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Epigenetic regulation of hemoglobin switching in non-human primates ☆

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ABSTRACT

Human hemoglobin switching describes the highly regulated, sequential expression of the 5 β-like globin genes (HBE, HBG2, HBG1, HBD and HBB) of the human β-globin gene complex. The sequential activation of these β or β-like globin genes during human development from early embryonic through late fetal ('adult') stages, and during erythroid maturation, occurs in an order corresponding to their 5′ to 3′ location on chromosome 11. The β-hemoglobinopathies are the most common inherited diseases in humanity, and are diseases of mutated HBB or its altered regulation. Since the other β-like globin genes can potentially substitute for defective HBB, much translational research is directed toward understanding and manipulating sequential activation at the human β-globin gene complex to treat β-hemoglobinopathies. Non-human primates provide a vital contribution to such efforts because of their recapitulation of the developmental/maturational switch in hemoglobin production as observed in humans (mice do not model this switch). Valuable insights into druggable epigenetic forces that mediate the switch have been thereby gained. We review important lessons learned in non-human primates, complemented by other studies, and suggest rational next steps.

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Introduction

Human hemoglobin switching describes the highly regulated expression of the 5 β-like globin genes (*HBE*, *HBG2*, *HBG1*, *HBD*, and *HBB*, that produce ε, γ, δ, β globin chains respectively) of the human β-globin gene complex. The activation of these β or β-like globin genes occurs sequentially, in an order corresponding to their 5' to 3' location on chromosome 11 (*HBE-HBG2- HBG1- HBD- HBB*), during human development from early embryonic through to late fetal ('adult') stages. Sickle cell disease (SCD) is an inherited blood disorder caused by a point mutation in the adult β-globin gene (*HBB*), resulting in an amino acid substitution that favors polymerization of deoxygenated sickle hemoglobin (HbS) molecules within the red blood cell (RBC). SCD significantly decreases quality of life and increases mortality of approximately 100,000 people in the United States and millions worldwide. Clinical observations showed that SCD patients who co-inherit elevated levels of fetal hemoglobin (HbF;α_2 γ_2) have reduced disease severity. Basic laboratory investigations revealed that the mechanism was inhibition by HbF of deoxygenated HbS polymerization. In β-thalassemias, increased expression of the γ-globin chain (HbF) can correct α/β hemoglobin chain imbalance, to also alleviate symptoms of these diseases. Thus, there are strong justifications for studies of the regulation of developmental globin gene switching that could lead to in vivo strategies to increase HbF levels, to treat both SCD and β-thalassemias [1].

Nonhuman primates provide a vital contribution to such efforts because of their recapitulation of the developmental switch in hemoglobin production as observed in humans [1]. Valuable insights into the epigenetic forces that mediate this switch, and into pharmacologic methods to manipulate this switch for therapeutic purposes, have been thereby gained. We review lessons learned, and suggest rational next steps.

Development of the baboon model

The early search for an effective method to increase HbF for the therapy of SCD and β-thalassemia was severely hampered by the lack of a suitable animal model to study the regulation of γ-globin gene expression and test possible strategies to increase HbF. This limitation was successfully addressed by studies showing that expression of HbF in the baboon was developmentally regulated in a manner identical to humans [2]. Early investigations of HbF regulation in baboons revealed 3 key conclusions: (1) HbF could be increased in adult animals by erythropoietic stress induced by phenylhydrazine hemolysis, phlebotomy, or hypobaric hypoxia and

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Table 1
Baboon γ-globin chain expression [7,8,12,18].

Sample	Condition	$\gamma/\gamma+\beta$	Iγ/Vγ chain ratio
Fetal Cord Blood ($n=2$)	58d gestation	0.819	1.85
Adult baboon ($n=3$)	Normal nonanemic	0.04+0.01	ND
Adult baboon ($n=3$)	Anemic, phlebotomized	0.07+2.77	0.65+0.10
CD34+ BM	14d Methylcellulose culture	0.57+0.09	ND
CD34+ BM ($n=6$)	14d Liquid culture	0.47+9.44	1.69+0.23
CD34+ BM	14d AFT024 co-culture	0.06+0.04	ND
Adult baboon ($n=5$)	Decitabine (anemic)	0.52+5.8	0.75+0.32
Adult baboon ($n=3$)	Decitabine (normal nonanemic)	0.41+0.15	ND
Adult baboon	RN-1 (2.5mg/kg/d; 3d; anemic)	0.78	2.62
Adult baboon	RN-1 ((0.5mg/kg/d; 3d; anemic)	0.68	2.75
Adult baboon	RN-1 (0.25mg/kg/d; anemic)	0.49	1.18
Adult baboon ($n=5$)	RN-1 (0.25mg/kg/d; normal nonanemic)	0.27+0.02	4.63, 20

the level of HbF induced was directly related to the degree of anemia; (2) the level of HbF induction was genetically determined in a species-related manner among baboons; and (3) the level of HbF induction was dependent upon age [3–5]. Although these studies clearly established that HbF levels could be experimentally manipulated in vivo in adult baboons, it remained unclear how these results could be translated into a treatment that would increase HbF levels for the therapy of SCD and β-thalassemia patients.

Although the structure of the β-globin gene complex and the pattern of developmental regulation of the β-like globin gene are now known to be conserved among all Catarrhine species (Old World Monkeys) [6], the bulk of experimental studies have been performed using baboons. The 2 baboon γ-globin genes can be differentiated by an amino acid difference at aa75 where the 5' gene contains isoleucine while the 3' gene contains valine. The ratio of expression of the Iγ and Vγ-globin genes varies with development, with the 5' Iγ-globin gene expressed at higher levels in the fetal period and the 3' Vγ-gene predominating in adults [5,7], thus mirroring the developmental pattern of expression of the human Gγ- and Aγ-globin genes. Alterations in the Iγ/Vγ-globin chain ratio that shift it toward that characteristic of fetal development are observed in cultured adult-derived CD34+ BM cells [7] and adult baboons treated with HbF inducing drugs decitabine [5] and the RN-1 [8] (Table 1) that likely reflect effects on erythroid differentiation.

An advantage of the baboon model for studies of globin gene regulation is the ability to obtain and purify large number of erythroid cells highly enriched for varying stages of erythroid differentiation from bone marrow aspirates of adult animals and also from fetal liver and yolk sac of fetuses of precise gestational ages from timed matings. Using commercially available baboon-specific antibodies and antibodies to human hematopoietic cells that are cross-reactive with baboons, cells representative of specific stages of erythroid differentiation, including CFUe and BFUe, can be highly purified using immunomagnetic column methods in combination with FACS. Using purified erythroid cells, analysis have been performed to characterize changes in epigenetic modifications during development [9,10] and following treatment with HbF-inducing drugs (Fig. 1) [8,11].

Although the baboon is an excellent in vivo model for studies of globin gene regulation and pharmacological HbF induction, experiments seeking to use in vitro cultures of baboon CD34+ BM cells induced to differentiate along the erythroid pathway as screening assays to identify potential HbF-inducing drugs were limited by high levels of HbF expression in these cultures. High HbF expression persists in baboon CD34+ cultures even when culture conditions are employed that result in low adult-stage level expression in human CD34+ cells. However, co-culture of baboon CD34+ BM cells with the mouse fetal liver AFT024 stromal cell line reduced HbF expression to low, near physiological, adult-stage levels [12] (Table 1). Differences in γ-globin expression between CD34+ cells grown in liquid cultures and stomal cell line co-cultures were associated with differences in the rate of erythroid differentiation and γ-globin promoter DNA methylation. The use of the AFT024 stromal cell line co-culture system has allowed the testing of new pharmacological HbF inducers in a more convenient and easily manipulated system prior to testing using the in vivo model [9].

DNMT1 inhibitors

Interrogation of the DNA methylation status of the globin genes in erythroid and non-erythroid tissues of varying developmental stages was initially performed using methylation-sensitive restriction enzymes in combination with Southern blot analysis. These studies provided the initial evidence that cytosines in the context of CpG residues within promoter region of the γ-globin gene were not methylated when the gene was highly expressed in fetal liver but were highly methylated when the gene was inactive in adult bone marrow. These results suggested the hypothesis that decreasing the level of cytosine methylation would increase γ-globin expression in adults [13]. This first direct test of this hypothesis in vivo was performed by treatment of anemic baboons with the pharmacological inhibitor of DNA methyltransferase, 5-azacytidine (5-aza). In this experiment baboons were initially phlebotomized for an extended period to induce a level of anemia and reticulocytosis similar to that of SCD patients and establish a baseline, stable HbF level. This was followed by administration of a 2-week course of 5-aza while the degree of anemia was maintained by periodic phlebotomy. The results were dramatic, with high levels of HbF induced in the treated baboons. Indeed, the level of HbF induction was so high that it constituted the majority of the total Hb, completely reversing developmental silencing of the γ-globin gene and thus inducing a "reverse switch" [14]. These experiments, the first that demonstrated that any drug could increase HbF expression, initiated a new experimental field of investigation of pharmacological therapies to increase HbF. These results in baboons were rapidly and successfully translated in clinical studies in both β-thalassemia and SCD patents at the NIH [15]. While 5-aza was clearly a very powerful activator of HbF in vivo, controversy regarding its mechanism of action soon ensued. The debate focused on the relative roles of the contributions of erythropoietic stress due to the hematological toxicity of 5-aza vs direct effects of the drug on DNA methylation in the mechanism of HbF induction. Other known cytotoxic drugs such as hydroxyurea and cytosine arabinoside were tested in non-human primates and subsequently shown to increase HbF [16]. To directly address this issue, experiments were performed comparing the effects of 5-aza, HU, cytosine arabinoside and phenylhydrazine in baboons. These experiments showed that 5-aza produced elevations of HbF 2-3 times higher than the other treatments. Moreover, HbF induction by

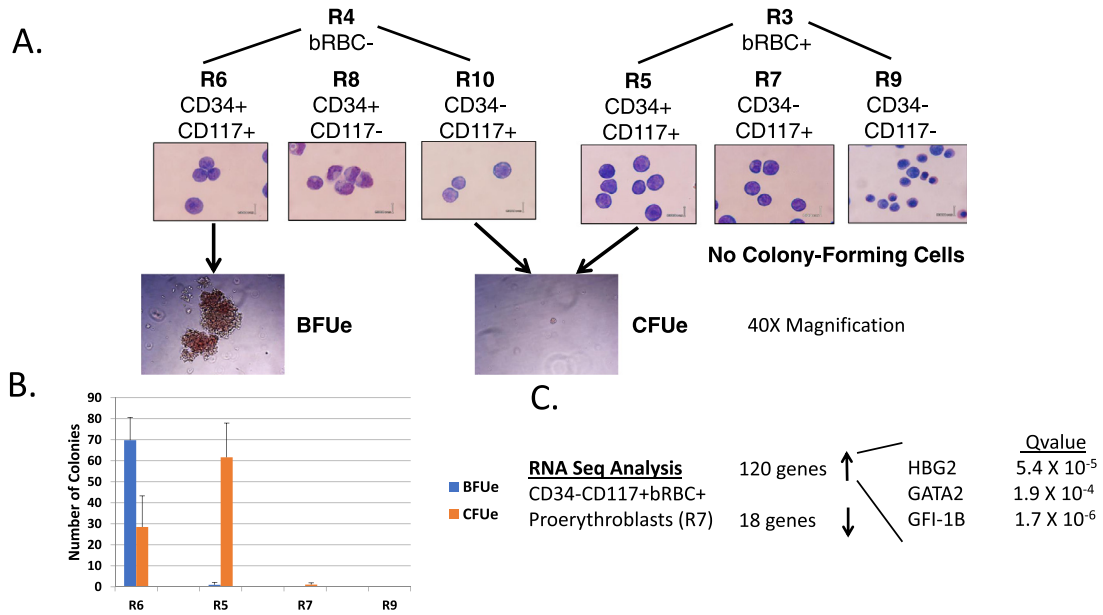

Fig. 1. A. Wright's stained erythroid subpopulations purified by immunomagnetic column enrichment and FACS from baboon BM aspirates. CD105+ (clone SN6) cells were enriched by immunomagnetic column chromatography followed by FACS purification using antibodies to CD34 (clone 563), CD117 (clone 104D2), and bRBC (clone E34-731). B. Methylcellulose colony assays showing enrichment of BFUe and CFUe colonies in FACS-sorted subpopulations. C. RNA seq analysis of the R7 FACs-purified proerythroblast subpopulation showed increased expression of 120 genes in RN-1 treated baboons. Among the genes exhibiting increased expression were HBG2, GATA2, and GFI-1B.

5-aza was not dependent on erythropoietic toxicity but was clearly associated with loss of DNA methylation of the γ-globin promoter region and throughout the genome [17]. High levels of γ-globin synthesis expression in normal, nonanemic baboons treated with DNMT1 inhibitors supported the hypothesis that HbF induction by DNMT inhibitors was not dependent on the anemia and erythropoietic stress produced [18,19]. Although 5-aza was recognized as the most powerful HbF inducing drug, HU was ultimately chosen for study in a large multi-center clinical trial to examine the effects of pharmacological induction of HbF in SCD due to unfounded fears of the possible carcinogenic effects of 5-aza. [15]

Although HU was the first FDA-approved drug to treat SCD, this drug did not induce significant increases in HbF in a large fraction of patients. Additional clinical studies to assess the effect of decitabine, the deoxy analog of 5-aza, in HU-refractory SCD patients showed that decitabine increased HbF to high levels [20]. Dose-limiting effects on platelets and neutropenia were observed in this study that were attributed to effects on hematopoietic differentiation rather than cytotoxicity.

Efforts were made to develop an oral decitabine therapy that would more easily allow chronic dosing and increase compliance. While low doses of decitabine effectively induced high levels of HbF when administered subcutaneously or intravenously, oral administration required high doses of drug (17-34 times the optimal subcutaneous dose) to achieve similar levels of HbF [21]. The inability of decitabine to be an effective oral drug is due to its rapid degradation by cytidine deaminase in the intestine and liver. Tetrahydrouridine (THU) is an effective inhibitor of cytidine deaminase. To better evaluate and enhance the translational development of this combination for oral therapy, the effect of THU on pharmacokinetics and pharmacodynamics of orally administered decitabine was investigated in baboons [22]. THU increased decitabine absorption time and widened the concentration time profile without high peak decitabine levels that caused DNA damage and cytotoxicity and decreased the interindividual variability in pharmacokinetics seen with decitabine alone. An oral THU-decitabine combination that produced a peak decitabine concentration of ~0.2 μM administered 2X/week for 8 weeks to baboons resulted in DNA hypomethylation of the γ-globin gene promoter and produced large increases in HbF. These experiments stimulated a clinical trial of an oral administration of the THU-decitabine combination in SCD patients. In patients treated at the highest decitabine dose (0.16mg/kg) in combination with THU, plasma concentration of decitabine peaked at 50nM and remained elevated for several hours. DNMT1 protein was decreased 75% in peripheral blood mononuclear cells, DNA methylation levels decreased 10%, and HbF increased 4% to 9% with substantial increases in F cells and total hemoglobin. Platelets increased and neutrophils decreased but remained within levels that did not require dose modification or treatment holds [23]. The lack of myelotoxicity in patients treated in this study is likely due to inhibition of conversion of decitabine to deoxyuridine by THU, a major mechanism of decitabine cytotoxicity. Therefore, the oral THU-decitabine therapy that had been initially optimized in baboons was shown to increase HbF a clinical trial in SCD patients with minimal hematopoietic toxicity suggesting that further studies to evaluate clinical efficacy be performed.

DNA methylation and co-repressor complexes

Phylogenetic footprinting studies have also provided additional powerful evidence that DNA methylation of CpG residues within the γ-globin gene promoter is involved in the mechanism of adult stage γ-globin repression [24]. These studies identified CpG residues within the γ-globin promoter region that are conserved in all Catarrhine simian primates where the gene is expressed throughout the fetal developmental stage. In prosimian primates such as *Galago crassicaudatus,* the γ-globin gene is expressed only in the embryonic stage in a manner similar to ε-globin and the CpG residues conserved in simians are absent from its promoter region. During primate evolution, the γ-globin gene evolved to a pattern of fetal-stage expression by escaping silencing at the end of the embryonic stage allowing continued expression throughout the fetal stage. This implies that a new mechanism evolved to silence its expression during transition to the adult stage. The appearance of highly conserved CpG residues within the γ-globin promoter co-

incided with evolutionary recruitment of the γ-globin gene to fetal stage-specific expression. Cytosine methylation targeted to these residues at the adult stage, repressing γ-globin gene expression, could thus provide the new mechanism of adult stage repression. To investigate this hypothesis, the level of epigenetic modifications of the globin gene promoters in highly purified erythroid cells of yolk sac, fetal liver, and bone marrow of baboon fetuses of known gestational ages obtained from timed matings at precise developmental stages was analyzed [9]. Bisulfite sequence analysis showed that DNA hypomethylation γ-globin promoters occurred during in yolk sac and fetal liver, corresponding with high levels of γ-globin expression, with rapid DNA methylation in late fetal stage bone marrow precisely at the time corresponding to γ-globin gene silencing and increasing β-globin expression. Additional studies of purified adult baboon BM and fetal liver cells of different stages of erythroid differentiation showed the presence of high levels of 5-hydroxymethylcytosine, an intermediate in the Tet-mediated DNA demethylation pathway, suggesting that active Tet-mediated DNA demethylation resulted in DNA hypomethylation of the γ-globin promoter during fetal liver erythroid differentiation, but that this process of demethylation and DNA hypomethylation was not sustained during adult erythroid differentiation. In vitro experiments in cultured baboon BM erythroid progenitors showed that the addition of Vitamin C, a Tet co-factor, increased γ-globin expression and decreased γ-globin promoter DNA methylation [11]. These experiments supported the hypothesis that DNA methylation directly repressed γ-globin gene expression in adult erythroid cells and that Tet-mediated DNA demethylation was involved in DNA hypomethylation of the γ-globin promoter and high-level expression of the γ-globin gene in fetal liver erythroid cells.

The recruitment of multi-protein co-repressor complexes targeted to the γ-globin promoter by site-specific DNA binding factors such as TR2TR4, BCL11A, and ZBTB7A has been shown to repress γ-globin gene expression in adults [25,26]. These co-repressor complexes contain multiple enzymes including DNMT1, HDACs, KDM1A, and EHMT1 that catalyze epigenetic modifications that silence gene expression. While multiple lines of evidence strongly suggest that reduction of DNA methylation by DNMT1 inhibitors is involved in the mechanism of HbF induction by these drugs, other effects of DNMT1 inhibitors that perturb the structure of these co-repressor complexes may also participate. Because DNMT1 functions not only as a DNA methyltransferase but also can be an integral component of the structure of these co-repressor complexes, its rapid depletion by proteolysis following decitabine treatment likely affects the integrity of the structure and function of the co-repressor [19]. Defining the exact molecular mechanism of DNMT1 inhibitors and the relative roles of DNA methylation and DNMT1 in γ-globin repression will require further investigation.

KDM1A (LSD1) inhibitors

Analysis of the protein composition of DRED, a TR2 and/or TR4-containg multi-protein co-repressor shown to repress γ-globin expression, first identified the histone demethylase H3K4 demethylase KDM1A (LSD1), as a component of the co-repressor [27]. SiRNA directed toward KDM1A and the drug tranylcypromine, a weak KDM1A inhibitor, each increased γ-globin expression in cultured human erythroid cells. Tranylcypromine failed to induce HbF in baboons in vivo. In the SCD mouse model a more potent tranylcypromine derivative, RN-1, increased HbF and F reticulocytes to levels similar to that of decitabine and was far more powerful than hydroxyurea, although the actual levels of HbF induced in the SCD mouse model is quite low [28]. However, subcutaneous administration of RN-1 to anemic baboons increased HbF to levels similar to that of decitabine [8], but was associated with dose-limiting neutropenia and thrombocytopenia. Analysis of erythroid cells purified from the BM of RN-1 treated baboons showed increased levels of histone acetylation and histone dimethyl and trimethyl lys 4 and decreased DNA methylation of the γ-globin promoter. Subsequent treatment of 2 normal nonanemic baboons with RN-1 (0.25mg/kg; 5d/wk) for over 270 days increased HbF and F cells with minor effects on neutrophil and platelet counts suggesting the relative safety of chronic, long term treatment with KDM1A inhibitors [29]. Oral administration of a highly selective KDM1A inhibitor, ORY-3001, also increased HbF, although decreases in neutrophils and platelets were again apparent [30]. The development of reversible LSD1 inhibitors that increase HbF while reducing less desirable hematological side effects is currently being pursued.

Conclusion and future direction

Experiments in the baboon model have made major contributions to the long-sought goal of an effective pharmacological therapy to increase HbF as therapy for SCD and β-thalassemia. While BM transplantation, lentiviral, and advanced gene editing techniques may appear substantially beneficial in results to date, it is unlikely that these strategies will sufficiently address the needs of the vast number of patients projected worldwide, particularly in developing nations, and the durability of benefits observed to date also remains to be seen. The baboon model thus remains highly important in the development of new small molecule drug strategies for HbF induction. Combinatorial therapy, including decitabine with inhibitors of other repressive epigenetic modifying enzymes such as KDM1A, EHMT1, and HDACs, and also pomalidomide that have demonstrated combinatorial effects on HbF in vitro culture systems can be evaluated first in the baboon model. New, more advanced, highly specific, inhibitors to known targets such as DNMT1 and KDM1A, and also to additional newly defined targets resulting from current basic studies analyzing the composition and the interactions of proteins within the recently identified co-repressor complexes, should greatly increase the number of new HbF-inducing drugs in the pipeline, all of which will benefit from testing in a suitable animal model such as the baboon.

Conflict of interest

The authors declare they have no conflict of interest.

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