



## Molecular control of hemoglobin switching

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### A B S T R A C T

The human *beta* ( $\beta$ )-*globin* locus contains five  $\beta$ -like *globin* genes, arranged in the order of their developmental expression pattern 5'- $\epsilon$  (embryonic) -  $G\gamma$  -  $A\gamma$  (fetal) -  $\delta$  -  $\beta$  (adult). The transition from fetal to adult  $\beta$ -like *globin* expression, known as hemoglobin switching, has attracted most attention. In patients with  $\beta$ -thalassemia or sickle cell disease, persistent expression of the endogenous  $\gamma$ -*globin* genes ameliorates the clinical manifestation of the disorders. Clinicians, epidemiologists and molecular biologists have studied hemoglobin switching for decades, applying novel tools and knowledge developed by the scientific community. In the 1990s, the field had exhausted the then available technologies without achieving a fundamental understanding of the molecular control of hemoglobin switching. This changed with the advent of the '-omics' era, sparked by the completion of the human genome sequence. Genome-wide association studies, comprehensive gene expression profiling, protein interaction screens and knockdown of genes using short interfering RNAs have propelled the field forward in the first decade of the 21st century. While we are still some way off understanding all the details of hemoglobin switching, there are a number of leads for the development of safe, affordable, off-the-shelf pharmacological compounds targeting the  $\gamma$ -*globin* suppression pathway.

#### Learning goals

At the conclusion of this activity, participants should be able to:

- describe developmental regulation of the human *globin* genes and hemoglobin switching;
- understand the challenges to achieve reactivation of  $\gamma$ -*globin* expression as a means to ameliorate the symptoms of  $\beta$ -thalassemia and sickle cell disease patients;
- discuss recent progress in knowledge of the molecular control of hemoglobin switching.

### Thalassemias and sickle cell disease

Diseases affecting red blood cells are by far the most common hereditary disorders in the human population. The symptoms vary from relatively mild, for instance deficiency for the metabolic enzyme glucose-6-phosphate dehydrogenase (G6PD) or erythrocyte structural proteins such as ankyrin (ANK1),  $\alpha$ - or  $\beta$ -spectrin (SPTA and SPTB), to lethal when left untreated. The latter is the case in severe forms of the thalassemias, a group of diseases caused by insufficient  $\alpha$ - or  $\beta$ -*globin* production. Hemoglobin, a tetramer composed of 2  $\alpha$ -like and 2  $\beta$ -like *globin* proteins, is responsible for the gas transport functions of the erythrocytes, and comprises more than 90% of the soluble protein in these cells. Each erythrocyte contains approximately  $250 \times 10^6$  hemoglobin molecules, and balanced production of the  $\alpha$ -like and  $\beta$ -like *globins* in terminally differentiating erythroid progenitors is important to achieve a 1:1 ratio. Excess  $\alpha$ -like or  $\beta$ -like chains are unable to form functional hemoglobin tetramers and will damage the erythroid cells. Insufficient production of  $\alpha$ -*globin* leads to  $\alpha$ -thalassemia. Severely compromised  $\alpha$ -*globin* production results in prenatal lethality, a condition known as hemoglobin Barts hydrops

foetalis. The symptoms of  $\beta$ -thalassemia, when  $\beta$ -*globin* expression is compromised, develop during the first year after birth. Humans have two specialized fetal  $\beta$ -like *globin* genes, *HBG1* and *HBG2*, which encode the  $A\gamma$ - and  $G\gamma$ -*globins*, respectively (Figure 1). These fetal genes are gradually silenced while expression of the adult  $\delta$ - and  $\beta$ -*globin* genes is activated (Figure 2). Malfunction of the *HBB* gene resulting in  $\beta$ -thalassemia becomes manifested during the first year of life following the switch from fetal-to-adult *globin* expression. There is no fetal  $\alpha$ -like gene, and in severe  $\alpha$ -thalassemia cases dysfunctional  $\gamma$ -*globin* tetramers are formed (hemoglobin Barts) at fetal stages. The presence of  $\gamma$ -*globin* protects the developing fetus from the effects of pathological  $\beta$ -*globin* variants. The most common mutation substitutes glutamic acid for valine (Glu6Val or E6V) in the 6<sup>th</sup> codon of  $\beta$ -*globin* and is the cause of sickle cell disease (SCD). The substitution of a glutamic acid by a valine residue (HbS) affects the biophysical properties of the hemoglobin tetramers. The change from a charged (E) to a non-polar (V) side chain enables polymerization of hemoglobin molecules under low oxygen conditions. This deforms the erythrocytes into the characteristic sickled shape. This has profound effects on

the physiological properties of the cells. Abnormal adhesion to blood vessel walls and microvascular occlusion result in local hypoxia aiding further sickling that leads to lasting organ damage. The lifespan of sickle erythrocytes is reduced from approximately 120 days to 16-20 days, placing the erythroid system under constant stress. Even with the best medical care, the life expectancy of SCD patients is still reduced by 2-3 decades. Hemoglobinopathies are a worldwide burden on the human population, with over 300,000 new patients born every year. The majority of these (83%) are SCD patients.<sup>2</sup>

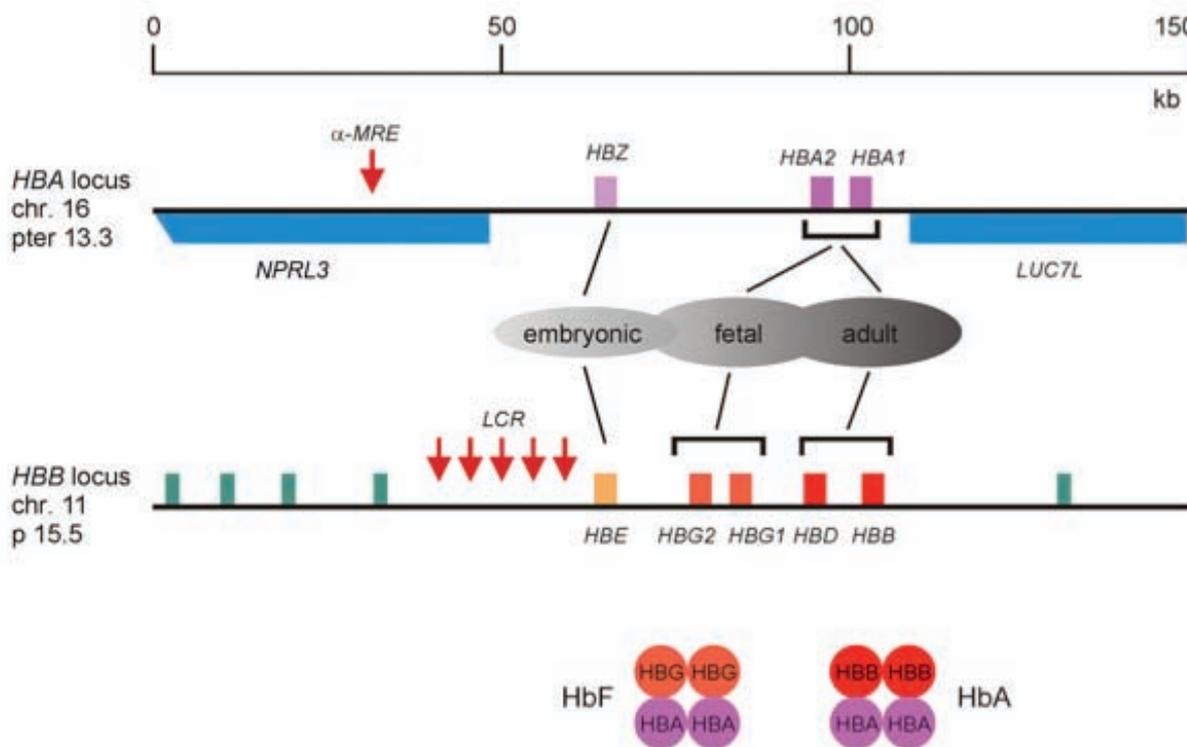
### Potential cures for hemoglobinopathies

Current standard medical care for thalassemia and SCD patients is aimed at alleviating the symptoms as much as possible. Progress has been impressive and has improved the quality of life for many patients, allowing them, to a large extent, to participate in society like any other individual. Carefully timed transfusion regimens, chelation therapy to remove excess iron from vital organs such as the heart and the liver, and early detection of potential adverse events have greatly contributed to the management of these diseases. However, this level of care is not available to the large majority of patients who live in parts of the world lacking such a sophisticated health care sys-

tem. Furthermore, the sheer number of patients would be an unbearable burden on any health care system. For example, Nigeria, with a population of 90 million, has an estimated 1-1.5 million SCD patients. Alternative treatments are, therefore, urgently needed. A limited number of patients have been cured by bone marrow transplantation. Clearly, this costly procedure is not available to the large majority of patients, since it requires a compatible donor and life-long follow up after transplantation.

### Gene therapy

An attractive alternative would be to correct the defective gene in the patients' cells through the intermediary action of induced pluripotent stem cells (iPS cells). Proof of principle for this approach has already been obtained in a mouse model for SCD.<sup>3</sup> A more straightforward approach is gene addition, in which a missing or corrected globin gene is added to the hematopoietic stem cells of the patients. In the past 25 years, formidable obstacles have been overcome to make gene therapy of hemoglobinopathies a realistic option. Globins must be expressed at very high levels in the erythroid cells, and this requires the addition of carefully selected elements of the *LCR* to the gene therapy vectors. The globin gene itself was also fine-tuned for use in this specific context. For instance,

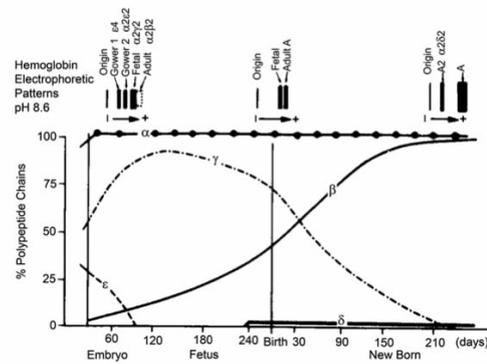


**Figure 1.** The human hemoglobin loci. The  $\alpha$ -like globin genes (*HBA1* and *HBA2*) are flanked by ubiquitously expressed genes (*NPRL3* and *LUC7L*). The major regulatory element ( $\alpha$ -MRE) is located upstream of the embryonic  $\zeta$ -gene (*HBZ*). The  $\beta$ -like globin genes are flanked by putative odorant receptor genes (green). The Locus Control Region (LCR), comprising 5 DNaseI hypersensitive sites required for high-level expression of the  $\beta$ -like globin genes, is located upstream of the embryonic  $\epsilon$ -globin gene (*HBE*). The developmentally regulated expression patterns and the composition of fetal (HbF,  $\alpha_2\gamma_2$  (*HBA2*, *HBG2*)) and adult (HbA,  $\alpha_2\beta_2$  (*HBA2*, *HBB2*)) hemoglobin are indicated.

splicing is a requirement for efficient expression and the introns of the globin gene were minimized to meet these demands. While this work was going on, it became clear that gene therapy vectors based on lentiviruses were superior to those based on the classically used  $\gamma$ -retroviruses. Unlike  $\gamma$ -retroviruses, lentiviruses are able to transduce non-dividing cells such as hematopoietic stem cells with high efficiency. The synthesis of all these efforts is the first demonstration of globin gene therapy in mice in 2000,<sup>4</sup> and the first report on a patient treated with a globin gene therapy vector in 2010.<sup>5</sup> While cautious optimism is justified, there are still a number of issues to be solved. Achieving globin expression at therapeutic levels is still challenging, and will require further optimization of the gene therapy vectors. An immediate concern is integration of the gene therapy construct in the genome of the patient's cells, which may interfere with the expression of neighboring genes. In a gene therapy trial for X-SCID, the  $\gamma$ -retroviral vector had integrated in proximity to the promoter of the *LMO2* proto-oncogene, leading to aberrant expression of *LMO2* and uncontrolled exponential clonal proliferation of mature T cells.<sup>6</sup> The patient who received globin gene therapy displayed outgrowth of a hematopoietic clone in which the lentiviral gene therapy vector had integrated close to the *HMGA2* gene leading to deregulated expression of this gene.<sup>5</sup> Although no adverse effects of this integration have been reported to date, it raises long-term safety issues. Targeted integration to 'safe harbors', which are apparently genetically empty areas of the genome, might be a step towards solving this problem.<sup>7</sup> It is encouraging that targeted site-specific integration of a globin transgene has recently been demonstrated.<sup>8</sup> This was achieved using zinc finger nucleases engineered to generate a double strand break at a specific location in the genome.<sup>9</sup> In conclusion, significant progress has been made in the development of cellular therapies involving gene correction or addition, and clinical trials of globin gene therapy have started. It is unlikely that such cellular therapies will become available to the large majority of patients within a reasonable time frame.

### Reactivation of fetal globin expression

Almost all  $\beta$ -thalassemia and SCD patients will have normal fetal  $\gamma$ -globin genes, which were expressed before birth but switched off during the first year after birth. This gradual change from  $\gamma$ - to  $\beta$ -globin expression is referred to as hemoglobin switching, and 'reversing the switch' is thought to ameliorate the symptoms of  $\beta$ -thalassemia and in particular SCD patients. Higher levels of  $\gamma$ -globin correlate positively with reduced pathology and clinical events in SCD patients. SCD patients with  $\gamma$ -globin levels higher than 20% of total  $\beta$ -like chains are often virtually event free and require little hospital care. The reason is that  $\gamma$ -globin acts as a chain breaker, stopping sickle hemoglobin from forming long polymers under low oxygen conditions. For  $\beta$ -thalassemia patients, reactivation of  $\gamma$ -globin would have to be more quantitative, since it needs to compensate for the absence of  $\beta$ -globin chains. In both cases,  $\gamma$ -globin is a well-known disease modifier and understanding hemoglobin switching at the molecular level has, therefore, been the subject of intense research efforts since the 1970s.



Kitchen, H. and Brett, I. *Annals New York Academy of Sciences* 241, 653-671 (1974)

**Figure 2. Expression of hemoglobins during human development. The developmental expression patterns of the human hemoglobins are shown. (Modified from Kitchen and Brett.<sup>1</sup>)**

### Hereditary persistence of fetal hemoglobin (HPFH)

Sustained expression of HbF in otherwise healthy individuals is termed hereditary persistence of fetal hemoglobin (HPFH). Initially, mutations in the  $\beta$ -globin locus were found that correlated with increased  $\gamma$ -globin levels. In most cases, this involved deletions of sequences 3' to the  $\gamma$ -globin genes, suggesting removal of repressor elements. Such a function was specifically allocated to the intergenic region between the  $A\gamma$ - and  $\delta$ -globin genes.<sup>10,11</sup> In some cases, very large deletions were found which are thought to bring novel enhancer elements in close proximity to the  $\gamma$ -globin genes.<sup>12</sup> Of particular interest are point mutations and small deletions that affect  $\gamma$ -globin levels in adults, since these potentially provide mechanistic insight into the hemoglobin switching mechanism. These are confined to the  $\gamma$ -globin promoters and alter expression of the linked gene only, suggesting a direct effect on promoter accessibility in adult erythroid cells. The most common variant is a single nucleotide polymorphism (SNP) C>T at position -158 in the *G\gamma* promoter, known as the *XmnI* polymorphism.<sup>13</sup> This is a common sequence variant in all population groups, found at a frequency of 0.32-0.35.<sup>14</sup> Presence of the T allele is associated with increased HbF levels. Quantitatively, a rare variant in the  $A\gamma$  promoter, the -117 G to A mutation, has the most dramatic effect. Heterozygotes display 10-20% HbF, containing only  $A\gamma$  chains.<sup>15</sup> The HPFH condition was mimicked in mice carrying a human  $\beta$ -globin locus transgene in which the -117 G to A mutation was introduced.<sup>16</sup> This provided unambiguous evidence that the HPFH phenotype was caused by the point mutation in the  $A\gamma$  promoter. This raised the hypothesis that the -117 G to A mutation would alter direct binding of a regulatory protein to the  $A\gamma$  promoter, either allowing binding of an activator or preventing binding of a repressor, or both. Unfortunately, systematic testing of this hypothesis through the combination of *in vitro* DNA binding assays, introduction of novel point mutations in the -117 area, and functional analysis of these engineered promoters in the transgenic mouse assay consistently failed to identify the factor(s) involved.<sup>17</sup> Similarly, very

interesting models were developed for the -202 C>G or C>T HPFH mutations. This area is capable of forming an intra-molecular triplex termed H-DNA, and the HPFH mutations were shown to destabilize this structure. A 'cold shock' domain protein YBX1 binds specifically to the -202 region, and binding affinity is reduced by the HPFH mutations.<sup>18</sup> However, an impact on globin regulation was not found in *Ybx1* knockout mice,<sup>19</sup> possibly due to functional redundancy with the closely related MSY4 factor. Thus, while the HPFH phenotype could be reproduced in transgenic mice carrying the human  $\beta$ -globin locus and biochemical assays revealed potential molecular mechanisms, unambiguous identification of proteins directly involved in globin switching remained elusive.

## The dawn of the “-omics” era

Not all variation in  $\gamma$ -globin expression is due to mutations in the  $\beta$ -globin locus. In rare HPFH families, the phenotype is not linked to the  $\beta$ -globin cluster or chromosome 11. An early example is provided by a large Indian kindred. Linkage analysis showed that the genetic determinant for HPFH segregated independently from the  $\beta$ -globin cluster,<sup>20</sup> suggesting involvement of a *trans*-acting factor. Via a painstaking mapping exercise the genomic location of this factor was mapped to chr6q23,<sup>21</sup> and could eventually be pinpointed to variants in the region between the *HBSIL* and *MYB* genes.<sup>22</sup> Recent work has shown that this intergenic region contains distal enhancers required for *MYB* gene activation.<sup>23</sup> The completion of the human genome sequence in 2001<sup>24,25</sup> enabled the development of genome-wide association analysis in population studies (GWAS). Application of GWAS led to the identification of *BCL11A* on chromosome 2p15 as a potential modifier of  $\gamma$ -globin levels.<sup>26,27</sup> Functional studies in primary human erythroid progenitors<sup>28</sup> and mice<sup>29</sup> demonstrated that *BCL11A* is a transcriptional repressor protein essential for the timing of the transition of fetal-to-adult globin expression. These discoveries have sparked an enormous interest in *BCL11A* as a target for  $\gamma$ -globin reactivation. This interest was further boosted by the observation that inactivation of *BCL11A* in the adult erythroid system corrects hematologic and pathological defects in a mouse model of SCD through induction of  $\gamma$ -globin expression.<sup>30</sup> Thus, within the time frame of a few years, *BCL11A* has been firmly established as the first realistic molecular target for reactivation of  $\gamma$ -globin expression in adults. It should be realized that tinkering with *MYB* is likely to adversely affect hematopoiesis and erythroid differentiation.<sup>31</sup> Unfortunately, both *MYB*<sup>32</sup> and *BCL11A*<sup>33</sup> potentially have been implicated in human malignancy, and mice transplanted with *Bcl11a*-deficient cells died from T-cell leukemia derived from the host.<sup>34</sup> Erythroid-specific ablation of *BCL11A* in mice did not result in any oncogenic events,<sup>30,35</sup> indicating that erythroid-specific inactivation of *BCL11A* in humans might be safe. To achieve this will be challenging; transcription factors such as *BCL11A* are viewed as highly unattractive drug targets *per se*, even without the confounding requirement for cell type-specific targeting. However, transcription factors perform their functions as part of multi-protein complexes, and *BCL11A* is known to interact with several other nuclear factors.<sup>28</sup> Identification of an essential partner in erythroid

cells may provide a handle on developing pharmacological compounds blocking these protein-protein interactions. Cell type-specific delivery might be achieved through erythroid-specific receptors.<sup>36</sup> Clearly, development of such novel therapeutic approaches will be the topic of intense research efforts in the coming years.

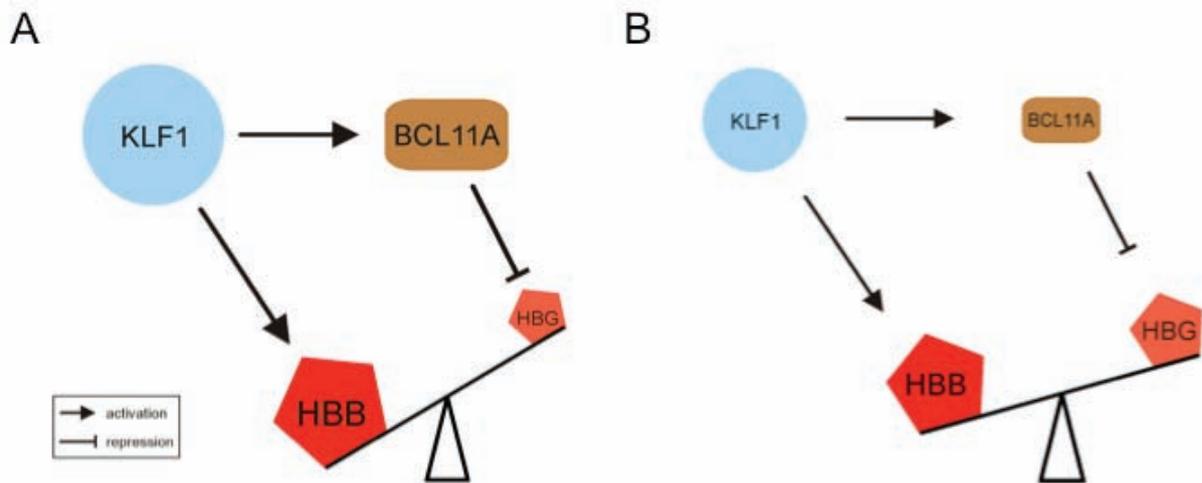
Variants in the *HBB*, *BCL11A* and *HBSIL-MYB* loci together account for approximately 50% of the variation in  $\gamma$ -globin expression.<sup>37</sup> The remaining variation could be accounted for by loci with relatively small impact, and by rare variants with significant quantitative effects on  $\gamma$ -globin expression that are typically missed by GWAS population studies. An example of the latter is provided by the identification of the *KLF1* gene as a  $\gamma$ -globin modifier through the study of a Maltese family in whom HPFH was found in 10 of 27 members.<sup>38</sup> A genome-wide SNP scan followed by linkage analysis identified a candidate region on chromosome 19p13.12–13. Sequencing revealed a nonsense mutation in the *KLF1* gene, p.K288X, which ablated the DNA-binding domain of this key erythroid transcriptional regulator.<sup>39</sup> Only family members with HPFH were heterozygous carriers of this mutation, suggesting that haploinsufficiency for *KLF1* was the cause of the HPFH phenotype. The *KLF1* p.K288X carriers displayed high HbF levels, although with considerable variation (mean 8.4%; range 3.3–19.5%). Part of this variability could be explained by SNP haplotypes at the *BCL11A* locus. Importantly, *BCL11A* expression was reduced in the *KLF1* p.K288X carriers and *KLF1* was shown to be a direct activator of *BCL11A* expression.<sup>38,40</sup> Knockout studies in mice had previously established that *KLF1* is essential to activation of  $\beta$ -globin expression.<sup>41,42</sup> Remarkably, expression of embryonic and fetal  $\beta$ -like globin genes was fully activated in the absence of *KLF1*.<sup>43,44</sup> Collectively, this has led to the proposal of the 'double whammy' model<sup>38</sup> (Figure 3). Firstly, *KLF1* acts on the *HBB* locus as a preferential activator of the  $\beta$ -globin gene.<sup>43</sup> Secondly, it activates expression of *BCL11A*, which in turn represses the  $\gamma$ -globin genes.<sup>28,38,40</sup> This dual activity ensures that, in most adults, HbF levels are less than 1% of total Hb. Notably, *MYB* is thought to be an activator of *KLF1* expression in human adult erythroid progenitors<sup>45</sup> and, therefore, the rough contours of a  $\gamma$ -globin suppression network are appearing.

Mutations in *KLF1* were first described to cause the rare 'inhibitor of Lutheran antigens' (In(Lu)) blood group phenotype,<sup>46</sup> and more recently a steady stream of novel mutations found across different populations has been reported.<sup>47–52</sup> Similar to the observations in the Maltese families, HbF levels associated with *KLF1* mutations were found to be highly variable.<sup>53</sup> This may be explained by the *KLF1* expression level derived from the remaining intact *KLF1* allele, but also suggests interplay with other modifier loci such as *BCL11A*.<sup>38</sup> Remarkably, all the mutations reported to date affect the DNA binding domain of *KLF1*. In many cases, a premature stop codon completely ablates the DNA binding domain, as was the case in the Maltese family. In other cases, amino acid substitutions are found in critical residues of the three zinc fingers comprising the DNA binding domain. These either interfere with the three dimensional structure of the zinc finger domains, or directly affect specificity of DNA target site recognition.<sup>53,54</sup> The latter mutations result in most cases in partially functional proteins. This is illustrated by the remark-

able discovery of two compound heterozygotes for the p.S270X nonsense and p.K332Q missense mutations in a Sardinian family<sup>51</sup> who displayed 22.1% and 30.9% HbF, respectively. HbF levels in the parents were unremarkable. The same study noticed that zinc protoporphyrin levels were increased in these two individuals, consistent with the notion that KLF1 regulates several enzymes in the heme synthesis pathway.<sup>55</sup> In separate studies, delayed hemoglobin switching<sup>49</sup> and increased HbA2<sup>50</sup> were reported in heterozygous carriers of *KLF1* mutations. This is clinically important since increased HbA2 (>3.9%) is a distinguishing feature of  $\beta$ -thalassemia carriers. Borderline cases require an extensive laboratory workup to exclude  $\beta$ -thalassemia carrier status; screening for *KLF1* mutations in such cases will aid the identification of couples at risk. One mutation with a dominant phenotype has been reported. The p.E325K missense mutation causes congenital dyserythropoietic anemia.<sup>52</sup> It changes a negatively charged (E-glutamic acid) into a positively charged amino acid (K-lysine) of an absolutely conserved residue in the second zinc finger which is directly involved in DNA sequence recognition.<sup>54</sup> The p.E325K *KLF1* mutant has a dominant-negative effect on the transcriptional activation properties of wild-type *KLF1*. This affected globin expression but also expression of other *KLF1* target genes, such as the water channel *AQP1* and the adhesion molecule *CD44*. Remarkably, HbF levels were 37.3% in one patient, who also expressed detectable levels of embryonic hemoglobin (Hb Portland,  $\zeta_2\gamma_2$ ). There are remarkable parallels with the phenotype of the mouse *Nan* mutant, which carries a missense mutation in the corresponding residue in mouse *KLF1*,<sup>56,57</sup> even though the *Nan* mutation p.E339D does not introduce a positively charged amino acid but leaves the negative charge intact (D-aspartic acid).

Clearly, it will be of great interest to investigate the impact of *KLF1* missense mutations on erythroid gene

expression and terminal differentiation at the molecular level. Unlike *BCL11A*, expression of *KLF1* is largely, although not exclusively,<sup>58,59</sup> restricted to erythroid cells and no association of *KLF1* mutations with malignancy has been reported. In principle, attenuating *KLF1* activity would, therefore, provide a safe approach to raise HbF levels in individuals with  $\beta$ -type hemoglobinopathies. Our recent analysis of compound *KLF1::BCL11A* mouse mutants showed that erythroid-specific ablation of *BCL11A*, alone or in combination with *KLF1* haploinsufficiency, only mildly affected steady-state erythropoiesis.<sup>35</sup> Furthermore, expression of  $\gamma$ -globin from a single-copy human  $\beta$ -globin locus was markedly increased in adult mice, lending further support to the role of the *KLF1*-*BCL11A* axis in globin switching. An important observation from the mouse studies is that in the complete absence of *BCL11A*, even in combination with *KLF1* haploinsufficiency, the  $\gamma$ -globin genes are not expressed to the full extent.<sup>30,35</sup> I propose that the tight repression of the  $\gamma$ -globin genes in mice provides a window of opportunity for identification of additional factors involved in the silencing mechanism at the adult stage. Enforcement of repression of the embryonic/fetal program in adult erythropoiesis may be executed by, for instance, the transcription factors MYB<sup>22</sup> and SOX6,<sup>60</sup> the chromatin-bound FOP/CHTOP protein<sup>61</sup> and NuRD complex,<sup>62,63</sup> the orphan nuclear receptors TR2/TR4<sup>64</sup> and the protein arginine methyl transferase PRMT5,<sup>65</sup> and is likely to include additional epigenetic mechanisms such as polycomb group (PcG) complex recruitment and DNA methylation. Future work should, therefore, be aimed at further clarifying the multi-layered repressive network of the embryonic/fetal program in the adult erythroid environment. The first steps to clarify the molecular differences between the developmental stages of erythroid cells have been taken in mouse<sup>66</sup> and human,<sup>67</sup> and have revealed many differentially expressed genes. These data have not yet been



**Figure 3.** Model for regulation of hemoglobin switching by *KLF1* and *BCL11A*. (A) *KLF1* preferentially activates the adult *HBB* gene. It also activates the *BCL11A* gene, and the *BCL11A* protein silences the fetal *HBG1/HBG2* (*HBG*) genes. (B) *KLF1* activity is reduced in member of the Maltese family carrying the *KLF1* K288X mutation. This decreases expression of *BCL11A* and the diminished amount of *BCL11A* protein alleviates repression of the *HBG1/HBG2* genes. The combined reduction of *KLF1* and *BCL11A* activity shifts the balance towards expression of the *HBG1/HBG2* genes.

explored in the context of globin switching. Another recent development is the potential role of micro-RNAs, more specifically micro-RNA-15a and 16-1 which are believed to exert their function via MYB.<sup>68</sup> There are, therefore, many leads that need to be followed up.

### Therapeutic reactivation of fetal hemoglobin

The ultimate goal of all these efforts is to develop safe pharmacological compounds targeting the  $\gamma$ -globin suppression pathway specifically. Currently, hydroxyurea,<sup>69</sup> 5-azacytidine<sup>70</sup> and short-chain fatty acids (butyrates)<sup>71</sup> go some way in increasing HbF levels, but none of these agents are specific and long-term safety is a concern. Of these, only hydroxyurea has FDA approval for treatment of SCD patients and it is used with considerable success.<sup>69</sup> Its beneficial effects are only partly due to increased HbF levels; it also reduces cell deformability and improves hydration status of sickle erythrocytes.<sup>72</sup> The majority of patients increase HbF production upon HU treatment;<sup>73</sup> however, HbF baseline and response magnitude among the patients is highly variable. In the 1990s, a screen of pharmacological compounds was conducted by OSI Pharmaceuticals, in which approximately 186,000 defined chemicals and fungal extracts were evaluated.<sup>74</sup> Eleven distinct classes of compounds were identified, many of which activated the stress response suggesting this was part of the mechanism of  $\gamma$ -globin induction. An activated stress response also appears to have a part in the distinction of responders and non-responders to hydroxyurea treatment.<sup>75</sup> Many other compounds with  $\gamma$ -globin inducing properties have been reported in the literature. Resveratrol<sup>76</sup> (a compound found in red wine), anthracy-

clines,<sup>77</sup> statins<sup>78</sup> and thalidomide derivatives<sup>79</sup> are just a few examples. Drugs already in use for treatment of other conditions, such as statins and thalidomide, are particularly attractive since these could in principle be adapted relatively quickly for therapy for  $\beta$ -thalassaemia and SCD. Unfortunately, HbF levels are not routinely determined in patients receiving long-term medication, and hence HbF induction as a side effect is essentially a chance discovery. Collectively, it can be concluded that random screening of compounds has as yet not yielded any alternative to hydroxyurea. It, therefore, appears that better screening systems integrated with approaches designed to directly target the  $\gamma$ -globin suppression pathway should be developed.

### Challenges for the future

Recent progress has been fuelled by the application of “-omics” technologies, but seemingly incremental improvements in other laboratory techniques have been equally important. Culture of primary erythroid cells, initially pioneered by Eitan Fibach,<sup>80</sup> were adapted to completely defined synthetic media by the late Hartmut Beug<sup>81,82</sup> (Figure 4). Using a buffy coat from as little as 15 mL of peripheral blood, we can now expand erythroid progenitors from healthy individuals<sup>38</sup> and patients<sup>61</sup> and use these cells for functional experiments. There is no need to select CD34-positive cells, since the majority of the *in vitro* erythroid expansion potential resides in CD34-negative cells.<sup>83</sup> The development of recombinant lentiviruses enabled efficient transduction of these cells, allowing shRNA-mediated knockdown and expression of exogenous proteins.<sup>38,61</sup> Lentiviral shRNA libraries targeting

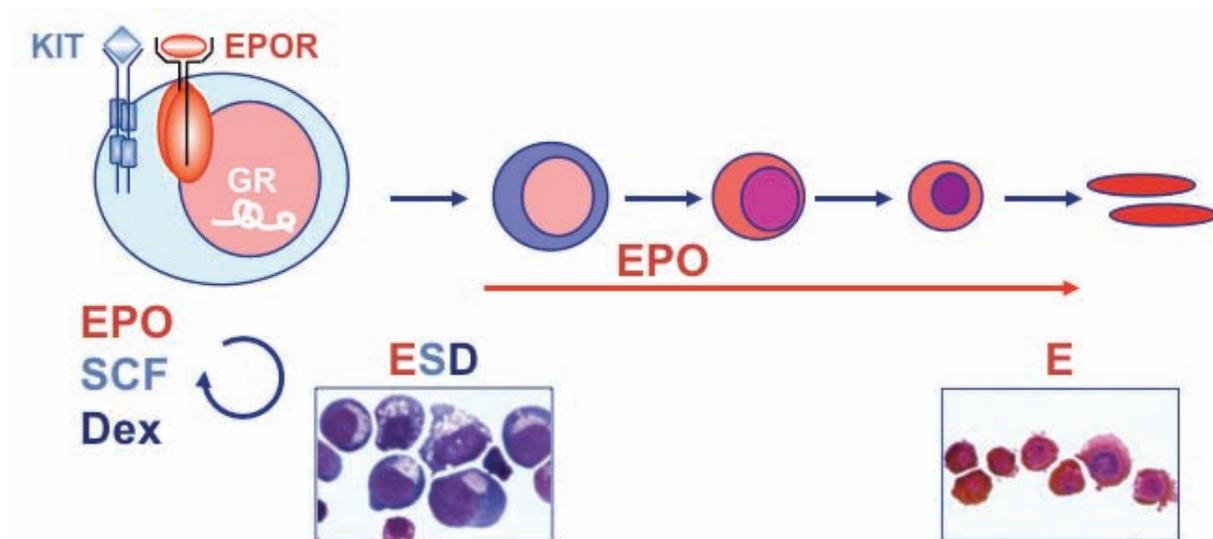
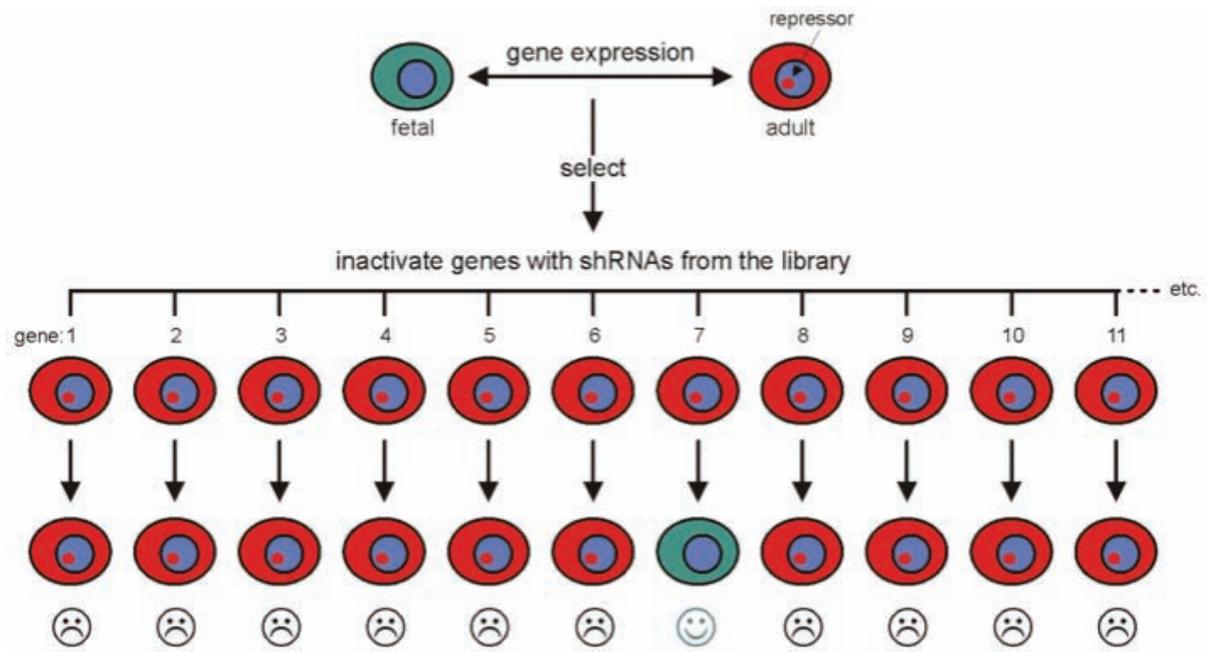


Figure 4. Culture of primary human erythroid progenitors. Erythroid progenitors can be expanded in synthetic medium containing erythropoietin (EPO, ligand for the erythropoietin receptor (EPOR)), stem cell factor (SCF, ligand for KIT) and dexamethasone (Dex, ligand for the glucocorticoid receptor (GR)) as survival- and growth factors. After approximately seven days under these conditions (ESD) the cultures are composed predominantly of proerythroblasts. Transfer to medium containing EPO but lacking SCF and Dex (E conditions) forces the proerythroblasts into terminal differentiation, leading to hemoglobinization (brown staining) and enucleation.



**Figure 5.** Proposed shRNA screen for identification of  $\gamma$ -globin repressors. Genome-wide gene expression profiles of cultured human fetal liver- and adult blood-derived proerythroblasts are compared, and genes overexpressed in adult cells (which should include  $\gamma$ -globin suppressors) are selected for lentiviral shRNA-mediated knockdown. Knockdown of a  $\gamma$ -globin repressor should result in increased  $\gamma$ -globin expression.

every protein-encoding gene in the human genome are available<sup>84</sup> and these can be used to identify  $\gamma$ -globin suppressors<sup>28,38,61</sup> (Figure 5). But there is a catch: *in vitro* cultures mimic stress conditions, and human erythroid progenitors respond to this by increasing  $\gamma$ -globin expression. HbF levels of approximately 5% are observed with most cells cultured from healthy donors. A further increase in HbF is easily achieved by applying additional stress to the cells, for instance lentiviral transduction. Typically, HbF levels may reach 30%. It is, therefore, highly recommended to rescue knockdown experiments by expression of shRNA-resistant versions of the genes of interest.<sup>61</sup> In adult mouse erythroid progenitors containing a complete human  $\beta$ -globin locus, transgene silencing of the  $\gamma$ -globin genes is much tighter.<sup>35</sup> This will, therefore, likely provide a much more stringent system for screening purposes. Building reporter loci in which expression of fluorescent proteins is dependent on activation of the  $\gamma$ -globin genes, and creation of immortalized erythroid progenitor lines<sup>81</sup> from mice carrying such reporter loci, will provide much improved tools for high-throughput screening of  $\gamma$ -globin activating molecules. Such systems are currently being developed.<sup>85,86</sup>

It will remain important to investigate HbF variation in the human population. Currently, no mutations affecting the MYB or BCL11A proteins have been reported, but it would be very interesting to know what the consequences of haploinsufficiency for these factors are. In addition, we have only begun to identify the players in the  $\gamma$ -globin suppression pathway. A locus on chr8q has been associated with HbF in the context of the *XmnI-G $\gamma$*  polymorphism,<sup>87</sup> but no causative link to a specific gene on chr8q has been made. We have also largely ignored the potential stromal contribution to hemoglobin switching, even

though hemoglobin switching is paralleled by the transition of the site of erythropoiesis from the fetal liver to the bone marrow. More likely than not, the erythroid progenitors in these two tissues. Identification of extracellular factors that promote the transition from fetal to adult erythropoiesis could be a major step forward in globin switching research. A recent paper implicating reduction of hedgehog signaling with developmental progression of hematopoiesis throughout human ontogeny might provide an example of such a factor.<sup>88</sup> Any factor identified as potentially involved in hemoglobin switching will have to be rigorously tested using conditional knockouts and well-established mouse models for human globin switching<sup>19,29,30,34,35,40,62,63</sup> before they are taken forward as targets for  $\gamma$ -globin reactivation in adults.

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