, many years later, to advances in the management of a serious disease; in this case, the extraordinary accomplishment of a gene therapy for SCD. As this is still a demanding procedure, all of us would prefer that, instead of requiring high-tech *ex vivo* manipulation of a cell suspension enriched in the patient's HSC, it could be done by a simple injection of nanoparticles encapsulating a gene-editing device. Such *in vivo* gene therapy has been already successful in hemophilia with the AAV vectors targeting hepatocytes. It appears at the moment more difficult to target HSCs, but there is no *a priori* reason to regard it as impossible. Such an approach would greatly help with the difficult ethical dilemma that must be addressed in order to obtain that not only ameliorating therapy but also curative therapy for SCD, using gene therapy, is accessible and affordable in Africa.

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There has been no financial support for this article. We declare an interest in providing a cure for sickle cell disease to patients in Africa. We are grateful to all those—including Stuart Orkin—from whom, through reading and listening, we have learnt about hemoglobin, hemoglobinopathies, and the switch. We are equally grateful to all patients from whom we have learnt about sickle cell disease in real life.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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