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Ugo Testa. Fetal hemoglobin chemical inducers for treatment of hemoglobinopathies. *Annals of Hematology*, 2008, 88 (6), pp.505-528. 10.1007/s00277-008-0637-y . hal-00477991

HAL Id: hal-00477991

<https://hal.science/hal-00477991>

Submitted on 30 Apr 2010

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Fetal hemoglobin chemical inducers for treatment of hemoglobinopathies

Ugo Testa

Received: 25 August 2008 / Accepted: 27 October 2008 / Published online: 15 November 2008
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Abstract The switch from fetal ($^G\gamma$ and $^A\gamma$) to adult (β and δ) globin gene expression occurs at birth, leading to the gradual replacement of HbF with HbA. Genetic regulation of this switch has been studied for decades, and the molecular mechanisms underlying this developmental change in gene expression have been in part elucidated. The understanding of the developmental regulation of γ -globin gene expression was paralleled by the identification of a series of chemical compounds able to reactivate HbF synthesis in vitro and in vivo in adult erythroid cells. Reactivation of HbF expression is an important therapeutic option in patients with hemoglobin disorders, such as sickle cell anemia and β -thalassemia. These HbF inducers can be grouped in several classes based on their chemical structures and mechanisms of action. Clinical studies with some of these agents have shown that they were effective, in a part of patients, in ameliorating the clinical condition. The increase in HbF in response to these drugs varies among patients with β -thalassemia and sickle cell disease due to individual genetic determinants.

Keywords Hemoglobin · Thalassemia · Sickle cell disease · Anemia

Introduction

In all species that contain β -globin genes, a switch in globin gene expression coincides with changes in morphol-

ogy of the erythroid cells, the site of erythropoiesis, and hemoglobin composition. The understanding of the regulation of fetal hemoglobin (HbF) expression is of fundamental importance both for unveiling the mechanisms responsible for hemoglobin switching and for the study of fetal hemoglobin reactivation in adult erythroid cells. Furthermore, there is a strong rationale for understanding the mechanisms responsible for hemoglobin switching, as reversing it can provide major therapeutic benefits to people affected by β hemoglobinopathies.

The genes present at the level of the human β -globin locus undergo two different developmental switches in their expression. The *embryonic switch* occurs at approximately 6 to 8 weeks of gestation and involves the switching from the expression of embryonic ϵ -globin gene to the expression of the two γ -globin ($^A\gamma$ and $^G\gamma$) genes: As a result of this switch, ϵ -globin synthesis is silenced, while γ -globins expression is considerably up-regulated and remains elevated during all the fetal life. The *fetal switch* (here simply referred as the hemoglobin switching) occurs in the perinatal period and is characterized by the progressive switch from the expression of γ -globin genes, whose synthesis is almost completely silenced, to the expression of β - and δ -globin genes, whose synthesis is up-regulated. As a consequence, HbF ($\alpha_2\gamma_2$) synthesis declines in adult erythroid cells to <1% of the total globin expression, while the major hemoglobin in adult life becomes hemoglobin A ($\alpha_2\beta_2$). Hemoglobin A₂ (HbA₂, $\alpha_2\delta_2$) is a minor hemoglobin whose expression is usually <2% of the total hemoglobin.

Studies carried out during the last three decades have in part clarified the molecular mechanisms governing globin gene expression. The human β -globin locus, consisting of about 100 kb located on chromosome 11, is composed of five functional genes ϵ , $^G\gamma$, $^A\gamma$, δ , and β , arranged in the order corresponding to their progressive expression during

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development (Fig. 1)(Fig. 2). The human β -globin locus contains also a locus control region (LCR) that contains five DNaseI hypersensitive sites (HS) and is located 5' with respect to β -like globin genes (Fig. 1). These sites represent regions devoid of nucleosomes and are particularly accessible to interactions with transcription factors. The LCR plays a major role in the control of globin gene expression, and its deletion produces β -thalassemia characterized by complete absence of β -globin expression. The β -like globin genes have three coding regions (exons) separated by two intervening sequences (introns). The regulatory elements that regulate the expression of globin genes during development and in adult erythroid cells are represented by promoters, enhancers, and silencers, which flank the genes in *cis* and are responsible at a large extent for the erythroid-specific expression of these genes and their ordered sequence of expression during development.

The two γ -globin genes are the results of a 5-kB tandem duplication estimated to have occurred about 35 million years ago. The coding regions of these two genes differ by only a single nucleotide located in exon 3 within the codon 136, where the $^G\gamma$ gene codon is GGA (coding for glycine) and the $^A\gamma$ gene codon is GCA (coding for alanine). It is important to note that the appearance of γ -globin genes during evolution was recent. In fact, γ -globin genes and HbF synthesis have been observed only in primates, while mammalian species such as mice have no γ -globin genes. Starting from about 18–24 months of age, circulating HbF levels reach the adult level of <1% of the total Hb. In adult erythrocytes, HbF is composed by about 40% $^G\gamma$ and 60% $^A\gamma$, while in the newborn by about 70% $^G\gamma$ and 30% $^A\gamma$ (Fig. 2).

Regulation of γ -globin gene expression

Gene transcription of the γ -globin genes, as well as of other globin genes present in the β -globin locus, is controlled by complex molecular mechanisms involving: (1) *cis*-acting

elements, represented by specific nucleotide sequences, such as the β LCR and (2) *trans*-acting elements, such as transcription factors and chromatin remodeling activities [1].

The human β -globin LCR was functionally defined as a DNA regulatory region that allows high levels of erythroid-specific expression to a *cis*-linked gene in a copy-dependent manner, which is independent of the integration site in the host genome [2]. The human β -globin LCR consists of five HSs, each composed by about 200–300 bp and containing binding sites for several transcription factors, including NF-E2, EKLF, GATA-1, and Sp1, whose binding to their recognition sites is required for DNase hypersensitivity [3]. The role of *cis*-acting elements in the regulation of γ -globin gene expression was explored through RNA-TRAP and chromosome conformation capture (3-C) experiments in transgenic mice carrying the entire or selected regions of the human β -globin locus. RNA TRAP experiments provided evidence that HS2 is in close proximity to the actively transcribed β -globin gene, in agreement with genetic data suggesting that HS2 is the most prominent enhancer element in the β -globin LCR [4]. In this context, particularly significant were the 3-C experiments carried out in transgenic mice carrying the entire human β -globin locus: These mice show a switch in the association of HS2 and HS3 sites in the β LCR from contacts with embryonic and fetal genes in yolk sac cells to contacts with the adult human β -globin in adult-type erythroid cells [5]. The same interactions between the adult β -globin gene and HS2 and HS3 sites in the β LCR were obtained by the 3-C analysis using human adult bone marrow erythroid cells [5]. Deletion of the HS3 site in these transgenic mice did not affect γ -globin gene expression in fetal cells, while it destabilizes normal chromatin structure at the β -globin locus in adult-type cells [6]. These observations strongly suggest that contacts between the LCR and the various genes of the β -globin locus are developmentally controlled and are required for the LCR to control globin gene expression rates.

In other studies, using the 3-C technology, the spatial organization of the β -globin locus was analyzed in

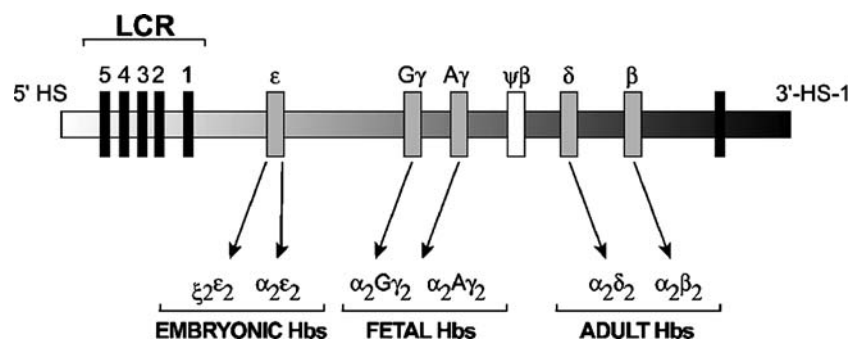


Fig. 1 Schematic representation of the human β -globin locus. Locus control region (LCR) with 5' DNaseI hypersensitivity sites (HS) is presented as black bars, the genes are presented as gray bars, and the

β pseudogene $\Psi\beta$ is shown as a white bar, in relative location along the locus. The types of hemoglobins formed by these various genes are also indicated

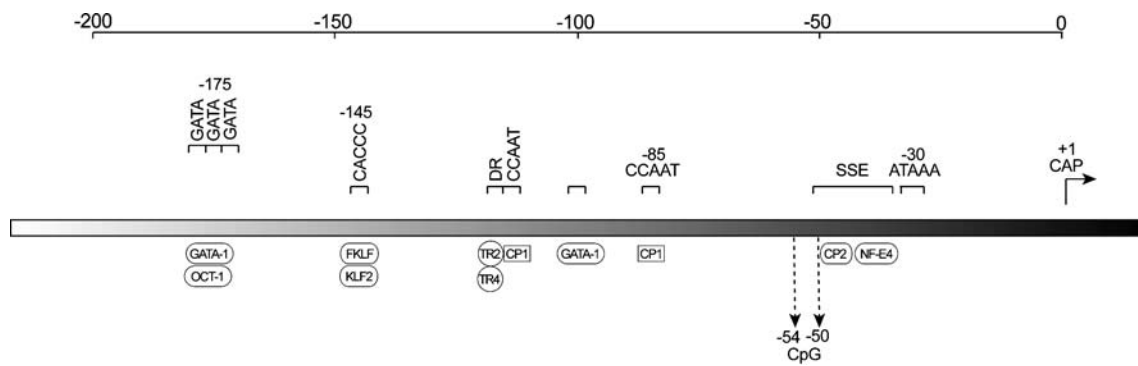


Fig. 2 Schematic representation of the human γ -globin genes proximal promoter. Shown are the DNA binding proteins that have been demonstrated to bind to the proximal γ -globin gene promoter.

The DNA sequences to which these protein bind are also indicated. The two methylable CpG residues at -50 and -54 are shown. The promoter is drawn to scale

expressing erythroid and nonerythroid brain cells. In brain cells, the inactive β -globin locus adopts a linear conformation, while in erythroid cells, the HSs of the β -globin LCR are in close proximity to the active genes [7]. The 40–60 kb of intervening chromatin containing the inactive globin genes loops out. This spatial clustering of transcriptional regulatory elements was referred to as an active chromatin hub [7]. Therefore, the active chromatin hub may be defined as a spatial chromatin configuration in which the LCR, together with other regulatory sequences, loops toward the active β -globin-like genes.

The recent development of the 4-C technology, which combines 3-C technology with microarray or sequencing approach, should allow the unbiased identification of DNA elements that interact with a target sequence in the nuclear space. In a 4-C study based on the analysis of the β -globin locus, it provided clear evidence that the active β -globin locus in erythroid cells contacted a completely different set of loci than the inactive locus in brain cells [8].

GATA-1, EKLF, and NF-E2 are the best-characterized tissue-specific transcription factors involved in the transcription of β -like globin genes. All these three factors bind to DNA elements in the β -globin genes. GATA-1 and EKLF are essential for active chromatin hub (ACH) formation: In fact, frequent contacts between the LCR and the β -globin-like genes are lacking in their absence [9]. In contrast, NF-E2, is dispensable for β -globin ACH formation [10]. The essential role of GATA-1 in inducing β -globin LCR chromatin structure remodeling is also supported by recent studies of enforced GATA-1 expression in non-erythroid cells [11]. Recent studies have provided evidence that long-range interactions between LCR and the active β -globin genes requires the binding to the LCR of a multimer transcriptional complex formed by the nuclear protein Nuclear LIM Interactor (NLI) and its erythroid-binding partners GATA-1/TAL-1/LMO2 [12].

The position of the genes in the β -globin locus is important to determine the sequence of expression of these

genes during ontogeny. In the human β -globin locus, five functional globin genes lie on the chromosome in the order 5'- ϵ - γ - α - δ - β -3'. Since the spatial arrangement of the β -like globin genes parallels their temporal expression pattern, it has been suggested that gene order or proximity to the LCR may be a determinant of the order of expression during ontogenesis [13–15]. In a more recent study, Harju et al. have carefully analyzed the effect of gene order on the temporal expression of the human β -like globins using β -yeast artificial chromosome (YAC) transgenic mice [16]. The results of these experiments showed that: (1) an adult β -globin gene located proximally to the LCR is preferentially expressed throughout all the development; (2) when an α -globin gene was placed at the same location, this α -globin gene was expressed during embryonic erythropoiesis and the fetal liver stage of definitive erythropoiesis, but was silenced during the adult stage and the downstream wild-type γ -globin genes were not expressed; (3) when the ϵ -globin gene was placed between the δ - and β -globin genes, it remained silent during embryonic erythropoiesis, while only the LCR-proximal wild-type ϵ -globin gene was expressed; and (4) finally, placement of a β -globin gene upstream of the γ -globin gene resulted in expression of this β -globin gene in embryonic cells [16]. These results clearly indicate that distance from the LCR is a major determinant of temporal gene expression during development [16].

Other important additional informations on *cis* elements regulating γ -globin gene expression derive from the analysis of the mutations occurring at the β -globin gene locus in patients with increased HbF [hereditary persistence of fetal hemoglobin (HPFH)]. Two types of HPFH have been described: non-deletion HPFH and deletion-type HPFH. Non-deletion HPFH forms are due to mutations occurring in either the γ - or α -globin gene promoters, resulting in continued HbF synthesis in the adult. Sequence analysis studies have provided evidence that non-deletion HPFH point mutations are clustered in three regions of the

γ -globin gene promoters, centered on positions -200 , -175 , and -115 relative to the transcription start site [17]. Two hypotheses have been proposed to explain increased HbF synthesis in adults carrying non-deletion HPFH mutations: (1) these point mutations decrease the binding of transcription repressors inhibiting γ -globin gene expression in adults and (2) these point mutations may generate binding sites that potentiate the binding of a transcriptional complex increasing γ -globin gene transcription in the adult.

The functional role of these point mutations was supported through the analysis of transgenic mice carrying the -200 , -175 , and -115 mutations [18–20]. Some of the mutations modify binding sites, present in the γ -globin promoter regions, for the transcription factors GATA-1 or NF-E4 or occur at the level of the distal CCAAT box upstream. Thus, the mutation at position -117 of the γ -globin gene determines the loss of GATA-1 to the γ -promoter region around -117 : This finding has led to the speculation that GATA-1 might act as a negative regulator of γ -globin gene in normal adults [18, 19]. The T to C substitution of the γ -globin gene has been identified in some individuals with non-deletion HPFH. In vitro experiments provided evidence that -175 mutations significantly reduced binding of the transcription factor Oct-1, but not GATA-1, whereas -173 mutations markedly decreased binding of GATA-1 but not of Oct-1 [20].

The -200 region is a highly GC-rich region known to be the target of five different types of point mutations affecting either the $^G\gamma$ promoter at position -202 and the $^A\gamma$ promoter at positions -202 , -198 , -196 , and -195 . Biochemical studies have characterized the nuclear proteins able to bind to the HPFH -198 γ -globin gene promoter and include a protein complex formed by DNMT1, the transcriptional co-activator p52, the protein SNEV, and RAP74 (a subunit of the transcription factor IIF) [21].

In addition to these mutations, also mutations occurring at the level of the CCAAT box of the human γ -globin gene produce the phenotype of HPFH. Because HPFH mutations in the CCAAT region activate the partially silenced γ -globin gene in the adult, it was suggested that the CCAAT-binding proteins may act as repressors [22].

Various forms of deletion-type HPFH have been identified. In the African-type of deletion HPFH, large deletions have been observed, beginning just 3' to the $^A\gamma$ gene and extending for 3' of the β -globin gene: Therefore, the deletion involves intergenic γ - δ sequences and the entire β and δ genes. To explain the phenotype of this HPFH form, it was proposed that the juxtaposition of enhancer elements, normally located downstream of the globin locus and the HPFH breakpoint, exerts a markedly positive effect on γ -globin transcription [23].

Other deletions affecting HbF synthesis are represented by $\delta\beta$ -thalassemias. Individuals with $\delta\beta^o$ -thalassemia

mutations have severely decreased HbA expression and moderately increased HbF expression. In these cases, HbF expression is heterogeneously distributed in red blood cells. In the $\delta\beta^o$ -thalassemias, the deletions at the human β -globin locus begin further 3' to the γ -globin gene than the HPFH African-type deletion in the γ - δ intergenic region and extend 3' to delete the δ - and β -globin genes. The differences between the clinical syndromes of $\delta\beta$ -thalassemia and HPFH, associated with different levels of HbF synthesis, as well as differences in the extent of the deletions, has led to suggest a key role for DNA sequences located between the human γ - and δ -globin genes in the γ -globin gene expression in these disorders and, more generally, in normal human globin switching. Particularly, the putative 1-kb region that represents the minimum region of difference between HPFH and $\delta\beta$ -thalassemia deletions is located approximately 3 to 4 kb upstream of the δ gene.

The important role of this DNA region in γ -gene regulation is directly supported by the analysis of patients affected by the $\delta\beta^o$ Corfu deletion. The 7.2-kb Corfu deletion extends from the γ - δ region upstream of the δ gene to involve the 5' end of the structural δ -globin gene [24]. Patients homozygous for the Corfu deletion have very high HbF levels (around 90%), only mild anemia, and no transfusional requirement [24]. The erythroid cultures issued from hemopoietic progenitors of Corfu deletion patients showed a very high level of γ -globin transcription [24]. These observations strongly support the notion that the intergenic γ - δ sequences play a key role in γ -globin reactivation in adult.

In addition to genetic elements present in the β -globin locus, also genetic elements present outside this locus control the level of HbF synthesis during adult life. The identification of these genetic traits was obtained through the study of heterocellular HPFH. HbF levels greatly vary in normal health individuals: In fact, the majority (~10%) has HbF levels comprised from 0.8% up to 5%. These individuals with relatively high HbF levels have heterocellular HPFH. Twin studies show that 89% of the quantitative variation of HbF (and of F cells) in normal subjects is genetically controlled, with 50–60% of the F-cell variance being due to genetic factors not linked to the β -globin locus [25, 26].

Among the genetic determinants linked to the β -globin locus, it has to be mentioned the C a T transition at position -158 in the promoter of the $^G\gamma$ -globin gene (known as the Xmn I- $^G\gamma$ site) [27]. This polymorphism, which is common in all population groups with a frequency of about 0.35 [25], has been shown to increase the $^G\gamma/(^G\gamma+^A\gamma)$ ratio and to be associated with increased HbF levels under conditions of erythroid expansion, as observed in β -thalassemia and sickle cell anemia [28]. As the result of two genetic conversion events, the -158 C a T change was observed

in the promoter of both $G\gamma$ and $A\gamma$ genes in healthy subjects with slightly elevated HbF levels [29]. The $-158\text{ C}\rightarrow\text{T}$ mutation in the $A\gamma$ gene has a significant effect on expression of the $A\gamma$ gene, since it restores the $G\gamma/A\gamma$ ratio, and its effect on HbF production seems to be stronger than that of the same mutation in the $G\gamma$ gene.

Linkage analysis and high throughput genotyping platforms and improved bioinformatics tools [30] have facilitated the localization of the unlinked three quantitative trait loci (QTLs) present on regions of the genome outside the β -globin locus at the level of chromosome 6q23, 8q, and Xp22.2-3 [31–33]. Five protein-coding genes (ALDH8A1, HBS1L, cMYB, AHI1, and PDE7B) have been identified in this 1.5 Mb 6q23 chromosome region [34]. The quantitative expression profiles of these five genes in individuals with normal and elevated HbF levels showed that only two genes, cMYB and HBS1L, were down-modulated in the latter ones compared to the former ones [34]. Overexpression of cMYB, but not of HBS1L gene, in erythroleukemic K562 cells induced a decrease of HbF levels [35]. In a subsequent high-resolution association study, the same authors identified multiple genetic variants within and 5' to HBS1L at 6q23 that are strongly associated with F-cell levels in families of Northern European ancestry [35]. Studies on erythroid progenitors in vitro have clearly shown that only HBS1L expression correlates with high F-cell alleles [36]. It is of interest to note that the HBS1L-MYB intergenic region on chromosome 6q23.3 influences not only F-cell frequency but also a series of hematological parameters, including erythrocyte, platelet, and monocyte counts in humans [37].

Recently, additional QTL affecting F-cell production have been identified. Thus, Menzel et al. genotyped 179 unrelated individuals from the extreme upper and lower tails of the F-cell distribution, drawn from a database of 5,184 phenotyped individuals from an adult twin registry. In addition to 6q23, they identified two additional QTLs on chromosomes 2p15 and 11p15 [38]. The 2p15 is a new locus mapping to the gene encoding the CH2-type zinc-finger protein BCL11A on chromosome 2p15 [38]. It was suggested that deregulated BCL11A expression may influence F-cell production by affecting the kinetics of erythropoiesis [38]. These observations have been confirmed through the analysis of single nucleotide polymorphisms at the level of the 2p15 locus and F-cell production in a population of Sardinian β -thalassemic patients. Particularly, it was observed that the SNP rs11886868 variant is significantly associated with Hb [39].

The mechanism by which these unlinked QTLs affect HbF levels is largely unknown. Some of the QTLs seemingly encode for a *trans*-acting factors interacting with other transcription factors active on the β -globin gene cluster and, through this mechanism, affect the rate of

γ -globin gene transcription [30]. Other QTLs, like the HIMP locus on chromosome 6q23, stimulates Hb F production by altering the kinetics of erythropoiesis, as observed in stress erythropoiesis of hemoglobinopathies [35].

The studies on LCR and on hereditary persistence of HbF patients have lead to identify a dual mechanism for regulation of globin gene expression and of hemoglobin gene switching: autonomous gene control and gene competition for the direct interaction with the LCR.

Chromatin epigenetic changes play an important role in the control of globin gene expression. The main chromatin epigenetic changes occur at the level of core histones and consist in covalent modifications, such as acetylation, methylation, and phosphorylation. The biologic effects of these covalent modifications of chromatin core histones are of two orders: (1) they modify the physical properties of chromatin, such as stability, flexibility, and compactness (i.e., histone acetylation makes the chromatin more flexible and helps it to adopt an open conformation) and (2) they allow the chromatin to become more accessible to the interaction with certain transcription factors.

Among these modifications, a relevant role is played by histone acetylation in the control of γ -globin gene expression. Histone acetylation studies of the β -globin locus in various animal species have clearly shown that the active genes are highly acetylated, while the inactive genes are scarcely or only mildly acetylated [40–43]. However, the majority of these studies have been carried out in human erythroleukemic cell lines, and none of them explored primary erythroid cells that undergo normal hemoglobin switching. Only recently, the histone acetylation of the genes present at the level of the β -globin locus was explored in primary erythroblasts corresponding at various ontogenic stages (fetal vs. adult stage). This analysis showed that the LCR was acetylated to the same level at all stages, whereas acetylation levels at the individual gene regions correlated with the state of transcription [44]. In the active genes, the promoters were less acetylated compared with the coding regions. Finally, the level of acetylation per histone at the active γ and β promoters was clearly higher than that at the inactive ϵ promoter [44].

Another covalent modification of histones associated with regulation of gene expression is represented by histone methylation. Lysine residues of histones can be mono-, di-, or trimethylated. There is evidence, based on various cellular systems, implicating changes in globin-locus DNA methylation with globin gene switching [45]. Furthermore, studies carried out in baboons, which have a fetal-to-adult globin switch that is similar to the one seen in humans, show a correlation between gene expression and globin promoter (promoter methylation and globin gene expression are inversely related) [46]. Finally, in

transgenic mice carrying a human β -globin YAC, targeted deletion of the methyl-CpG binding protein MBD2 gene delays γ -globin developmental silencing [47]. Recently, the pattern of γ - and β -globin promoter methylation was explored in primary human fetal liver and adult bone marrow erythroid cells. γ -Promoter methylation was low in fetal erythroblasts and high in adult erythroid cells [48]. The pattern of γ -globin promoter methylation was explored during human adult erythroid differentiation showing that: (1) γ -globin promoters are initially hypermethylated in CD34⁺ cells and during the initial stages of erythroid differentiation; (2) the upstream γ -promoter CpGs becomes hypomethylated during the subsequent step of erythroid differentiation, from day 7 to day 10 of culture, corresponding to the development of early erythroid precursors and to the phase of fetal hemoglobin production that occurs during early adult erythropoiesis; (3) at later stages of erythroid differentiation, the γ -globin gene promoters become remethylated [48].

A last important element in the regulation of γ -globin gene expression is represented by the transcription factors binding to the γ -globin promoters. The proximal region of the γ -globin promoters extends over a region composed by about 200-bp region, where various sequences able to bind various transcription factors have been identified. This 200-bp region contains a canonical TATA box, a stage selector element (SSE) at -34, two methylable CpG residues at -50 and -54, two CAAT boxes at -85 and -115, one CACCC box at -145, and a GATA binding site at -175.

The 5' untranslated region of the γ genes occupies a region of about 50 nucleotides between the Cap site, the start of transcription, and the initiation (ATG) codon. This region includes the conserved sequence GCAGTCCA-CAC of the capping box (+1 to -10).

The TATA box of the human γ -globin promoters determines the initiation site but not the efficiency of transcription on the γ -globin genes [49].

A SSE binding site is present at the level of -53 to -34 of the γ -globin gene promoter. This SSE element binds a heterodimeric complex between CP2, an ubiquitous transcription factor, and NF-E4 a protein present in erythroid cells, called stage selector protein (SSP). Alternatively, the SSE site may bind the transcription factor Sp1; the binding of Sp1 and SSP to SSE is mutually exclusive [50, 51]. Jane et al. proposed that the presence of this SSE in the γ -globin promoter would be important for the silencing of the β -globin in *cis* [52]. This hypothesis was supported by the observation that SSE confers a competitive advantage to the γ gene over the β gene in the interaction with HS2 in transient transfection assays. However, a functional role for the SSE was not supported by *in vivo* studies in transgenic mice, thus suggesting that the SSE site does not affect silencing of

the γ -globin gene [53]. More recent studies have shown that two NF-E4 components are present at the level of SSP, a p22 NF-E4 and a p14 NF-E4; importantly, the p14 NF-E4 subunit is present at higher levels in bone marrow than cord blood [54]. The p14 isoform acts in direct contrast to p22 NF-E4, suppressing γ -gene expression: It interacts with CP2, resulting in diminished association of CP2 with SSE and it inhibits the p45 NF-E2 recruitment to the γ -promoter [55].

In this region of the promoter, CpG dinucleotides at -55 and -50 are also present in the γ -promoter. The methylation of these two dinucleotides is involved in the mechanism of γ -gene silencing in adult erythroid cells [56].

At position -85 and -115 of the γ -globin gene promoter, there are two CCAAT boxes. Several mutations occurring at the level of the CCAAT box region of the human γ -globin promoter produce the phenotype of HPFH. The CCAAT box of the γ -globin promoter has been identified as a positive *cis* element by *in vitro* transcription assays. Several transcription factors have been identified to interact with γ CCAAT boxes: NF-Y(CP-1), CCAAT/enhancer-binding protein (cEBP), CCAAT transcription factor/NF1, CCAAT displacement protein (CDP), and GATA-1. However, of all of these, only NF-Y has been confirmed as an *in vivo* γ CCAAT-binding factor [57]. Experiments in a transgenic mouse model provided evidence that the CCAAT box of the γ -globin gene promoter is important for the expression of the γ -globin gene in adult erythroblasts but not in embryonic erythroid cells [58].

Near to the distal CCAAT box, Tanabe et al. identified the Direct Repeat (DR) element at the level of which binds Direct Repeat Erythroid Definitive (DRED) repressor complex [58]. The DRED is a 540-kDa complex, composed of TR2 and TR4, two nuclear orphan receptors believed to bind DNA and recruit other repressors to achieve γ -gene silencing in definitive erythroid cells [59]. The DRED complex binds to DR sequences observed in the promoter of γ - and ϵ -globin genes. Silencing of fetal β -type globin genes is delayed in definitive erythroid cells of TR2 and TR4 null mutant mice, whereas transgenic mice that express dominant-negative TR4, human fetal γ -globin is activated in definitive, but not in primitive erythroid cells [60]. These findings strongly suggest that TR2/TR4 acts as a stage-selective repressor. The TR2 and TR4 orphan nuclear receptors act also as repressors of GATA-1 transcription [61]. The DRED complex acts by inhibiting the binding of EKLF to the ϵ - and γ -globin CACCC sites.

The DR element, if located around four CCTTG motifs (two upstream and two downstream), and the mutation of the DR motif, but not of the CCTTG motifs, reactivate γ -globin gene expression in adult erythroid cells [62].

A CACCC box is present upstream the CCAAT boxes. A CACCC box is present also in the promoter of the

β -globin gene: The binding of the transcription factor EKLf to this CACCC box is strictly required for β -globin expression. The function of the γ -globin CACCC box has been investigated in the context of the competition model of Hb switching, based on the assumption that the expression of the γ -globin genes precludes the expression of the β -globin gene in embryonic/fetal erythroid cells by competing for the enhancing activity of the LCR. In transgenic mice models, it was demonstrated that disruption of the γ -globin promoter CACCC box results in the loss of β -globin gene developmental specificity [63, 64]. Analysis of the effect of mutations of the CACCC box on γ -globin expression in transgenic mice showed that this box is not required for γ -globin expression in primitive erythropoiesis, but it is necessary for γ -gene expression in the cells of definitive erythropoiesis [65]. To explain these findings, Li et al. proposed that the transcription factors recruited by the CACCC, CCAAT and TATA boxes present on the γ -globin promoter interact each other to form a large molecular complex: In embryonic erythroid cells, the function of transcription factors failing to be recruited into the complex because a mutated promoter box is compensated by the other transcription factors recruited by the other cis elements; in contrast, in adult erythroid cells, an intact transcriptional complex is absolutely required for γ -globin promoter activity [65]. According to this hypothesis, it was suggested that in embryonic erythroid cells, LCR is able to interact also with an incomplete γ -globin promoter complex, while in adult erythroid cells, a complete γ -globin promoter is required to mediate its interaction with LCR.

Kruppel-like factors (KLFs) are a large family of transcription factors able to interact with CACCC promoter elements. The most important of these KLFs is represented by EKLf, whose inactivation is lethal due to the absence of β -globin gene expression in definitive erythroid cells [66]. EKLf concentrations increase ~3-fold from primitive to definitive erythroid cells [67]. However, EKLf deficiency does not affect ϵ - and γ -globin gene expression, thus indicating that this transcription factor is dispensable for the expression of embryonic and fetal globin genes. In addition to EKLf, other KLFs are expressed in erythroid cells, including KLF2, KLF4, KLF5, and KLF12, whose expression increases with erythroid maturation [68]. KLF2, KLF6, KLF7, KLF10, KLF12, KLF15, and KLF16 are expressed higher in fetal erythroid than in adult erythroid cells [69]. The silencing of KLF8 or Sp1 induced elevated levels of γ -globin expression in K562 cells [69]. Among these KLFs, particularly interesting are fetal Kruppel-like factor (FKLF, also known as KLF11) and KLF2 for their capacity to bind CACCC and stimulate γ -globin gene promoter [70, 71]. Finally, recently, it was shown that ectopic expression of FKLF can also induce fetal hemoglobin expression in the setting of adult erythropoiesis in vitro as well as in vivo [72].

Around the -175 of the γ -globin genes, there are two consensus recognition motifs for the GATA-1 transcription factor. An Oct-1 binding motif is found between the GATA-1 sites and overlaps the downstream GATA-1 site. As mentioned above, the T to C mutation at position -175 of the γ -globin gene has been identified in some individuals with nondeletion HPFH. Studies on WT and mutant -175 γ -globin promoter region showed that this promoter region binds a multiprotein complex containing GATA-1, Oct-1, and other transcription factors, contributing to the formation of a repressive chromatin structure that silences γ -globin expression in adult erythroid cells [73].

A more upstream region of the γ -globin gene proximal promoter, located between -730 and -378 relative to the mRNA start site, plays a role as an adult stage-specific silencer of the γ -globin genes. A recent study has provided evidence that the silencing function of this promoter region is exerted by the binding of a protein complex containing GATA-1, FOG-1, and Mi2 at the $-566/-567$ GATA site [74].

In addition to transcription factors binding to the γ -globin gene promoter, also transcription factors interacting at the level of the intergenic sequence between γ and β genes affect γ -globin gene expression. Thus, O'Neill et al. [75] described a large protein complex that binds a pyrimidine rich sequence intergenic between γ - and β -globin genes, which contains the transcription factor Ikaros as one of its components. Importantly, deletion of this region of the human β -globin locus in transgenic mice delays adult globin switch [76]. Ikaros null mice display only a small increase in the γ/β globin ratio [77]. Mice harboring a point mutation in the third zinc finger of Ikaros (the resulting protein retains its ability to dimerize with isoforms and family members, but is defective in DNA binding) die of anemia in late gestation with a much more severe phenotype [78]. In these mice, a marked downmodulation of β -globin and upregulation of γ -globin expression was observed; furthermore, chromatin conformation capture data demonstrated that Ikaros facilitates long-distance DNA looping between the LCR and a region upstream of δ -globin gene [79].

The ensemble of these studies on chromatin conformation studies, globin gene promoters, and transcription factors has led to the formulation of the “competition model” to try to explain at the molecular level the developmental changes occurring in globin gene expression. The competition theory of hemoglobin switching proposes that transcription factors specific of embryonic (yolk sac), fetal (fetal liver), and adult (bone marrow) erythroid tissues (or combinations of stage-specific transcription factors) bind to globin gene promoters and provide individual genes with a competitive advantage for interaction with the LCR at the appropriate developmental stage. Developmental stage-specific interactions between

the LCR and embryonic/fetal and adult β -globin genes have been demonstrated *in vivo* by RNA tagging experiments [80, 81] and chromosome conformation capture experiments [82]. The long-distance looping between the LCR and individual genes of the β -globin locus determines the formation of an ACH. The transcription factors EKLF, GATA-1, and FOG-1 are essential for LCR interactions with adult β -globin genes in definitive erythroid cells [82, 83]; however, other transcription factors, such as p45 NF-E2, although indispensable for appropriate β -globin expression in adult erythroid cells, are not required for long-distance DNA looping [84]. The transcription factors that promote LCR interactions with embryonic/fetal globin genes in primitive erythroid cells are in large part unknown.

Chemical inducers of HbF synthesis

Insights into our understanding of the molecular mechanisms controlling hemoglobin switching and, particularly, γ -globin gene expression have greatly contributed to the identification and study of chemical compounds able to activate HbF synthesis in adult erythroid cells. A fundamental input to study chemical inducers of HbF synthesis came from the observation, made several decades ago, that HbF may have a protective effect in sickle cell anemia [85]. The same applies to β -thalassemias. Thus, a considerable number of studies have led to the identification of different classes of pharmacological agents able to reactivate HbF synthesis both *in vitro* and *in vivo*. Some of these agents have been introduced in therapy for the treatment of sickle cell anemia and β -thalassemia. These compounds are analyzed below, with particular emphasis to the analysis of their mechanism of action and of their impact in the treatment of hemoglobin disorders, such as sickle cell disease and thalassemia. For the analysis of the chemical structure of HbF inducers, the readers may refer to an excellent recent review by Gambari and Fibach [86].

Hypomethylating agents

DNA methylation is a mechanism of gene silencing that occurs when methyl groups become covalently attached to the 5-carbon position of a cytosine residue at a “CpG” site. This epigenetic process occurs during S phase and is catalyzed by the DNA methyltransferase family of enzymes. Genes with heavily methylated promoter regions cannot be transcribed and are effectively silenced. DNA methylation and associated silencing has been shown to occur during development at the level of globin genes. Thus, analysis of γ - and β -globin promoter methylation showed that, in general, promoter methylation and globin gene expression are inversely related [48]. Furthermore,

the γ -globin promoter methylation changes during erythroid differentiation of adult erythroid progenitors: γ promoters are initially hypermethylated in CD34⁺ cells, become hypomethylated during the pre-erythroid phase, and then return to be hypermethylated at later stages of erythroid differentiation [48]. In fetal erythroid progenitors, the γ -globin promoter is hypermethylated and becomes progressively less methylated as erythroid differentiation progresses and is hypomethylated in the mature erythroid progeny [87]. These observations support a model in which differences in the methylation pattern of the γ -globin gene in differentiating erythroblasts at different stages of development is the result of fetal stage-specific demethylation associated with transcription activation.

5-Azacytidine (5-Aza) was the first agent able to reactivate HbF synthesis in humans used for the treatment of β -thalassemia [88] and sickle cell disease [89] patients. The mechanism through which 5-Aza stimulates HbF synthesis is unclear. 5-Aza acts both as a cytotoxic anticancer chemotherapy agent and as an inhibitor of DNA methyltransferase enzymes. Given these multiple effects of 5-Aza, two theories have been proposed to explain the stimulatory effect of 5-Aza on HbF synthesis. The first hypothesis implies that the stimulatory effect of 5-Aza on HbF synthesis is related to the capacity of this drug to exert an initial cytotoxic effect on erythroid progenitors and precursors, followed by a compensatory erythroid cell production corresponding to a wave of stress erythropoiesis. This compensatory erythroid response is characterized by the presence of an increased proportion of erythroid elements synthesizing HbF [90]. The second hypothesis is based on the capacity of 5-Aza to inhibit DNA methyltransferase enzymes, resulting in inhibition of DNA methylation [91]: It was supposed that demethylation of DNA in the promoter region of fetal globin genes leads to a transcriptional derepression of these genes [92, 93].

However, a recent study based on the analysis of the effect of 5-Aza on cultures of purified erythroid progenitors showed that 5-Aza induction of HbF is not the result of global DNA methylation or of changes in kinetics of differentiation of erythroid precursors, but involves an alternative, unrecognized mechanism [94]. This conclusion was based on the observation that: doses of 5-Aza inducing HbF synthesis *in vitro* did not alter the growth and differentiation kinetics and agents able to induce reduced promoter methylation, such as small interfering RNA silencing DNA methyltransferases, failed to induce HbF synthesis [94]. Analysis of γ -globin mRNA and protein levels showed that the former one was increased of about 2-fold by 5-Aza, while γ -globin protein was increased of about 10–50-fold over control, thus indicating that post-transcriptional mechanisms play a major role in 5-Aza

induction of HbF synthesis [94]. Other authors have questioned these data arguing that small-interfering RNA treatment may not have reduced γ -globin promoter DNA methylation to sufficient levels to an appropriate stage of erythroid differentiation to increase γ -globin expression [95].

The capacity of 5-azacytidine to stimulate HbF synthesis *in vivo* was demonstrated in 1982 in an anemic baboon [96]. These observations stimulated the development of clinical trials of 5-azacytidine in a small number of sickle cell anemia and β -thalassemia patients. These trials showed that 5-azacytidine treatment elicited in these patients an increase of HbF synthesis and reduced the level of anemia [88, 97, 98].

However, concerns over the carcinogenic potential of 5-Aza [99] hindered its development for human treatment until the safer derivative decitabine (5-aza-2'-deoxycytidine), with low carcinogenic potential, was discovered. Decitabine produces equivalent elevations of HbF in baboons and in patients with sickle cell disease at molar doses 10% to 20% that of 5-azacytidine. The analysis of the effects of decitabine on hemopoietic differentiation provided evidence that this agent favors erythroid/megakaryocytic commitment at the expense of the granulocyte/macrophage lineage [100]. Interestingly, in these studies, 100% of sickle cell disease patients showed an increase in HbF production, including patients who had previously failed to respond to hydroxyurea. SCD patients treated with low-dose decitabine had improved total hemoglobin levels and a decrease in reticulocytes due to less red blood cell hemolysis [101, 102]. The clinical effectiveness of decitabine was recently proven in a phase II study in severe sickle cell disease patients [103]. Increased HbF levels were also observed in an oncologic study combining decitabine and carboplatin in advanced solid tumors [104].

Histone deacetylase inhibitors

Gene expression is controlled by alterations in chromatin structure produced by acetylation and deacetylation of histone tails, resulting in gene activation or repression, respectively. Histone deacetylase (HDAC) enzymes determine deacetylation of histone tails, causing chromatin condensation and repression of transcription. It was shown that histone deacetylase inhibitors bind a central zinc atom in HDAC to produce hyperacetylation of histone H3 and H4 that determines an open chromatin configuration and binding of transcription factors with consequent induction of gene transcription.

The comparative analysis of the histone acetylation profiles of the human β -globin cluster between the fetal and adult stages of development showed that: the LCR was acetylated at the same level in both fetal and adult

erythroblasts, while the acetylation in the globin gene regions correlated to the state of transcription (the promoter in an active globin gene was acetylated to a lower level compared with the coding region); all promoters, irrespective of the state of transcription, were acetylated to the same steady-state levels in both fetal and adult erythroblasts, but, concerning the acetylation levels per histone, the active γ and β promoters are more acetylated than the inactive ϵ promoter [105].

Many experimental evidences have indicated that inhibition of the activity of HDAC causes an increased HbF synthesis. Particularly, inhibitors of HDAC determine the hyperacetylation of ϵ -amino groups of lysine residues of histones [106], and this modification in turn leads to a reduced interaction between core histone proteins and DNA in the promoter region of certain genes determining their transcriptional activation. Several HDAC inhibitors, such as sodium butyrate, adipicin, scriptaid and trichostatin A (TSA), have been shown to induce HbF synthesis *in vitro*; butyrate induced HbF synthesis in humans, and it will be analyzed below in a separate chapter. Particularly, *in vitro* studies carried out both in erythroleukemia cell lines and in primary erythroid cultures have shown that TSA [107, 108], MS-275, and scriptaid [106, 109] and apicidin [110] stimulate HbF synthesis.

However, these molecules do not seem to act directly on globin gene promoters, but their effects seem to be indirect and mediated through induction of the p38 MAPK. Four major MAPK pathways have been characterized: ERK-1/ERK-2; ERK-5/BMK1; c-JUN amino-terminal signal kinases; and p38. Several studies, including analysis of knockout mice and *in vitro* grown erythroid cells, have led to the conclusion that p38 is a major mediator of globin gene regulation [111]. Studies carried out on thicostatin [107] and apicidin [112] have shown that both these agents induce p38 phosphorylation, whose activity is required to induce histone hyperacetylation.

Using computations modeling and screening of a chemical library, novel short-chain fatty acid derivatives have been identified, of which, a few demonstrate higher potency than the prototype butyrate. Particularly, one of these compounds, RB7, exhibited a high capacity to stimulate HbF synthesis in cultured erythroid progenitors [113]. Molecular studies have shown that RB7 induces dissociation of HDAC3 and its adaptor protein NCoR, from the γ -globin gene promoter, thus promoting the coincident and proportions recruitment of RNA polymerase II to this promoter [113].

Analysis of the effectors molecules activated by p38 MAPK has suggested as possible targets CREB1 and ATF-2, whose phosphorylation was increased by Trichostatin A [114]. These activated transcription factors bind to cyclic AMP responsive elements (CRE) present in the promoter

regions of the γ -globin genes and transactivate these genes [114].

Some studies have been carried out using valproic acid (2-propylpentanoic acid), a compound related to short fatty acid derivatives, used in the treatment of seizure disorders in childhood. Clinical studies reported increased HbF levels in patients treated with valproic acid for epilepsy [115, 116], and subsequently, this compound was used in small series of patients with sickle cell anemia [117–119]. However, the stimulatory effect of valproic acid on HbF synthesis was only weak, reflecting the relatively modest HbF inducing potency of valproic acid in erythroid cells in vitro [120, 121]. Recently, valproic acid derivatives have been synthesized exhibiting a higher HbF-inducing activity in vitro, but the activity of these compounds in vivo remains to be evaluated [122].

Recently, two additional histone deacetylase inhibitors, compounds 9 and 24, have been identified, able to stimulate HbF synthesis in erythroid cultures from normal and β -thalassemic patients [123].

Butyrate

Butyrate was shown to promote cell differentiation and to enhance globin gene expression in human erythroleukemia cells [124], to increase embryonic globin gene expression in chicken adult erythroid cells pretreated with 5-azacytidine [125], to cause higher HbF levels at birth in infants born from diabetic mothers (infants of diabetic mothers have a delay in γ to β globin switch after birth associated with elevated plasma α -amino butyric acid levels) [126, 127], to enhance HbF synthesis in cultures of primary adult erythroid progenitors/precursors [128], and to increase HbF synthesis in adult baboons [129, 130]. Furthermore, it was shown that butyrate infusions in the ovine fetus delay the switch from fetal to adult hemoglobin [131].

The mechanism through which butyrate stimulates HbF synthesis in adult erythroid cells remains unclear. Since butyrate is an inhibitor of histone deacetylase, it was proposed that butyrate increases HbF levels by enhancing the transcription rate of the γ -globin gene via changes in histone acetylation at the level of critical promoter regions. Studies of butyrate regulation of γ -globin expression in erythroleukemic K562 cells showed that the regulation occurred at least in part at the transcriptional level because butyrate can increase expression of a γ -globin promoter construct fused to a reporter gene in a transient transfection assay [132]. Subsequent studies have shown that γ -globin gene activation by butyrate and other short-chain fatty acids was dependent on a duplicated CCAAT box sequence, located just upstream of the transcription start site [133, 134].

In vivo footprinting studies performed in erythroblasts of patients exhibiting induction of HbF synthesis by butyrate

treatment revealed alterations in DNA-binding proteins at the level of four regions of the proximal γ -globin gene promoter, designated butyrate response elements γ 1 to 4 (BREG1–4) [135]. Additional studies have shown that stimulatory effect of butyrate on differentiation of erythroleukemic cell lines [136] and on HbF synthesis [114] requires p38 activation. Butyrate stimulated p38 MAPK phosphorylation via a mechanism involving generation of reactive oxygen species (ROS): inhibitors of ROS formation blocked both butyrate-induced p38 phosphorylation and HbF induction [137]. Finally, other studies have suggested that butyrate stimulates HbF synthesis acting also through a different mechanism involving an increased efficiency of translation of γ -globin mRNA [138].

Recently, using primary erythroid cultures derived from normal CD34⁺ cells or from CD34⁺ cells of sickle cell disease patients, the epigenetic changes induced by butyrate at the level of the β -globin locus have been analyzed [139]. This analysis showed that butyrate exposure resulted in a true reversal of the normal developmental switch from γ - to β -globin expression: This is associated with increased histone acetylation and decreased DNA methylation of the γ -globin genes, with opposite changes in the β -globin gene [139].

Given the promising in vitro and in vivo studies on the stimulation effect of butyrate on HbF synthesis and the low toxicity profile of this drug, in 1993, a pharmaceutically suitable preparation of butyrate, Arginine Butyrate, was administered in six patients, three with sickle cell disease and three with β -thalassemia [140]. This initial study provided evidence that Arginine Butyrate infusion rapidly increases HbF production up to levels suitable to ameliorate the clinical condition of these β -hemoglobinopathies.

However, a subsequent study showed that the continuous infusion of Arginine Butyrate did not result in a sustained HbF production and did not improve the hematologic condition of both sickle cell disease and β -thalassemic patients [141].

The decrease in HbF levels observed after prolonged Arginine Butyrate administration was related to the growth-inhibitory activity of this drug on hemopoietic cells, and it was therefore suggested that this drawback could be bypassed through the intermittent administration of the drug. According to this view, Arginine Butyrate was given intermittently for 4 days every 4 weeks: This drug regimen resulted in a sustained HbF production in the majority of sickle cell disease-treated patients [142].

Not all sickle cell disease patients responded to butyrate treatment: Particularly, those exhibiting >2% HbF baseline levels responded to treatment, while those with lower HbF levels were resistant to this treatment [143].

Sodium phenylbutrate is an alternative compound that is absorbed enterally and has been reported to increase HbF levels in patients with sickle cell disease [144]. However,

major limitations of this therapy are that high doses (15–20 g/day) of this drug need to be administered each day to achieve a marked HbF stimulation and that the drug had a short half-life. This compound was tested also at lower dosages (1–11 g/day) in children with sickle cell disease: At these doses, phenylbutyrate induced an increase of HbF synthesis that was, however, not sustained with continuous therapy [145].

Butyrate was assayed in β -thalassemia patients. Given the limited effect of Arginine Butyrate, orally administered butyrate compounds have been tested to treat these patients. Both sodium phenylbutyrate [146] and isobutyramide [147] achieved a significant stimulation of HbF synthesis. Interestingly, hematologic responses to sodium phenylbutyrate occurred only in those patients who had high endogenous erythropoietin levels and was unrelated to any particular pattern of globin gene mutation [146]. Particularly, isobutyramide treatment elicited a significant enhancement of HbF synthesis, increased hemoglobin levels, and reduced transfusion requirements in transfusion-dependent β -thalassemia patients. Importantly, response to treatment was associated with high pretreatment HbF levels (>4.5%) and high parental HbF [147].

Hydroxyurea

Hydroxyurea (HU) is a well-known drug used for treatment of myeloproliferative disorders. HU acts as an agent blocking DNA synthesis through the inhibition of ribonucleoside diphosphate reductase. Ribonucleotide reductase provides the cell with a balanced supply of deoxyribonucleosides triphosphates for DNA synthesis. Mammalian cells possess three non-identical subunits of ribonucleotide reductase: one homodimeric large subunit R1, carrying the catalytic site and two variants of the homodimeric small subunit, R2 and p53-inducible p53R2, each containing a tyrosyl free radical essential for catalysis. In vivo HU is converted to a free radical nitroxide and transported by diffusion into the cells, where it quenches the tyrosyl-free radical of the R2 subunit of ribonucleotide reductase, thus inactivating the enzyme.

Initial studies carried out in 1984 in anemic monkey [148] and in sickle cell anemia patients [149] showed that HU could stimulate HbF production. However, not all sickle cell patients treated with HU showed increases in HbF production [150]. F-cell responsiveness in these patients was related to HbF pre-therapy levels [151].

The studies concerning the analysis of the cellular and molecular mechanisms through which HU enhances HbF production were considerably stimulated by the observation that its enhancing effect on HbF synthesis is observed also in vitro in erythroid cell culture systems [152]. An initial study carried out by Fibach et al. [152], using a two-phase

liquid erythroid cell culture system, showed that HU increased the level of HbF synthesized by erythroblasts, decreased their proliferation and increased cell size and Hb content per cell.

The mechanism through which HU stimulates HbF synthesis has been explored in a large set of studies. Most of these studies suggest that HU exerts its stimulatory effect on HbF synthesis through guanosine 3',5'-cyclic monophosphate (cGMP) signaling pathways. Previous studies have shown soluble guanylyl cyclase (sGC) and cGMP-dependent protein kinase signaling pathways [153]. HU was shown to be able to induce the nitric oxide (NO)-dependent activation of sGC in cultured CD34⁺ human progenitors [154]; on the other hand, HU treatment increases NO and cGMP levels in the blood of patients with sickle cell anemia [155, 156].

Recent study further provided evidence that HU was able to nitrosylate and activate sGC in human erythroid cells: particularly, it was shown that HU can directly interact with the dooxy-heme of sGC by a free-radical nitroxide pathway [157].

Other studies have shown that HU, in addition to cGMP, induces also an increase of cAMP levels [158]. Agents that enhance either cGMP or cAMP levels in primary erythroid cells induce an increase of HbF production [158, 159]. It was suggested that cGMP and cAMP stimulate γ -gene expression, acting through different molecular mechanisms, the former one at post-transcriptional level and the latter one at transcriptional level. A recent study showed a pivotal role of the HU-induced small GTP-binding protein, secretion-associated and RAS-related (SAR) protein in γ -globin induction by HU [160]. This protein exerts its effect by causing cell apoptosis and G1/S phase arrest by reduction of PI3K and ERK phosphorylation with increased p21 and GATA-2 expression [160].

After the initial studies carried out in baboons showing the capacity of HU to induce in vivo HbF synthesis, the efficacy of this drug to enhance HbF synthesis in sickle cell disease was tested in many small clinical trials (reviewed in [161]). After these initial studies, a large multicenter controlled trial was carried out. The Multicenter Study of Hydroxyurea Trial (MSHT) was designed to test the hypothesis that HU will reduce the occurrence of painful crises and episodes of acute chest syndrome in adults with moderate-severe sickle cell disease [162]. The results of this trial provided evidence that HU reduces the frequency of painful crises and episodes of acute chest syndrome and a reduction in transfusion requirements and hospitalizations in adults with sickle cell disease [163, 164]. As a consequence of this placebo-controlled study, HU was approved by the FDA for the treatment of sickle cell disease patients.

Analysis of these patients participating to the MSHT study showed that about 50% of the HU-treated patients had long-term increments of HbF; furthermore, initial HbF levels were not associated with HbF response to treatment [163]. A long-term observational (9 years) follow-up study of mortality in these patients provided several interesting findings: (1) a 40% reduction in mortality was found when individuals were compared based on total duration of hydroxyurea therapy without attention to the original study grouping; and (2) cumulative mortality at 9 years was 28% when HbF levels were lower than 0.5 g/dL after the trial was completed, compared with 15% when HbF levels were 0.5 g/dL or higher [164]. However, as recently pointed out, this observational study had several intrinsic limitations: (1) it is an observational study, not a randomized trial; and (2) study participants self-selected whether they wanted to continue, discontinue, or start hydroxyurea treatment [165].

In addition to this study, another report analyzed the quality of life in the patients participating to the MSH trial. Thus, health quality of life measures were assessed in these patients as a secondary endpoint to determine if the clinical benefit achieved with HU treatment could translate into a measurable benefit perceptible to the patients [166]. The results of this study showed that over 2 years of treatment, the benefit of HU treatment on quality of life parameters, other than pain, was limited to the group of patients taking HU who maintained a high HbF response, compared to those who achieved only a low HbF response or those on placebo [166].

Patients who die while on HU therapy represent a subgroup of older sickle cell anemia patients, with more severe disease and organ damage [167].

In addition to this randomized trial, 12 observational studies that enrolled adults reported a relative increase in fetal hemoglobin of 4% to 20%, a relative reduction in crisis rates by 68% to 84% and a decrease of hospital admission by 18% to 32% reviewed in [168].

Studies have been carried out in children with sickle cell anemia, showing the clinical efficacy and short/middle-term safety of HU [169–172]. In this context, particularly important were the two Hydroxyurea Safety and Organ Toxicity (HUSOFT) studies. These two studies, the original HUSOFT study and the HUSOFT extension study were prospective, multicenter, open-label, single-arm studies designed to determine the toxicities, to assess the hematological effects and to evaluate the effect on splenic function of HU administration to a very young age group of sickle cell disease patients [169, 172]. The HUG-KIDS study, carried out in 68 children and adolescent with sickle cell anemia, showed that hydroxyurea treatment had no adverse effect on height or weight gain or pubertal development [171]. Hydroxyurea was shown also to be able to reduce the incidence of secondary stroke in children with sickle cell

anemia and stroke [173]. The results of these two studies provided evidence that infants with sickle cell anemia tolerate HU therapy (up to 6 years of treatment) with sustained hematologic benefits, improved growth, and possibly preserved splenic function [169, 170]. Importantly, the studies carried out in children showed that HU induces HbF levels to twice the average level achieved in adults [169–172]. Interestingly, in children with sickle cell anemia, higher baseline HbF predicted a better response to HU [172] and higher HbF levels under HU treatment were associated with a better hematological response [173]. Finally, a recent study showed that hydroxyurea treatment decreased transcranial Doppler flow velocities, thus suggesting that this drug may be an effective treatment for the prevention of primary stroke in children with sickle cell anemia [174]. The ensemble of these studies strongly supports the use of hydroxyurea for treatment of children with sickle cell disease [175].

According to all these considerations, hydroxyurea is one of the key therapeutic approaches to sickle cell anemia and is recommended for patients who have frequent pain episodes, history of acute chest syndrome, other severe occlusive events, or severe symptomatic anemia [176]. In these patients, hydroxyurea prolongs survival and improves the quality of life [176].

As mentioned above, the response to HU treatment is heterogeneous for two reasons: first, the majority, but not all patients respond to HU treatment with an increase in HbF; second, among the responders, the magnitude of the HbF response to HU is variable. It was suggested that genetic elements linked to the β -globin gene-like cluster and QTL, involved in the physiologic regulation of HbF levels, could also modulate the response to HU [177]. The analysis of 29 candidate genes within loci reported to be linked to HbF levels was carried out in 137 sickle cell anemia patients treated with HU in the MSH trial showing the existence of single nucleotide polymorphisms in genes within the 6q22.3–23.2 and 8q11–q12 associated with the HbF response to HU [177]. A recent study suggested also a possible role of SNPs present at the level of the promoter of the SAR gene as a determinant in the differential HbF response to HU treatment observed in sickle cell anemia patients [178].

HU is a cytotoxic agent, and some concerns have been raised about its safety, particularly in view of its chronic administration to young sickle cell anemia patients. So far, no malignancies have been reported in adults with sickle cell disease enrolled in the MSH trial or in the 226 adults with sickle cell disease treated at the Medical College of Georgia and followed for 15 years [179]. However, case reports [180] have described the development of acute leukemia in three patients with sickle cell disease receiving HU therapy, two of whom developed leukemia 6 to 8 years

after starting of treatment [180]. Analysis of DNA damage by the alkaline Comet assay in blood leukocytes of individuals with sickle cell disease treated with HU showed that this drug causes DNA damage, the extent of this damage being related to drug dosage and treatment duration. However, a recent National Institutes of Health Consensus Conference concluded that the risk for cancer association with hydroxyurea therapy in sickle cell disease does not appear to be higher than the baseline rate for this patient population.

HU response in β -thalassemia has been extensively studied and, according to the majority of the reports, its administration was safe and able to significantly improve transfusion requirements (Table 1). Early studies have documented only a moderate hemoglobin increase following HU therapy [181–185]. However, in these initial studies, it was remarked the capacity of HU to clearly improve symptomatic cord compression occurring in some β -thalassemic patients as a consequence of paraspinal extramedullary erythropoietic tissue [186–189].

Significant clinical improvements have been obtained using HU in HbE/ β^o -thalassemia patients (Table 1). Thus, in a multicenter trial of 42 patients with HbE/ β^o -thalassemia treated with hydroxyurea (with a starting dose of $7 \text{ mg kg}^{-1} \text{ day}^{-1}$, increased to a maximum dose of $20 \text{ mg kg}^{-1} \text{ day}^{-1}$), about 50% of patients demonstrated a clear increase in steady-state hemoglobin levels [190]. The increase in hemoglobin level induced by HU was mainly due to its capacity to stimulate HbF synthesis [190]. In these patients, pre-therapy HbF levels are the best predictor of response to treatment [190]. Similar observations on smaller series of HbE/ β^o -thalassemia patients have been also reported [191, 192], thus confirming the efficacy of this drug in these populations of β thalassemic patients.

More recent studies have confirmed in various types of β -thalassemia major and β -thalassemia intermedia patients a significant clinical benefit following HU therapy, with elimination of transfusion requirements in a significant proportion of transfusion-dependent β -thalassemic children [193]. These findings have been confirmed in large studies carried out on Iranian, Indian, and Algerian β -thalassemic patients, showing good responses in a significant proportion (i.e., >50%) of β -thalassemic patients, without any major toxicity related to HU administration [194–200].

Two association studies investigated β -globin locus-related SNPs in 136 and 45 β -thalassemia Iranian patients [193, 194], respectively, indicating that the most significant modulating factor involved in good and moderate response to HU was positively correlated with the HBG2-158T allele.

It is of interest to note that the analysis of globin chain synthesis in some β -thalassemia intermedia patients responding to the treatment with HU showed a stimulatory

effect caused by drug treatment at the level of β -globin synthesis and not of γ -globin synthesis [201].

Stem cell factor

Erythropoiesis is regulated by a number of growth factors, among which erythropoietin (Epo) and stem cell factor (SCF), also known as Kit ligand (KL), play an essential, non-redundant function. SCF exerts its biologic effects through interaction with its membrane receptor c-kit. The essential role of SCF and c-kit in erythroid cell development is supported by studies carried out utilizing White Spotting and Steel mutant mice [202, 203]. These mice exhibit erythroid and other lineage-associated defects (macrocytic hypoplastic anemia, reduction in pigmented cells, mast cell deficiency, and sterility) due to inherited mutations within the c-kit and SCF genes, respectively [202, 203]. Particularly, mice that lack the expression of c-kit (the c-kit white spotting mutant, c-kit^w, encodes a shortened protein lacking the transmembrane portion, which therefore, fails to be expressed on the cell surface) exhibit a severe reduction of CFU-E number in the fetal liver and die of anemia around day 16 of gestation [202]. Importantly, the transgenic expression of Epo can overcome the lethality caused by the c-kit^{w/w} mutation: In the mutant mice rescued by Epo, CFU-Es are rescued to normal frequencies [204]. This important observation suggests that EpoR signals can partially bypass the strict requirement for c-kit signaling in erythropoiesis in the absence of c-kit *in vivo* [204].

Two isoforms of SCF are generated from a single gene through mRNA splicing: A glycoprotein of 248 aminoacids is rapidly cleaved from the cell to release a soluble active protein of 164 amino acids; a glycoprotein of 220 amino acids, which lacks the proteolysis cleavage site, encoded by differentially spliced exon 6 sequences, remains predominantly anchored to the cell membrane [205]. Mutant mice that selectively lack the expression of membrane-associated SCF show macrocytic anemia, in spite of the presence of secreted SCF [206].

The biologic response to erythroid cells to SCF is markedly enhanced when this growth factor is supplied in combination with Epo. The marked synergy between SCF and Epo is supported by a large number of *in vitro* studies. In fact, clonogenic studies have shown an increase in erythroid colony formation (with an increase of both colony number and colony size) co-administering SCF and Epo. On the other hand, studies in liquid suspension cultures of CD34⁺ cells grown under erythroid cell culture conditions have shown a marked enhancement in the output of the erythroid cell progeny, associated with delayed erythroid maturation. The marked amplificatory effect of SCF on erythroid cell progeny was not due to amplification of the

Table 1 Clinical trials for the treatment of β -thalassemia based on the use of hydroxyurea

Genotype/ phenotype	Number of patients	Percent of responding patients	HU doses	References
β -thal intermedia	5	80% (2 patients showed a good response with a Hb total increase of about 3 g/dL; ; 2 had a moderate response and 1 did not respond to treatment. Response was related to HbF increase.	3–10 mg kg ⁻¹ day ⁻¹	Hoppe et al. [184]
β -thal intermedia	7	43% (only 1 out 7 patients showed a substantial increase of Hb level, leading to transfusion withdrawal).	10–20 mg kg ⁻¹ day ⁻¹	De Paula et al. [185]
β -thal major	4	25% (only 1 out 4 patients showed an increase of Hb level of 1.3 g/dL).	10–20 mg kg ⁻¹ day ⁻¹	De Paula et al. [185]
β -thal intermedia	2	100% [marked increase (about 4 g/dL) of total Hb content, associated with marked improvement of the clinical condition. Pediatric patients.	15–20 mg kg ⁻¹ day ⁻¹	Bradai et al. [193]
β -thal major	5	100% [marked increase (about 4 g/dL) of total Hb content, associated with marked improvement of the clinical condition]. Pediatric patients.	15–20 mg kg ⁻¹ day ⁻¹	Bradai et al. [193]
HbE/ β^o -thal	13	92% (the increase of total Hb level was modest, <1 g/dL; 33% increase of HbF level; improvement of α /non- α globin ratio).	10–20 mg kg ⁻¹ day ⁻¹	Fucharoen et al. [191]
β -thal major	36	69% (responding patients became transfusion-free).	20 mg kg ⁻¹ day ⁻¹	Alebouyeh et al. [195]
β -thal major	133	84% [61% patients exhibited a good response (transfusion-free condition); 23% a moderate response (decrease of the transfusion frequency from 1 to 6 months)].	10–15 mg kg ⁻¹ day ⁻¹	Yavarian et al. [194]
β -thal intermedia	37	70% (46% of the patients displayed a good response with a rise of total Hb content of about 2 g/dL and a marked decrease of transfusion frequency).	10–15 mg kg ⁻¹ day ⁻¹	Dixit et al. [196]
β -thal intermedia	163	91.5% (83 of 106 transfusion-dependent patients became completely transfusion-free; 17/43 patients on long-interval transfusions became transfusion-free).	8–12 mg kg ⁻¹ day ⁻¹	Karimi et al. [197]
HbE/ β^o -thal	45	100% (40% of patients displayed a good response with an increase of total Hb of about 1.3 g/dL; 13 of these patients remained transfusion independent during long-term follow-up).	7–20 mg kg ⁻¹ day ⁻¹	Singer et al. [190]
β -thal major	45	64.5% [44.5% patients displayed a good response (>70% decrease of transfusion rate); 20% a partial response (40–70% decrease of transfusion rate).	15–20 mg kg ⁻¹ day ⁻¹	Bradai et al. [199]
β -thal major	11	82% (responding patients became transfusion-free).	11 \pm 3 mg kg ⁻¹ day ⁻¹	Koren et al. [198]
β -thal intermedia	7	100% (all patients became transfusion-free).	11 \pm 3 mg kg ⁻¹ day ⁻¹	Koren et al. [198]
β -thal intermedia	18	61% (responding patients had a total Hb increase of >1.5 g/dL).	5–30 mg kg ⁻¹ day ⁻¹	Mancuso et al. [245]
HbE/ β -thal	13	77% (responding patients showed a clear increase of HbF content).	5–10 mg kg ⁻¹ day ⁻¹	Watanapokasin et al. [192]
β -thal intermedia	9	90% (all these patients displayed a good response)	15–20 mg kg ⁻¹ day ⁻¹	Bradai et al. [199]
β -thal major	20	80% (reduction of transfusion requirement)	16 mg kg ⁻¹ day ⁻¹	Ansari et al. [200]

erythroid progenitor compartment but to an amplification of early erythroid precursors.

The synergistic effect of SCF and Epo in sustaining erythropoiesis implies some mechanisms of cooperation between c-kit and Epo-R. A large number of in vitro studies have suggested a major role for Stat5, Src family kinases and MAP kinases (ERK-1/ERK-2) in the mechanism of cooperation between c-kit and EpoR (reviewed in [207]. In addition to these effects, SCF potentiates the antiapoptotic effects induced by Epo in erythroid cells through a mechanism mainly involving upmodulation of the antiapoptotic proteins Bcl-X_L and Bcl-2 [208].

Given these pronounced effects of SCF on erythropoiesis, it seemed important to evaluate a possible effect of

this cytokine on HbF synthesis. The majority of these studies have been carried out by investigating in vitro the effect of SCF on HbF synthesis in cultures of adult erythroid cells.

The analysis of the effect of cytokines on HbF synthesis in cultures of erythroid progenitors requires a stringent methodology, involving the use of purified hemopoietic progenitors (usually human CD34⁺ cells purified either from bone marrow or peripheral blood) and of serum-free conditions [209–212]. In fact, fetal calf serum, routinely used for culture of human hemopoietic cells, contains factors able to stimulate HbF synthesis [209–212]. Furthermore, accessory cells (i.e., not-CD34⁺ cells present in cultures of unpurified hemopoietic progenitors) release

endogenous cytokines and may affect or modify the effect of exogenous cytokines on HbF synthesis.

In an initial study, Miller et al. observed a clear stimulation of HbF synthesis induced by SCF in normal (from 0.5% to 6%) and sickle cell anemia (from 4 to 6.8%) BFU-Es grown under serum-free conditions [213]. These findings were confirmed and extended by Peschle et al. using purified hemopoietic progenitors grown under serum-free conditions, either in semisolid medium to generate BFU-E or in liquid suspension cultures allowing the selective growth of erythroid cells [214]. Using these culture conditions, it was observed that SCF at optimal concentrations (i.e., 100 ng/ml) elicited a marked enhancement of HbF synthesis levels up to 20% [214]. The stimulatory effect of SCF on HbF production could be mediated either by an effect on the BFU-E HbF synthesis program or by the recruitment of BFU-Es with an elevated HbF production potential. Experiments carried out on single BFU-Es provided evidence in favor of a direct, dose-related stimulatory effect of SCF on HbF synthesis reactivation [215]. In this same study, it was shown that SCF was able to synergize with sodium butyrate to enhance HbF synthesis: In fact, the combined addition of the two agents together induced HbF synthesis levels up to 40–43% in normal adult erythroid cells [215].

Wojda et al. [216] comparatively analyzed 12 cytokines able to act on erythropoiesis and confirmed that, among them, only SCF was able to significantly enhance HbF synthesis. The capacity of SCF to induce HbF synthesis in adult erythroid cells was correlated to the presence of c-kit on erythroid progenitors/precursors: in fact, the delayed addition of SCF to erythroid cultures was active in inducing a marked enhancement of HbF synthesis at the condition that was added on erythroid cell progeny c-kit⁺ [216]. Other subsequent studies have shown the possible synergistic effect between SCF and other agents to stimulate HbF synthesis. Two molecules, transforming growth factor- β (TGF- β) and corticosteroids, potentiate the effect of SCF on HbF synthesis. Thus, despite their opposite effects on erythroid cell growth, SCF and TGF- β had synergistic effects with respect to HbF synthesis [217]. In erythroid cultures supplemented with Epo+SCF+TGF- β , about a 40% level of HbF synthesis was observed, with a pancellular distribution of HbF in erythroid cells [217]. The analysis of the effects of glucocorticoids on HbF synthesis in cooperation with SCF was prompted from studies showing that corticosteroids, such as dexamethasone, cooperate with Epo and SCF to enhance and sustain the proliferation of erythroid progenitors [218]. Dexamethasone when added together with Epo and SCF enhanced HbF synthesis up to 55%; furthermore, analysis of erythroid cultures of sibling BFU-Es showed that the stimulatory effect of dexamethasone+SCF was related to

the modulation of γ -globin expression rather than to recruitment of BFU-Es with elevated HbF synthetic potential [219].

The effect of SCF was recently investigated in cultures of β -thalassemic erythroid cells. In erythroid cultures of β -thalassemic patients, addition of SCF (in the presence of Epo) remarkably stimulated cell proliferation (3–4 logs over control cultures), decreased the percentage of apoptotic and dyserythropoietic cells, and markedly increased γ -globin synthesis, reaching levels 3-fold higher than in control cultures (from 27% to 81%) [220]. These studies indicate that in β -thalassemia, SCF induces an expansion of effective erythropoiesis and the reactivation of HbF synthesis up to levels observed in fetal levels and may hence considered as a therapeutic agent for this disease [220].

The mechanism through which SCF stimulates HbF synthesis in adult erythroid cells is largely unknown. It is evident that the mechanism of HbF induction by SCF must be related to one or several signaling pathways induced by the interaction of this cytokine with its receptor c-kit. In this context, Bhanu et al. [221] evaluated the effect of inhibitors of various signaling pathways, including JAK2, PKC, PI3K, p38MAPK, guanylate cyclase, and MEK inhibitors. Only MEK inhibitors elicited a pronounced inhibitory effect on HbF synthesis, thus indicating that phosphorylation of ERK-1/ERK-2 MAPK plays an essential role in the mechanisms through which SCF activates γ -globin gene expression in adult erythroid cells. Other studies have suggested a possible role of some transcription factors whose expression is induced by SCF, such as Id2, Tal-1, and FKLf, in mediating the transcriptional activation of the human γ -globin gene [215, 219]. These *in vitro* studies with SCF must be extended to *in vivo* studies to evaluate optimal *in vivo* doses, schedules of administration, and side effects. In this context, SCF and Epo in combination have been administered to baboons, achieving up to 20% HbF levels [222]. Preclinical studies in primates and mice suggested that SCF occasionally causes significant allergic side effects, related to induction of mastocytosis [223, 224]. This was confirmed by subsequent clinical trials in HIV and cancer patients [225, 226], aplastic anemia [226], cord blood transplantation [227], and postablative chemotherapy [228, 229]: specifically, mild allergic reactions (e.g., pruritus, urticaria, and cutaneous angioedema) were reported in a minority of patients, while severe ones (e.g., laryngospasm) were observed only in exceptional cases. In the most extensive phase 3 trial, SCF was used for stem cell mobilization: Patients received recombinant SCF at 20 $\mu\text{g kg}^{-1} \text{ day}^{-1}$ for five consecutive days and three of them reported systemic allergic reactions, resolved after treatment with steroids [230]. The potential use of SCF for treatment of β -thalassemic or sickle cell anemia patients

implies a chronic administration protocol, based on sequential intermittent therapy cycles: This protocol may amplify the potential risk of allergic reactions and must be considered cautiously in view of these possible side effects.

It is of interest to note that recent studies have involved the SCF/c-kit system in the mechanism of physiologic hemoglobin switching. First, it was observed that the level of c-kit and SCF expression is particularly elevated during embryonic/fetal life at the level of tissues involved in hematopoiesis (AGM region and fetal liver) [231]. Second, it was reported that cord blood serum contains clearly higher SCF levels than adult serum, a phenomenon seemingly related to the capacity of human umbilical vein endothelial cells to release large amounts of SCF [232–234]. Third, c-kit expression on cord blood CD34⁺ cells gradually declines: This decrease is directly related to the decline of HbF content. This decline of c-kit expression is paralleled by a concomitant gradual decrease of SCF levels (Testa U et al., unpublished observations). Altogether, these observations suggest that a decline of kit activity could play a role in the mechanism of perinatal HbF→HbA hemoglobin switching. In line with this hypothesis, addition of SCF in cord blood BFU-E cultures reactivates HbF production in a dose-dependent fashion, almost up to the pre-switch levels (Testa U et al., unpublished observations).

Other compounds

In addition to the compounds mentioned above, other molecules have been shown to stimulate HbF synthesis. The mechanism of action of many of these drugs was largely unknown.

Thalidomide, a synthetic glutamic acid derivative initially prescribed as a sedative and anti-nausea medicine, withdrawn from the market for its teratogenic activity and then readmitted as a drug for its immunomodulatory, anti-inflammatory, and antiangiogenic activity, was recently shown to activate HbF synthesis *in vitro* [235]. This conclusion was supported through the analysis of the effect of thalidomide on CD34⁺ cells induced to erythroid differentiation [235]. Preliminary evidence suggests that thalidomide induces increased expression of the γ -globin gene via ROS-dependent activation of the p38 MAPK signaling pathway and histone H4 acetylation [235].

Lenalidomide and pomalidomide are members of a class of immunomodulators used as anti-cancer agents. Particularly, lenalidomide reduces the need for transfusions in some patients with anemic myelodysplastic syndrome. Both these drugs are able to induce HbF synthesis in healthy and sickle cell disease CD34⁺-derived erythroblasts by regulating the transcription of γ - and β -globin genes [236]. Furthermore, when combined with hydroxyurea, pomalido-

midomide and, to a lesser extent, lenalidomide were found to have synergistic effects on HbF upregulation [236].

Conclusions and future directions

The experimental and clinical observations summarized above support the idea that agents enhancing HbF synthesis represent a rational approach for the treatment of sickle cell anemia and β -thalassemia. Although many studies showed clinical efficacy of some HbF inducers in patients with hemoglobin disorders, the impact of these new therapies on the natural history of sickle cell anemia was clear, but of only limited impact in β -thalassemia patients. The discrepancy observed in these two different disorders in the response to HbF inducers may be related to the higher level of HbF required in β -thalassemia to achieve clinical results compared to sickle cell anemia. Furthermore, these studies showed that only a part of the treated patients responded to these new drugs with increased HbF production, due to individual genetic determinants influencing HbF, unrelated to the mutated hemoglobin genes.

The limited/moderate effect of the available HbF inducers on clinical course of β -thalassemia is mainly related to the moderate capacity of these agents to stimulate HbF synthesis *in vivo*. Therefore, better agents that can induce higher levels of HbF are clearly needed. In this context, SCF seems to be a very promising HbF inducer for its capacity to greatly enhance HbF synthesis and to inhibit apoptosis of β -thalassemic erythroblasts. However, the toxicity concerns related to the use of this cytokine remain to be bypassed. Alternatively, the combination therapy based on two or more drugs, stimulating HbF synthesis through different complementary mechanisms, could be used for a strategy to achieve higher levels of HbF synthesis *in vivo*, sufficient to make the patients transfusion-free (i.e., two HbF inducers or an HbF inducer administered together with erythropoietin) [237].

It is important to note that therapy of hemoglobinopathies based on the administration of agents stimulating HbF synthesis cannot be curative and needs a chronic administration. The only curative treatments for hemoglobinopathies are based on hemopoietic stem cell transplantation or gene therapy. Hemopoietic stem cell transplantation is the only approach so far, that may lead to a definitive care for β -thalassemia. However, the use of hemopoietic stem cell transplantation as a routine treatment for β -thalassemia is considerably limited by the shortage of suitable HLA-compatible bone marrow donors (reviewed in [238]). Furthermore, bone marrow transplantation from an HLA-identical familial donor is associated with a 5% mortality rate; this mortality rate is higher (about 20%) for bone marrow transplantation from non-related HLA-matched

donors. Cord blood may be used as an alternative source of hematopoietic stem cells for curative treatment of hemoglobinopathies but had the same limitations described for bone marrow (reviewed in [239]).

Recent studies have created a great excitement showing that fully differentiated somatic cells (such as skin fibroblasts) can be reprogrammed to make cells similar to embryonic stem cells called induced pluripotent stem cells [240]. Hanna et al. have used these cells to correct a mouse model of sickle cell disease, thus opening the way to the eventual use of these cells to cure hemoglobinopathies [241]. Although this study offers an exciting advancement for the potential of pluripotent stem cells for the treatment of hemoglobinopathies, many problems remain to be solved before induced pluripotent stem cells could be used in human trials; particularly, the induced hemopoietic stem cells are not the equivalent of naturally occurring repopulating stem cells: in fact, they cannot restore all hemopoietic lineages [242].

The only other possible curative approach for β -thalassemia and sickle cell disease is represented by gene therapy. Hematopoietic stem cell-targeted gene therapy using replication-incompetent viral vectors holds promise for the treatment of hemoglobinopathies. During these last years, success has been obtained in a series of gene therapy trials for primary immunodeficiencies. However, in the setting of hemoglobinopathies, significantly higher levels of stem cell gene transfer are required to achieve clinical success [243, 244]. Clinical trials of gene therapy for hemoglobinopathies are in an initial phase of development, and there are evidences to believe that many years are required before a successful clinical trial for these disorders will be achieved.

It is therefore evident that at the moment (and in the near future), curative therapies of hemoglobinopathies have a limited impact because they may be used in a limited number of patients and/or because they still need to be developed in a clinically active approach. In this situation, it is important to offer to the clinician's therapeutic strategies an alternative to transfusional support and iron chelation. In this context, drugs able to stimulate HbF synthesis certainly represent an important therapeutic approach.

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