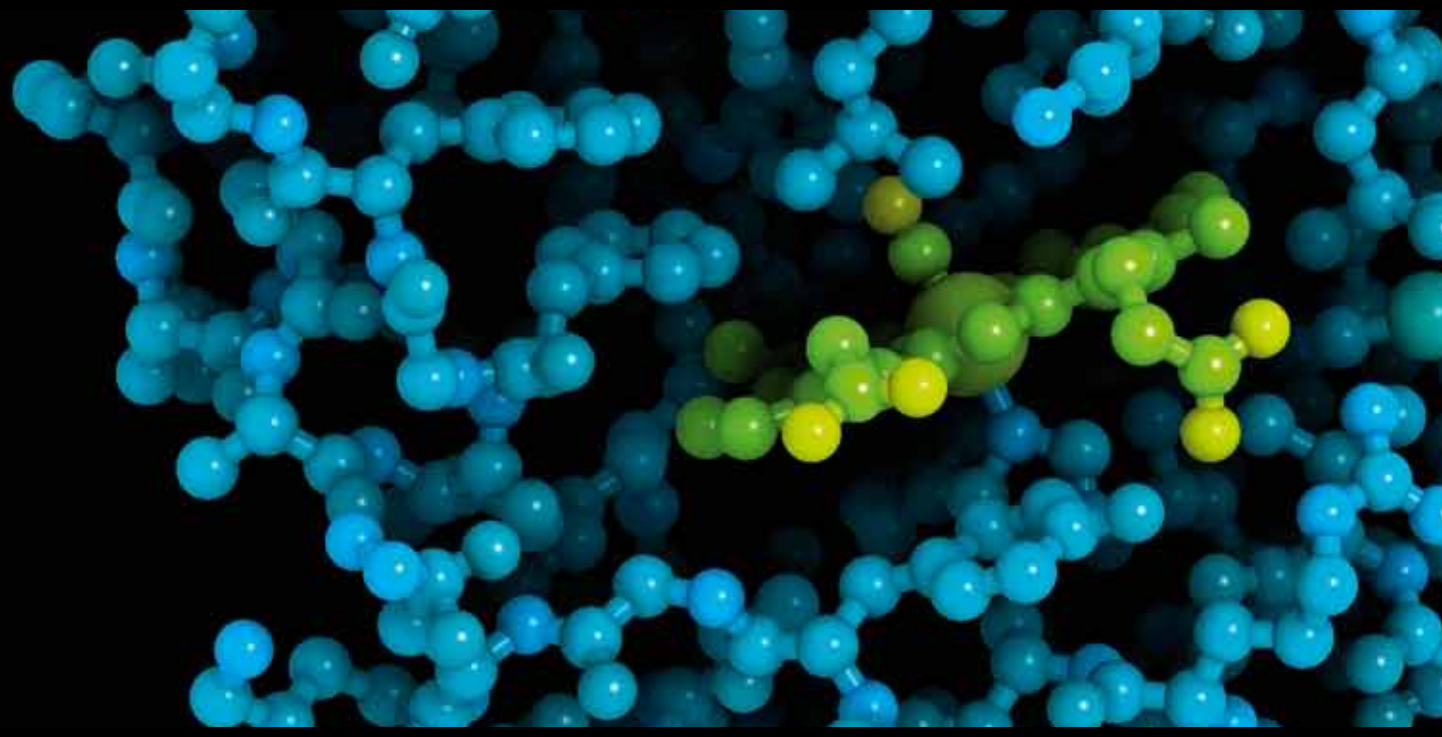
Sickle Cell Disease: Genetics, Cellular and Molecular Mechanisms, and Therapies

Guest Editors: Betty S. Pace, Solomon F. Ofori-Acquah, and Kenneth R. Peterson



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Editorial Board

Contents

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Editorial

Sickle Cell Disease: Genetics, Cellular and Molecular Mechanisms, and Therapies

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Sickle cell disease (SCD) is a global public health disorder that affects millions of people across the globe. It is a monogenic disorder caused by an A-to-T point mutation in the β-globin gene that produces abnormal hemoglobin S (Hb S), which polymerizes in the deoxygenated state, resulting in physical deformation or sickling of erythrocytes. Sickle erythrocytes promote vaso-occlusion and hemolysis, which are two major cellular hallmarks of the disease. Rapid advances made in understanding the molecular genetics of SCD in the early part of the 20th century have not been matched by comparable progress towards understanding its clinical complications, and developing effective therapies. Contemporary reevaluation of SCD as the product of multiple gene interactions promises to overcome the historical constraints of the single-gene disease paradigm that has inevitably impeded translation of research discoveries into clinical benefit.

Two landmark papers in the late 1940s by the Nobel laureates Linus Pauling and Janet Watson provided the molecular bases for SCD and a rational strategy to treat the disease. The publication by Pauling et al., *Sickle Cell Anemia, a Molecular Disease*, in *Nature* in 1949 established SCD as the first molecular human disease, and it established the inheritance pattern of the disorder and of monogenic diseases generally. In addition, that seminal work laid the foundation for the explosion of knowledge in human molecular genetics decades later that gave birth to a new discipline called *gene therapy*. The publication by Watson *The Significance of the Paucity of Sickle Cells in Negro Infants* provided the concept that fetal hemoglobin (Hb F) ameliorates the clinical presentation of SCD for the first time in 1948, ushering in one of the most intensely studied areas of SCD research.

Advancements in *Genetics, Cellular and Molecular Mechanisms, and Therapy of SCD* in the two decades following the seminal works by Pauling and Watson were driven primarily by studies on the erythrocyte, involving polymerization of Hb S and antisickling hemoglobin variants, rheology, and red cell membrane. A highlight amongst these studies was the landmark work by Kan and Dozy published in the article *DNA Sequence Adjacent to the Human Beta-Globin Structural Gene: Relationship to Sickle Mutation.* That study described the presence of single-nucleotide polymorphisms in the human genome for the first time, and it initiated a new avenue of research that led to the discovery of the multicentric origin of the sickle mutation, and it laid the foundations for genetic association studies in SCD, which are currently a major focus of several investigations.

The scope of SCD research expanded beyond the erythrocyte in the 1980s to encompass vascular biology, notably the endothelium, coagulation, and inflammation. Twenty years later, the most compelling evidence that these factors play a critical role in the pathogenesis of SCD is the demonstration that tumor necrosis factor induced adhesion of leukocytes to the vascular endothelium provides the initial cellular events of vaso-occlusion in a mouse model of SCD. Paradigm-shifting insights into the mechanisms of globin gene expression spearheaded by the discovery of the locus control region (LCR) by two groups in the 1990s heralded a new era in SCD research. First, these insights helped to create developmentally regulated and clinically relevant

transgenic mouse models of SCD. Second, they permitted the development of efficacious DNA vectors for gene therapy of SCD that continue to improve as novel elements of gene delivery systems become available and are incorporated into newer generation vectors.

The current special issue of *Anemia* contains original research articles about progress made towards Hb F induction using novel pharmacological agents and artificial zinc finger transcription factors, and a web-based tool to evaluate adherence to hydroxyurea therapy. The latter tool represents efforts to integrate new technology to improve the clinical care of individuals with SCD. Also included in this issue is the first report from a Congolese group of the association of Hb F levels with clinical severity in this population. Several articles report alteration in redox environment and link this phenomenon to impaired hematopoietic progenitor and stem cell function improved by treatment with n-acetyl cysteine in transgenic SCD mice, reduced migration of endothelial progenitors cells derived from children who have SCD, and lastly an association of oxidative stress markers with an atherogenic index in adults with sickle nephropathy. What is known about the deleterious effects of sickling on the genitourinary tract and the role of cyclic nucleotide signaling is reviewed. Finally, articles report two endothelial dysfunction including increased activity of the elastase cathepsin K, and age-dependent increase, in vascular permeability, that culminates in pulmonary edema in middle-aged SCD mice.

The wide variety of experimental studies in this special issue represents potentially new therapeutic tools, ranging from novel approaches for Hb F induction, improved stem cell function and a biomarker to predict risk for SCD nephropathy. Furthermore, the findings of endothelial dysfunction via upregulated cathepsin activity may represent a new pharmacologic target to block accelerated arterial disease observed in children with SCD. The reports in this issue will aid research efforts to close the gap between understanding SCD genetics and developing effective new clinical care approaches and therapeutic options.

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Research Article

Foetal Haemoglobin, Erythrocytes Containing Foetal Haemoglobin, and Hematological Features in Congolese Patients with Sickle Cell Anaemia

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High HbF levels and F cells are correlated with reduced morbidity and mortality in sickle cell disease (SCD). This paper was designed to determine the HbF and F cells levels in Congolese sickle cell anemia (SCA) patients in order to determine their impact on the expression of SCD. *Population and Method*. HbF levels were measured in 89 SCA patients (mean age 11.4 yrs) using a standard HPLC method. F cell quantitation was done in a second group of SCA patients ($n = 42$, mean age 8.9 yrs) and compared with a control group ($n = 47$, mean age 5 yrs). F cells were quantified by a cytofluorometric system (MoAb-HbF—FITC; cut off at 0.5%). *Results*. The mean value of HbF was 7.2% ± 5.0 with heterogeneous distribution, most patients (76%) having HbF < 8%. Mean values of F-cells in SCA patients and control group were 5.4% ± 7.6 (median: 2.19% ; range 0,0–30,3%) and 0.5% ± 1.6 (median 0.0, range 0–5.18), respectively. SCA patients with F cells >4.5% developed less painful crisis and had higher percentage of reticulocytes. *Conclusion*. Congolese SCA patients displayed low levels of HbF and F-cells that contribute to the severity of SCD.

1. Introduction

Fetal hemoglobin (HbF, a2.2) is a major contributor to the phenotypic heterogeneity of sickle cell anemia (SCA). A major ameliorating factor is an inherent ability to produce fetal hemoglobin; elevated levels are correlated with reduced morbidity and mortality in patients with SCA [1–3].

In normal adults, HbF levels are distributed in a nonuniform way in the red cells with a range varying from 0.1 to 7% of total hemoglobin (Hb). In red cells producing higher HbF (termed F cells), HbF is elevated (around 25% of cellular Hb) and genetically determined [2, 4, 5].

Genetic variation at three principal loci—the HBB cluster on chromosome 11p, HBS1L-MYB region on chromosome 6q and BCL11A on chromosome 2p—have been shown to influence HbF levels and disease severity in ß thalassemia and SCA. Taking into account these loci, there is still substantial residual variance in HbF levels, suggesting the importance of other quantitative trait loci (QTL) modulating HBG expression [4, 6].

Total Hb and HbF levels vary in SCA patients according to the βS haplotypes: values are greater in patients bearing the Arabo-Indian and Senegalese haplotypes and less in those with the Bantu or Central African haplotype [2, 4, 6–8].

Sickle cell disease (SCD) has a high prevalence in Sub-Saharan Africa where majority of the affected patients live. B^S gene prevalence in the Democratic Republic of Congo (DRC) is around 25% and about 1.7% of newborns are affected (50,000 births per year) [9].

Expression of SCA in Congolese patients displayed a severe form with high mortality and complications [10].

To our knowledge, there are no specific data on HbF and F cells reported in SCA patients living in DRC. We therefore present here the preliminary analysis of HbF and F cells

in two series of SCA patients and correlations with other hematological parameters and clinical data.

2. Population and Methods

All patients were SS homozygotes regularly followed up in comprehensive sickle cell programs in DRC.

A first study quantified HbF in steady state SCA patients followed up in Lubumbashi ($n = 48$) and Kinshasa ($n = 41$), a total of 89 patients (34 M, 48 F; mean age 11.4 yrs ± 5.4). No patient was on hydroxyurea treatment.

The second study involved 42 SCA patients (23F, 24 M; mean age 8.8 yrs ± 5.1) and a control group of 47 non-SCA patients (26 F, 21 M; mean age 5 yrs ± 5.1) recruited in Kinshasa. In this study, we assessed F Cells numbers and compared the results to hematologic parameters and clinical data.

Diagnosis of SCA was established using standard hemoglobin electrophoresis techniques (acetate electrophoresis or Isoelectric focusing-IEF) coupled to Itano solubility test. The percentage of HbF was determined by high performance liquid chromatography (HPLC).

HbF expression was evaluated using a previously described flow cytometric procedure [11, 12] with slight modifications. In brief, twenty microliters of whole blood were fixed with 1 mL ice-cold 0.05% glutaraldehyde in PBS pH 7.4 vortexed for 15 seconds (s), then incubated at room temperature (RT) for 10 min. The cells were washed twice with PBS, permeabilized by vortexing for 15 s with 0.5 mL ice-cold 0.1% Triton X-100 (Sigma, Milan) in 0.1% bovine serum albumin in PBS (BSA-PBS), and incubated at RT for 5 min. The cells were then washed once with 0.1% BSA-PBS and suspended in 0.5 mL 0.1% BSA-PBS.

Ten microliters of cell suspension were then mixed with 20 μL of 1-in-5 diluted MoAb-HbF-FITC (IQ products, Milan) in 0.1% BSA-PBS and 70 μL of 0.1% BSA-PBS and incubated in the dark at RT for 15 min. An irrelevant mouse antibody of the appropriate subclass was used as a negative control to determine background fluorescence. The cells were washed once with 0.1% BSA-PBS and immediately measured by flow cytometry (as described below).

The flow cytometer analysis reported the percentage of F+ cells in total counted red blood cells of each sample. The positive cut off point was set at 0.5% above negative population of isotype control staining cells.

HbF expression was, also, analyzed using the Kolmogorov-Smirnov statistic test (D-value), which allows the objective and accurate identification of small differences in fluorescence intensity [13]. Samples with $D < 0.15$ were considered negative, whereas those with a $D \geq 0.15$ were considered positive.

Modified technique for evaluating HbF expression: considering the complexity of the previous procedure for identifying the F+ cells, we applied a second flow cytometric technique to perform F+ cells evaluation. This method (routinely utilized for characterizing other cellular parameters, as for example MDR in patients affected by acute leukemias) enabled us to test the samples more conveniently, using fewer and simpler steps, and a precise identification of the red blood cells population in the flow cytometric dot plot, useful for a specific analysis. In addition, this technique led to increased capacity to analyze more samples together than the previous one.

Twenty microliters of whole blood were fixed (Fix and Perm permeabilization kit; Caltag Laboratories) with 100 μL of Medium A at room temperature (RT), in the dark, for 15 min; then cells were washed once with PBS, and then after incubated with 100 μL of Medium B and 4 μL of MoAb-HbF-FITC at RT, in the dark for 30 min. Finally, cells were washed once with PBS, and immediately measured by flow cytometry. The flow cytometric analysis was performed considering the same parameters used for the previously described technique [11, 12].

Flow cytometric analysis was conducted using a FACScan flow cytometer (Becton Dickinson), operated at 488 nm which detects green (MoAb-HbF-FITC) fluorescence. Data acquisition and analysis were performed with the CellQuest software (Becton Dickinson). We measured 50,000 events. The red blood cell area was gated by forward scatter signals (FSC) versus side scatter signals (SSC). The latter was measured using the logarithmic scales (log SSC).

Comparison of hematological parameters (Blood cell counts and HbF levels) were made with other reports of African SCA patients [14–17].

These studies were approved by the Local Ethnic Committees of the participating institutions, Campus Biomedico di Roma, and The CEFA/Centre Hopsitalier Monkole, in accordance with the Declaration of Helsinki.

Statistical analyses were conducted with a software program SPSS system (Version 12, Chicago). Results were expressed as the mean value and median value: standard deviation(SD). Comparisons of means were analysed by Students t-test, correlations by Pearsons test, and comparison between categorical variables by Chi square test or Fishers exact test (where appropriated).

HbF expression (D-value) and F+ cells were represented as dichotomized variable (positive versus negative). Data were analyzed using the two-sided Student's t-test to correlate results obtained by mean of the two different parameters of analysis and the two flow cytometric techniques, while Mann-Whitney U-test was used to measure the differences observed between positive and control groups.

Values were considered statistically significant when $P < 0.05$.

3. Results

3.1. Patients Population. In the first study of 89 SCA patients, the mean HbF% was 7.2% ± 5.0 (median 5.9; range 1–27.5%). It was 7% and 7.4% in the Lubumbashi and Kinshasa group, respectively ($P > 0.05$). Values of HbF were higher in females (7.4%, mean age 10.4 yrs) than in males (6.9%, mean age 9.2 yrs), but the difference was not statistically significant (Mann Whitney test chi square = 0.018, degree of freedom = 1, P = NS). Higher values were observed in children aged less than 3 yrs but no statistical differences were observed between the different age groups.

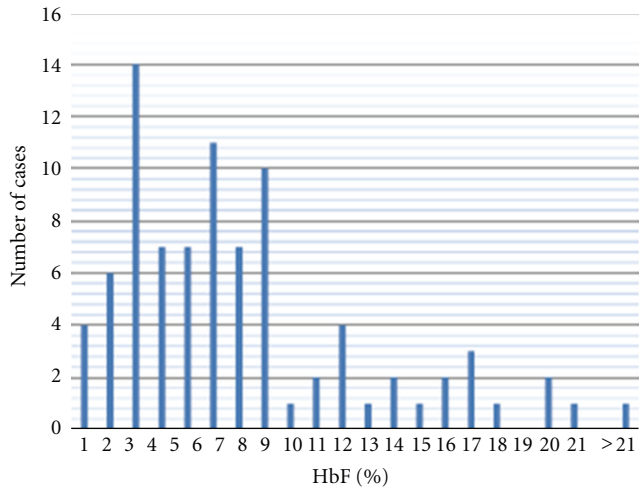

Figure 1: Population distribution of the HbF rate. Patient distribution related to HbF rate displayed a heterogeneous pattern with a predominant group (74%) with HbF% <8. GIobally, only 20/89 (or 22.5%) of patients displayed values higher than 10% of HbF.

Distribution of HbF rate displayed a heterogeneous pattern with a predominant group (66/89 or 74%) with HbF < 8% and two other groups with 9–13% and 14–17%. Globally, levels of HbF were less than 10% in 69/89 of cases (77.5%) and varied considerably; the distribution pattern was not normal even after log transformation of values (not shown), (Figure 1).

In the second study, enumeration of erythrocytes containing HbF (F cells) using the first, standard flow cytometric technique resulted in mean %. SD of F+ cells in 42 SCD samples of 5.44% ± 7.6 (median: 2.19%; range 0.00–30.3%). The mean *D*-value. SD was 0.21 ± 0.007 (median: 0.33; range 0.07–0.57). In the 47 controls, the mean %± SD of F+ cells was 0.50% ± 1.06 (median: 0; range 0–5.18%), and the mean *D*-value ± SD was 0.024 ± 0.034 (median: 0; range 0–0.15) (Figure 2).

Correlation among F+ cells % and *D*-value for the entire population was highly significant with a $r = 0.67$ ($P < 0.0001$). The comparison among SCD patients and controls as for the % of F+ Cell and *D*-value was also highly significant ($P < 0.001$ for both F+ cells % and *D*-value).

The evaluation of the samples with the Fix & Perm flow cytometric technique showed a mean %. SD of F+ cells of 8.67%. 13.48 (median: 4.63; range 0–57.75%), while the mean *D*-value. SD was 0.19. 0.17 (median: 0.15; range 0–0.7).

Comparison of the two flow cytometric techniques showed strong correlation for F+ cells values ($r = 0.63$; $P = 0.0005$) and for *D*-value parameters ($r = 0.53$; $P < 0.005$). 40/42 of SCA patients (95%) had values above the cut-off value of 0.5% while in the control group, only 12/47 subjects (25.5%) had values above the cut off.

Population distribution of % F cells were heterogeneous and displayed a nonnormal distribution even after log-transformation of values (Figure 3). Patients aged <12 yrs displayed higher values than older patients: mean values of 3.7 were observed in group 1 and 4.7 in group 2 versus 1.9 in group 3 (Table 1).

3.2. Comparison of %F Cells with Hematological Parameters and Clinical Issue. We found no significant correlations between the results obtained by cytometry system with the glutaraldehyde method with clinical and biological data; but with the Fix & Perm method, the number of vaso-occlusive crisis was significantly reduced in patients with F cells rate >4.5% ($P < 0.05$) while the reticulocytes number was significantly elevated ($P < 0.005$).

We did not observe significant differences between haematological parameters (Hb, MCV, MCH, and MCHC) in different age groups, although children aged >18 yr displayed higher value of RDW (Table 1).

Comparisons of hematological parameters in our patients with those described in other African SCA patients are depicted in Table 2.

4. Discussion

Hematological characteristics and clinical severity in SCA are variable and are influenced by environmental and genetic factors, including the presence of α-thalassemia, variation in Hb F level, and the haplotype background that is linked to the β globin gene [14]. The Bantu or CAR haplotype is considered as a major risk factor associated with clinically severe form of SCD and organ damage [7, 18, 19]. Most of the SCA patients living in central Africa and in DRC carry the CAR haplotype [20].

The protective role of HbF in the sickling of red cells and the clinical severity of SCD is evident. The HbF level has emerged as an important prognostic factor both for sickle cell pain and mortality; and a %HbF > 10% has been suggested as a threshold level for reduced clinical severity [5, 6, 19, 21, 22].

Different studies on HbF levels in SCA patients bearing CAR haplotypes reported levels values varying from 2 to 10.8%, but generally less than 10% [14, 18, 19, 23, 24]. To date, no values of HbF levels have been reported in Congolese SCA patients living in DRC. The mean value of 7.2% HbF observed in our study confirmed that patients bearing the CAR haplotype had levels of HbF less than 10%, the minimal level that permits a protective role on the sickling of red cells [2, 19]. We found no significant differences in Hb levels in our patients related to sex or age although recent studies confirmed that adult females have higher HbF and F Cells values than males because of the presence of an X-linked QTL (Quantitative Trait locus) [4, 6]. Mouele [16] reported similar data in the neigboring Congo Brazzaville.

Nagel et al. [19] suggested that the HbF level in SCA patients aged more than 5 yrs was dependent of the C-T mutation at position −158 Gγ in the promoter of the Gγ globin gene (known as the Xmn I-Gγ site) [23]. They also found mean levels of HbF at 6.4% and 12.4% in the groups with a rate of Gγ < 38% and Gγ > 38%, respectively. The presence or absence of alpha deletion did not modify these observations. Patients with CAR haplotype had a low Gγ globin gene expression in comparison to the other

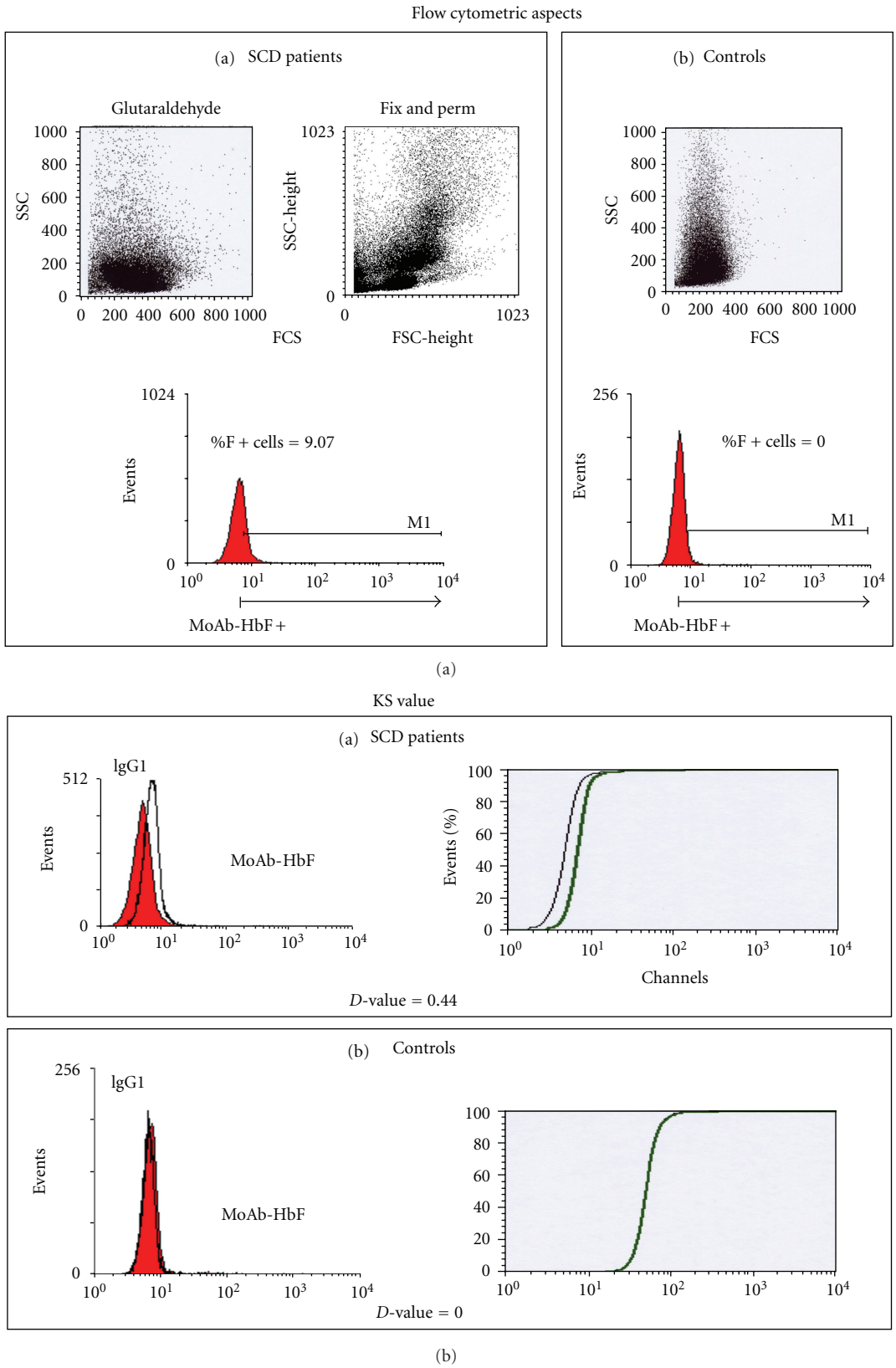

Figure 2: Illustration of flow cytometric and KS aspects of F cells values in a SCA patient and a control. HbF expression was, also, analyzed using the Kolmogorov-Smirnov (KS) statistic test (D-value), which allows the objective and accurate identification of small differences in fluorescence intensity. Samples were considered positive when $D \geq 0.15$.

Table 1: Hematological parameters related to F cells rate in different age groups.

Group (n)	age (yrs)	WBC (G/L)	RBC (T/L)	Hb (g/dL)	Pcv (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	Pts (G/L)	RDW-cv (%)	PDW (%)	MPV	F Cell (*D*-value)	F cell %
1 (12)	2–5 (12)	18.38	2.49	6.4	21.8	89,9	26.3	29.2	514.8	24.5	13.1	10.1	0.3	3,7
2 (21)	6–12 (21)	18.74	2.39	6.4	21.1	90,6	27.3	30.2	434.5	24.2	12.6	10.1	0.3	4,7
3 (7)	13–18 (7)	15.92	2.12	5.8	18.6	87,9	27.1	31.0	367.0	25.9	14.5	11.4	0.2	1,9
4 (1)	>18 (1)	14.80	1.68	4.5	16.7	99,4	26.8	26.9	375.0	32.0	14.1	10.5	—	—

Subjects were divided in 4 age groups (1, 2, 3, and 4) and compared each to others. Parameters that displayed significant differences concerned the F cells rate and the RDW. Significant differences were observed in the F cells rate between the group 1 and 2 versus group 3 ($P < 0.05$); RDW was significantly higher in a child aged >18 yrs than in the other groups.

Table 2: Comparison of hematological parameters in SCA patients from different African studies.

Countries	Nb	Mean age	Hb (g/dL)	PCV (%)	RBC (T/L)	MCV (fl)	MCH (pg)	MCHC (g/dL)	HbF(%)	Ref erences
Tanzania	12	10.7	6.42	24.7	2.27	108.8	28.8	26	8.6	[14]
Kenya	25	10.9	7.85	26	2.54	102.4	30.9	30.2	7.5	[14]
Angola	4	9.3	7.30	20.7	2.70	88.3	30.5	35.3	2	[14]
Nigeria	249	9.7	7.53	28	2.76	103	26.8	26.8	9.2	[14]
Nigeria	94		7.4	26	3.6				7.2	[17]
Nigeria	200	23.6	7.5	23.0	—	79.3	28.3	32.5	2.1	[15]
R Congo	116	9.4	6.6						8.8	[16]
DR Congo	115	8.7	7.0	23.2	2.47	95.3	28.3	30.3	7.4	Personal communication
DR Congo	42	8.9	6.2	20.7	2.3	89.6	26.8	29.7	7.2	Our data

Most of the African SCA patients have Hb less than 8 g/L and Hb F less than 10%. Large variations of HbF rate were observed in the same country like Nigeria probably because of the heterogeneous population who were tested. In DR Congo, values were almost s similar.

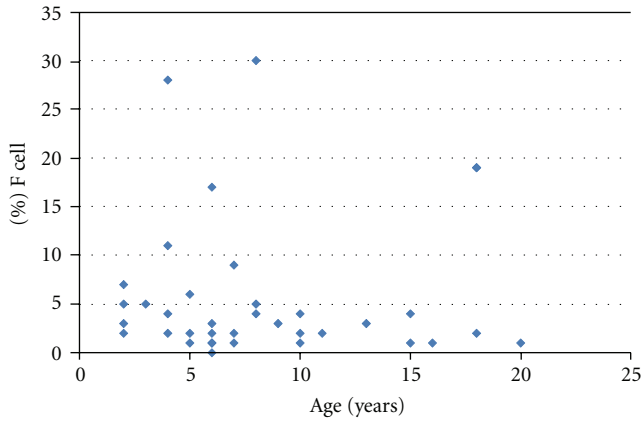

Figure 3: % F cells distribution in 42 SCA patients related to age. The mean value was at 5.44 ± 7.6 with a non gaussian pattern even after log-transformation of values (not shown).

African haplotypes (Senegalese and Benin types) [19]. This polymorphism has been associated with erythropoietic stress and expanded erythroid mass secondary to ineffective erythropoiesis or hemolytic process and preferential survival of the red cell precursor that contain HbF, as observed in β-thalassemia and sickle cell anemia [4, 6, 24].

Genetic variation at three principal loci—HBB cluster on chromosome 11p, HBS1L-MYB region on chromosome 6q, and BCL11A on chromosome 2p—have been shown to influence HbF levels and disease severity in β thalassemia and SCA [4]. A recent study revealed that all three principal HbF loci have a significant impact in Tanzanian patients with SCA; the strongest association being seen at the BCL11A locus on chromosome 2 [25].

We think that values observed in our study were probably due to the heterogeneity of Gγ globin gene expression in patient bearing the Bantu haplotype [19]. Comparison of the HbF values reported in other African SCA patients, displayed low values of 2,17 ± 1.81%, and 4.7 ± 2.9% in Nigerian patients described by Omoti [15] and Falusi and Olatunji [26], respectively. Mouele [16] reported values of 8.8 ± 5.8% in SCA patients in the neighboring Republic of Congo.

Differences observed in those African SCA patients may be due to the various age proportion of populations, the coinheritance trait of thalassemia gene, or other genetic components controlling the number of F cells and the clinical status of the patients [21, 24]. As HbF has been shown to be stable in SCA patients at 4–6 years [6, 19], comparison of HbF values should be determined in children aged >4 yrs and in steady state.

Levels of HbF and F cells vary considerably among different populations; this variability does not originate from a single genetic locus and HbF persistence is considered as a quantitative trait (QT) depending on multiple genes being expressed together with a small environmental component [4].

In normal adults, F cells % varied from 0.5 to 7% while in SCA patients, values have a much broader range [21]. We have no local reference values in Congolese SCA patients,

but 40/42 (95%) of patients and 12/47 (25.5%) of controls displayed values higher than the cut-off point (0.5%). Higher values displayed by some SCA patients and controls (Figure 2) would be due to the concomitant hereditary persistence of fetal hemoglobin (HPFH) as described in other studies [21]. The wide ranging distribution of % HbF and F cells observed in our population can be due to the small sample size in this study, and also due to genetic factors (α-thalassemia and the QTL traits) and to environmental factors (malaria, infections). This later condition coupled with a chronic inflammatory status that has been reported in majority of SCA patients living in DRC [27] would contribute to the hyperhemolytic status. Hemolysis can play a role in the "erythropoietic stress" due to malaria and other infectious complications as expressed by the high reticulocytes number in Congolese SCA patients [28]. Further studies are required to evaluate this hypothesis.

Elsewhere, a significant correlation has been reported between the F cells rate and the %HbF and also with some erythrocyte indices like MCV and Hb [5, 21]. In our study, we found no significant correlations between F cells and other hematological parameters, except the reticulocyte percentage.

Although comparison of hematological parameters in SCA patients from different African countries showed variability, globally, it appears that SCA African patients had low values of Hb (<8 g/L) and %HbF (<10). These data can explain the severity of phenotype of SCD in patients bearing African β^s haplotypes.

In spite of the wide individual variations of the %HbF and F cells rate, these two parameters would be used as a tool to monitor response to agents such as hydroxyurea, a drug that reduces the severity of SCD [4, 6, 21].

5. Conclusion

In spite of some limitations of this study, we have provided new data highlighting low HbF levels and clinical severity of SCA in Congolese patients which can be used to compare African patients located in different geographical area and genetic background. Moreover, comparison of both flow cytometric techniques for F+ cell quantitation resulted in a significant statistical correlations. To confirm its reliability, the less complex and quicker Fix & Perm technique should be further utilized for measuring the amount of F+ cells in SCA. Furthermore, Genome wide studies in different sub-Saharan SCA patients would contribute to the understanding of the complex role of HbF and F cells in the phenotype and the complex physiopathology of sickle cell disease.

Conflict of Interests

The authors have no conflicts of interest.

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Research Article

Induction of Fetal Hemoglobin *In Vivo* Mediated by a Synthetic γ-Globin Zinc Finger Activator

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Sickle cell disease (SCD) and β-thalassemia patients are phenotypically normal if they carry compensatory hereditary persistence of fetal hemoglobin (HPFH) mutations that result in increased levels of fetal hemoglobin (HbF, γ-globin chains) in adulthood. Thus, research has focused on manipulating the reactivation of γ-globin gene expression during adult definitive erythropoiesis as the most promising therapy to treat these hemoglobinopathies. Artificial transcription factors (ATFs) are synthetic proteins designed to bind at a specific DNA sequence and modulate gene expression. The artificial zinc finger gg1-VP64 was designed to target the -117 region of the $^{A}\gamma$-globin gene proximal promoter and activate expression of this gene. Previous studies demonstrated that HbF levels were increased in murine chemical inducer of dimerization (CID)-dependent bone marrow cells carrying a human β-globin locus yeast artificial chromosome (β-YAC) transgene and in $CD34^{+}$ erythroid progenitor cells from normal donors and β-thalassemia patients. Herein, we report that gg1-VP64 increased γ-globin gene expression *in vivo*, in peripheral blood samples from gg1-VP64 β-YAC double-transgenic (bigenic) mice. Our results demonstrate that ATFs function in an animal model to increase gene expression. Thus, this class of reagent may be an effective gene therapy for treatment of some inherited diseases.

1. Introduction

Human hemoglobin is a tetrameric molecule composed of two α-like and two β-like chains, located on chromosomes 16 and 11, respectively. The β-like chain is comprised of the product of one of five functional genes (embryonic ε-, fetal $^{A}\gamma$- and $^{G}\gamma$-, and adult δ- and β-globin) which are developmentally expressed in the order that they are arrayed in the locus [1, 2]. As human erythroid development proceeds, the proper β-like globin genes are activated or repressed, giving rise to the different hemoglobin chains expressed throughout development [2]. Hemoglobin switching from fetal γ-globin to adult β-globin gene expression begins shortly before birth and is usually completed within the first 6 months after birth. In some individuals, hemoglobin switching is not completed, resulting in a condition called hereditary persistence of fetal hemoglobin (HPFH), which is characterized by high expression of fetal hemoglobin (HbF, γ-globin) during adult definitive erythropoiesis [1, 2]. Sickle cell disease (SCD) and β-thalassemia patients are phenotypically normal if they carry compensatory mutations that result in HPFH as well [1, 2]. These genetic studies have indicated that increased HbF will help alleviate pathophysiology associated with these hemoglobinopathies, and thus, research has focused on elucidating the pathways involved in the maintenance or activation of γ-globin expression by drug or gene therapy.

Pharmacological agents such as butyrate, decitabine, and hydroxyurea are effective in inducing HbF *in vitro* and *in vivo* [3]. To date, hydroxyurea, a ribonucleotide reductase inhibitor, is the only drug approved for clinical use in sickle

cell patients [3]. Although it is effective in pediatric patients, the drug also has demonstrated effect on the induction of γ-globin in adult patients, but the long-term effect on organ damage, stroke, and carcinogenesis remains uncertain [3–5]. Thus, there is a need to develop new and more effective therapeutic drugs to treat SCD and β-thalassemia.

Many studies have demonstrated the role of stage-specific transcription factors in hemoglobin switching, indicating the potential therapeutic use of these transcription factors to treat hemoglobinopathies [6–9]. The zinc finger transcription factor *BCL11A* was recently shown to function as a repressor of HbF expression [6]. When erythroid Krüppel-like factor 1 (EKLF1, KLF1), an adult β-globin gene-specific zinc finger transcription factor, was knocked down in erythroid progenitor $CD34^+$ cells, γ-globin expression was induced [9]. DRED (direct repeat erythroid definitive) is a repressor complex that binds to the direct repeat (DR) elements in the ε- and γ-globin gene promoters, and two of the components in this complex are the orphan nuclear receptors TR2 and TR4 [8]. Enforced expression of TR2/TR4 increased fetal γ-globin gene expression in adult erythroid cells from β-YAC transgenic mice [7] and also in adult erythroid cells from the humanized SCD mice [10]. These studies clearly demonstrate that manipulation of transcription factors efficiently reactivates γ-globin expression during adult definitive erythropoiesis.

The use of synthetic zinc finger transcriptional activators designed to interact with a specific DNA sequence and activate gene expression has been well documented [11–14]. In fact, data from studies in cell lines indicated that synthetic activators targeted to the proximal promoter of the $^{A}\gamma$-globin gene have successfully induced γ-globin gene expression [11–15]. The artificial zinc finger gg1-VP64 was designed to interact with the -117 region of the $^{A}\gamma$-globin gene proximal promoter [12]. A 7–16-fold increase in γ-globin expression was observed in K562 cells stably transfected with gg1-VP64 [12]. Increased γ-globin gene expression was also observed following transfection of the gg1-VP64 construct into immortalized bone marrow cells isolated from human β-globin locus yeast artificial chromosome (β-YAC) transgenic mice [11]. More recently, the gg1-VP64 activator was reported to significantly increase HbF levels in $CD34^+$ erythroid progenitor cells from normal human donors and β-thalassemia patients [14, 15]. In this study we demonstrate that gg1-VP64 increased γ-globin gene expression during adult definitive erythropoiesis in β-YAC transgenic mice.

2. Materials and Methods

2.1. gg1-VP64 Construct. Enforced erythroid-specific expression of the gg1-VP64-HA fusion, consisting of the gg1 zinc finger moiety, the VP64 activator, and an HA tag for detection of the protein fusion was obtained by cloning it into the unique *Bgl*II restriction enzyme site of pμ'LCR-β pr-*Bgl*II-β int2-enh, a vector previously shown to confer erythroid/megakaryocytic-restricted expression upon a linked gene [11, 12]. A 0.8 Kb *Apa*I-*Hind*III gg1-VP64 fragment was made blunt-ended and ligated into *Bgl*II-cut, blunt-ended, and phosphatased pμ'LCR-β pr-*Bgl*II-β int2-enh. Transgenic mice were generated as previously described [16, 17]. These mice were crossed to β-YAC transgenic mice [16] to produce four bigenic lines bearing the gg1-VP64 construct and a β-YAC reporter (2, 7, 10, and 18). PCR was employed to genotype the transgenic lines using the following primer sequences: β-YAC: Hu ε-globin forward, 5′-TTCTTGGAAAAGGAGAATGGGAGAGAT-3′; Hu ε-globin reverse, 5′-GCAGTAAAATGCACCATGATGCCAGGC-3′ and gg1-VP64: TF-3, 5′-TTCTCCCGCAGCGATCAC-3′ and TF-4, 5′-CCAAAGCACCTGGGTCTGA-3′ [12].

2.2. Phenylhydrazine Treatment of Mice. Adult bigenic gg1-VP64 β-YAC and single transgenic β-YAC mouse lines at least 6 weeks old were given 60 mg phenylhydrazine (10 mg/mL in phosphate-buffered saline; P-6926; Sigma-Aldrich, St. Louis, MO, USA) per kg body weight via intraperitoneal injection for three consecutive days [18]. Mice were sacrificed 4 days posttreatment, and spleen, liver, and blood were harvested and processed for total RNA extraction and cellular lysate preparation.

2.3. Reverse-Transcriptase PCR (RT-PCR) and Real-Time Quantitative PCR (qPCR). Total RNA was prepared from adult blood and tissue lysates using the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). RT-PCR was performed using gg1-VP64 specific primers TF-3, 5′-TTCTCCCGCAGCGATCAC-3′ and TF-4, 5′-CCAAAGCACCTGGGTCTGA-3′ [12].

qPCR analysis was performed with SYBR Green dye using MiniOpticon or CFX96 instruments (Bio-Rad, Hercules, CA, USA). Expression of γ- and β-globin was calculated using the relative quantification method, as previously described [19, 20], using samples from β-YAC transgenics as a control. PCR primer sequences utilized for expression studies were: Hu-γ1, 5′-GACCGTTTTGGCAATCCATTTC-3′; Hu-γ2, 5′-GTATTGCTTGCAGAATAAAGCC-3′; β-globin FWD, 5′-GAGAAGTCTGCCGTTACTGCC-3′; β-globin REV, 5′-CCGAGCACTTTCTTGCCATGA-3′; Mo-Gapdh FWD, 5′-AGGTTGTCTCCTGCGACTTCA-3′; Mo-Gapdh REV, 5′-CCAGGAAATGAGCTTGACAAAG-3′; Mo-α-globin FWD, 5′-GATTCTGACAGACTCAGGAAGAAAC-3′; Mo-α-globin REV, 5′-CCTTTCCAGGGCTTCAGCTCC-ATAT-3′. Tripli-cate data sets were generated, and qPCR results were normalized to murine Gapdh or α-globin genes.

2.4. Western Blot Analysis. Chemical inducer of dimerization (CID)-dependent β-YAC bone marrow cell [11] and CID-dependent gg1-VP64 β-YAC bone marrow cell lysates were prepared as described [21, 22]. Protein concentrations were measured spectrophotometrically using the Bradford assay. Fifteen μg of cellular lysate was mixed with loading dye (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heated at 95°C for 5

minutes, followed by separation in a 10% SDS-12% polyacrylamide gel using Tris-glycine buffer. Western blotting was performed as previously described [22], according to standard procedures [21].

2.5. Antibodies. Anti-β-actin (sc-21757 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-HA probe (Y-11, sc-805, Santa Cruz Biotechnology), goat anti-rabbit HRP (sc-2030, Santa Cruz Biotechnology), and goat anti-mouse HRP (sc-2031, Santa Cruz Biotechnology) antibodies were used for western blotting.

2.6. HbF Detection by Flow Cytometry. Detection of HbF (F cells) was performed by flow cytometric analysis. Briefly, mouse blood was collected from the tail vein in heparinized capillary tubes. Ten μL of whole blood was washed in PBS and fixed in 1 mL 4% fresh paraformaldehyde (Sigma Aldrich, Saint Louis, MO, USA). The cells were centrifuged, the supernatant discarded, and the pellets were resuspended in 1 mL ice-cold acetone : methanol (4 : 1) for 1 minute. Cells were washed twice in ice-cold PBS/0.1% BSA and resuspended in 800 μL of PBS/0.1% BSA/0.1% Triton X-100 (PBT). One μg sheep anti-human hemoglobin F-FITC-conjugated antibody (A80-136F, Bethyl Laboratories, Montgomery, TX, USA) was added to 100 μL of the cell suspension and incubated for 40 minutes at room temperature. Cells were washed twice with 1 mL ice-cold PBS/0.1% BSA, and the pellets were resuspended in 200 μL of PBS. Cells were analyzed using a BD LSRII (BD Biosciences, San Jose, CA, USA) with a 530/30 nm emission filter (FITC/GFP). Data from 30,000 events was acquired for analysis using BD FACSDiva software (BD Biosciences, San Jose, CA, USA).

3. Results

3.1. Establishment of gg1-VP64 β-YAC Transgenic Lines. To evaluate the effect of the synthetic zinc finger gg1-VP64 on γ-globin gene expression during adult definitive erythropoiesis, gg1-VP64 transgenic lines were produced and bred to β-YAC transgenic mice [16, 17, 23]. Four gg1-VP64 β-YAC bigenic lines were obtained (lines 2, 7, 10, and 18), and samples from these lines were utilized in this study. The presence of the gg1-VP64 construct was confirmed by the presence of a PCR product amplified from a specific region of the gg1-VP64 construct. In addition, the presence of the human β-globin locus was confirmed by PCR amplification of the human ε-globin gene, to confirm the presence of the β-YAC transgene (see the Materials and Methods section). Expression of gg1-VP64 in adult blood samples of the gg1-VP64 β-YAC bigenic lines at the mRNA level was confirmed by RT-PCR (Figure 1(a)). Amplification of the gg1-VP64 fragment was observed exclusively in samples containing the gg1-VP64 construct.

To further demonstrate expression of the gg1-VP64 fusion at the protein level, CID-dependent BMCs were derived from gg1-VP64 β-YAC bigenic mice as previously described [11]. These BMCs maintained the same globin gene expression pattern observed in the adult transgenic mice. Western blotting was performed using an anti-HA tag antibody, which specifically recognizes the HA tag in the gg1-VP64 construct utilized to generate the transgenic lines [12]. A 29 KDa fragment corresponding to the HA-tagged gg1-VP64 fragment was detected in the gg1-VP64 β-YAC CID BMCs, but not in β-YAC CID BMCs lacking gg1-VP64 used as the control (Figure 1(b)). Together, these data confirm the expression at the protein level of the gg1-VP64 zinc finger construct in the gg1-VP64 β-YAC bigenic lines.

3.2. Expression of Fetal Hemoglobin in gg1-VP64 β-YAC Mice during Adult Definitive Erythropoiesis. To test whether the presence of gg1-VP64 induced γ-globin expression during adult erythropoiesis in β-YAC transgenic mice, human β-like globin gene expression was measured by qPCR in adult blood from F_2 or F_3 generation adult mice. Mouse α-globin and Gapdh served as internal controls to quantitate human β-like globin transgene expression levels. All values were normalized to these internal controls and corrected for transgene and endogenous gene copy number. A 5-fold increase in γ-globin gene expression was observed in the peripheral blood samples from the gg1-VP64 β-YAC bigenic line compared to the wild-type β-YAC mice (Figure 2(a)). The expression of the adult β-globin gene was demonstrated to be slightly increased in the adult blood samples from the gg1-VP64 β-YAC bigenic lines, but this increase was not significant (Figure 2(b)).

To further demonstrate that increased γ-globin mRNA expression in the gg1-VP64 β-YAC bigenic lines correlates with an increased percentage of HbF-containing cells, flow cytometry analysis was performed using an anti-human hemoglobin F-FITC-conjugated antibody. The gg1-VP64 β-YAC bigenic mice showed an 8.8% and 7.6% increase of F cells (Figures 3(c) and 3(d)) compared to a wild-type β-YAC transgenic control (0.8% F cells; Figure 3(a)). Positive controls included the previously characterized -117 Greek HPFH β-YAC mice (32.4% F cells; Figure 3(b)). We also performed staining of gg1-VP64 β-YAC bigenic mouse peripheral blood cytospins with the same antibody (Figure 4), which demonstrated a heterocellular distribution of F cells in the gg1-VP64 β-YAC animals (Figures 4(c) and 4(d)), compared to a pancellular distribution in -117 Greek HPFH β-YAC mice (Figure 4(b); [23]). Although only one representative microscope field is shown in each panel of Figure 4, the number of positively stained cells was approximately 10-fold higher compared to wild-type β-YAC transgenic mice (Figure 4(a); data not shown).

The effect of gg1-VP64 was also assessed in RNA samples extracted from spleens of phenylhydrazine-treated gg1-VP64 β-YAC bigenic mice. Phenylhydrazine treatment induces high levels of γ-globin gene expression due to the reticulocytosis resulting from hemolytic anemia [18]. qPCR was performed on RNA samples from gg1-VP64 β-YAC line 7, and a 100-fold increase in γ-globin expression was observed compared to the phenylhydrazine-treated β-YAC control mice (Figure 5). Together our data demonstrate that

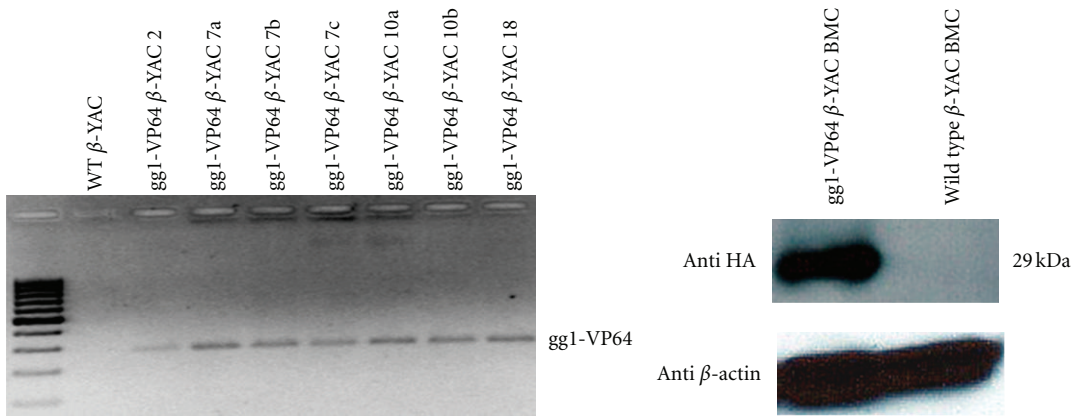

Figure 1: Expression of gg1-VP64. (a) Total RNA isolated from gg1-VP64 β-YAC bigenic line adult peripheral blood was analyzed by RT-PCR using gg1-VP64-specific primers. Each lane shows an individual from the established lines; numbers are indicated at the top of the panel; the gg1-VP64 product is indicated to the right side of the panel. (b) Cellular lysates from CID-dependent gg1-VP64 β-YAC BMCs were assayed by western blotting using an anti-HA tag antibody to detect the gg1-VP64-HA fusion (29 kDa, indicated to the right of the panel). CID-dependent β-YAC BMCs were used as the negative control. Anti-β-actin was employed as loading control. M, marker lane.

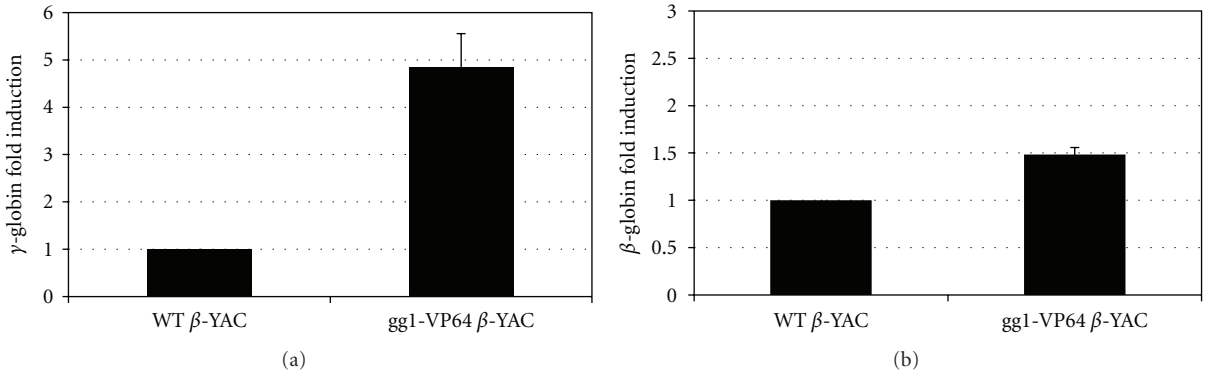

Figure 2: Human β-like globin gene expression in adult blood samples from gg1-VP64 β-YAC transgenic mice. Total RNA isolated from adult blood was subjected to qPCR analysis using SYBR Green. Primers for human γ- and β-globin were utilized, and the data was normalized to mouse α-globin or Gapdh gene expression. (a) γ-globin gene expression. (b) β-globin gene expression. Results are the average of 7 different gg1-VP64 β-YAC bigenic mice $\pm$ the standard error of the mean (SEM). Student's t-test values were $P < 0.01$ for γ-globin and $P > 0.1$ for β-globin.

the zinc finger gg1-VP64 construct increased γ-globin gene expression *in vivo* during adult definitive erythropoiesis.

4. Discussion

The use of synthetic gene-targeted transcription factors that bind to specific DNA sequences to regulate the expression of endogenous genes is an emerging field. Engineered zinc finger transcription factors in which zinc finger motifs are coupled to an activation domain provide new therapeutic venues to enhance gene expression and treat diseases such as hemoglobinopathies [14, 15, 24–26].

The transcription factor gg1-VP64 is a hexameric zinc finger-based DNA binding domain, designed to interact specifically with an 18-base pair target DNA sequence at the -117 nucleotide in the proximal promoter of the $^{A}\gamma$-globin gene [12]. Our study demonstrates increased γ-globin gene expression at both the mRNA and protein level *in vivo* during adult definitive erythropoiesis in gg1-VP64 β-YAC transgenic mice. Our data corroborate previously published data where γ-globin gene expression is increased in K562 cells, in CID-dependent β-YAC BMCs and human erythroid $CD34^{+}$ progenitor cells following transfection of the gg1-VP64 construct [11–15]. A G-to-A mutation at position -117 of the $^{A}\gamma$-globin gene is associated with high levels of fetal hemoglobin in the Greek population (Greek hereditary persistence of fetal hemoglobin or HPFH) [27]. This mutation alters a direct repeat element (DR1) in the $^{A}\gamma$-globin gene promoter [7, 8, 28]. Interestingly, a complex called DRED (direct repeat erythroid-definitive) binds this same region, silencing the fetal γ-globin gene [7].

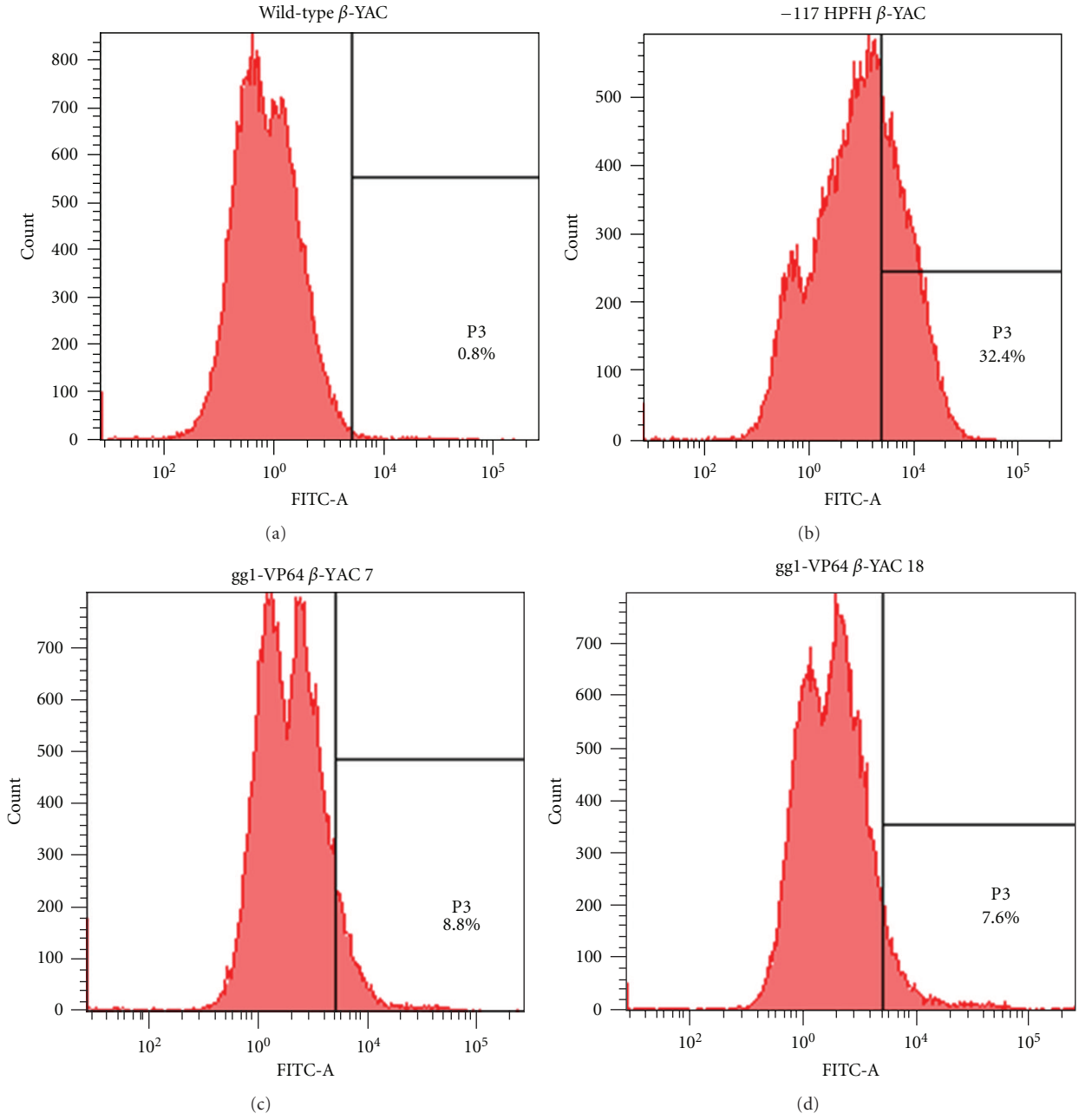

Figure 3: Flow cytometry analysis of F cells in blood from two adult gg1-VP64 β-YAC bigenic mice. A sheep anti-human hemoglobin F-FITC-conjugated antibody was used to determine the percentage of HbF-expressing cells. (a) wild-type β-YAC; (b) −117 Greek HPFH β-YAC; (c) gg1-VP64 β-YAC 7; (d) gg1-VP64 β-YAC 18.

Many studies have been performed in transgenic mouse models bearing human β-globin locus constructs [16, 29–31]. Unlike humans, mice do not have a fetal-stage-specific hemoglobin. However, the human $^{A}\gamma$-globin gene functions as a fetal gene in mice, and the HPFH phenotype is recapitulated in transgenic mice containing −117, −175, −195, or −566 $^{A}\gamma$-globin HPFH point-mutant globin constructs or β-YACs ([23, 27, 32–34], unpublished data). These models have been utilized extensively to understand the function of *cis*-acting elements and *trans*-acting factors within the γ-globin locus, including their potential effects in restoring γ-globin expression in adult erythropoiesis [23, 27, 32–34]. Recently, enforced expression of the *trans*-acting factor TR2/TR4 orphan nuclear receptor was shown to increase γ-globin gene expression in adult erythroid cells of the humanized SCD mouse model [10]. In another study, knockout of *BCL11A* in SCD mice was shown to increase γ-globin expression and red cell survival, thus correcting the SCD phenotype [35]. Taken together, these studies demonstrate the utility of mouse models for screening transcription factors that

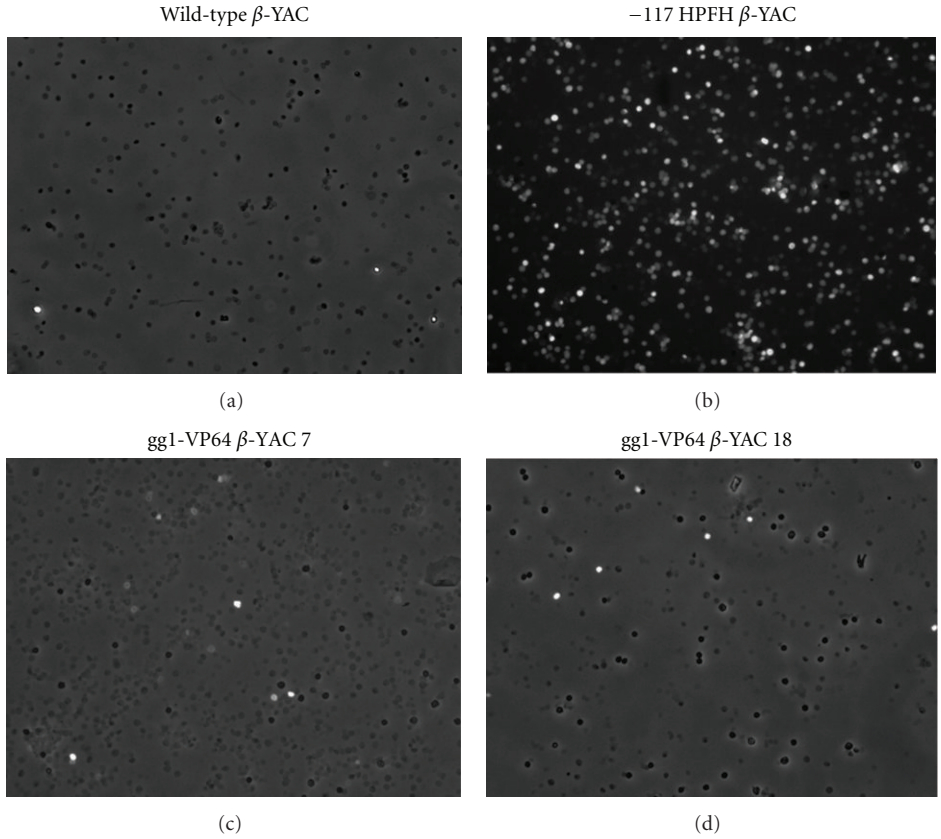

Figure 4: Staining of gg1-VP64 β-YAC bigenic mouse adult blood with anti-human hemoglobin F-FITC-conjugated antibody. Processing of peripheral blood cytospins was performed as described in the Materials and Methods section. (a) Wild-type β-YAC; (b) −117 Greek HPFH β-YAC; (c) gg1-VP64 β-YAC 7; (d) gg1-VP64 β-YAC 18.

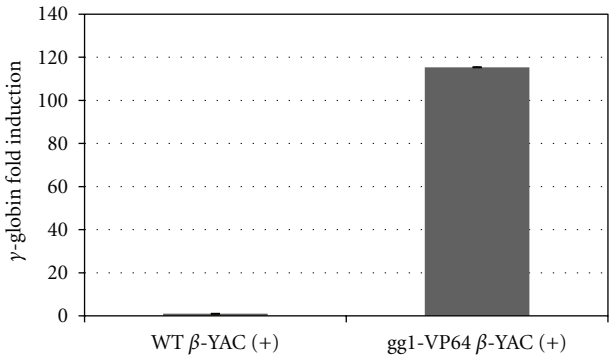

Figure 5: γ-globin gene expression in phenylhydrazine-treated samples from gg1-VP64 β-YAC bigenic mice. Total RNA isolated from adult spleen was subjected to qPCR analysis using SYBR Green. Primers for human γ-globin were utilized, and the data was normalized to mouse α-globin or Gapdh gene expression. Results are the average of 3 replicates of the gg1-VP64 β-YAC bigenic mouse $\pm$ the standard error of the mean (SEM).

can reactivate HbF *in vivo*. Finally, the data presented in this study indicates that a synthetic transcription factor can induce the expression of γ-globin gene expression and HbF *in vivo* during adult definitive erythropoiesis in transgenic mice and supports the use of these constructs as a potential new therapy to treat sickle cell disease and other hemoglobinopathies.

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Review Article

Sickling Cells, Cyclic Nucleotides, and Protein Kinases: The Pathophysiology of Urogenital Disorders in Sickle Cell Anemia

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Sickle cell anemia is one of the best studied inherited diseases, and despite being caused by a single point mutation in the *HBB* gene, multiple pleiotropic effects of the abnormal hemoglobin S production range from vaso-occlusive crisis, stroke, and pulmonary hypertension to osteonecrosis and leg ulcers. Urogenital function is not spared, and although priapism is most frequently remembered, other related clinical manifestations have been described, such as nocturia, enuresis, increased frequence of lower urinary tract infections, urinary incontinence, hypogonadism, and testicular infarction. Studies on sickle cell vaso-occlusion and priapism using both *in vitro* and *in vivo* models have shed light on the pathogenesis of some of these events. The authors review what is known about the deleterious effects of sickling on the genitourinary tract and how the role of cyclic nucleotides signaling and protein kinases may help understand the pathophysiology underlying these manifestations and develop novel therapies in the setting of urogenital disorders in sickle cell disease.

1. Introduction

Sickle cell anemia (SCA) has been first described over a century ago [1] and has become one of the best studied inherited human diseases. Despite being caused by a single point mutation in the *HBB* gene, multiple pleiotropic effects of the abnormal hemoglobin S production range from vaso-occlusive crisis, stroke, and pulmonary hypertension to osteonecrosis and leg ulcers [2–4].

Genitourinary tract function is also affected in SCA, and although priapism is most frequently remembered, other related clinical manifestations have been described, such as nocturia, enuresis, increased frequency of lower urinary tract infections, urinary incontinence, hypogonadism, and testicular infarction. Sickle hemoglobin S (HbS) polymerizes when deoxygenated, resulting in a series of cellular alterations in red cell morphology and function that shorten the red cell life span and lead to vascular occlusion. Sickle cell disease (SCD) vaso-occlusion constitutes a complex multifactorial process characterized by oxidative stress and recurrent ischemia-reperfusion injury in a vicious circle contributing to reduced blood flow and results, eventually, in complete obstruction of the microcirculation and organic dysfunction [3–6]. The exact pathogenetic mechanisms that tie genitourinary complications to the fundamental event of HbS polymerization and hemolytic anemia in SCA have just about started to be unraveled.

This paper focuses on how previous, sometimes poorly explained, clinical observations of urogenital disorders in patients with SCD relate to more recent discoveries on the role of cyclic nucleotides and protein kinases in the pathophysiology of sickle vaso-occlusion.

2. Priapism

Priapism is defined as a prolonged and persistent penile erection, unassociated with sexual interest or stimulation, and is one of the complications associated with sickle cell anemia (SCA) since early in 1934 [7]. Priapism reaches a frequency of up to 45% in male patients with SCA, and the rate of resulting erectile dysfunction (ED) exceeds 30% [8–10].

Although this complication has been previously reviewed in depth in this journal [11], the main concepts behind its pathophysiology will be summarized here for better understanding of the mechanisms discussed throughout the paper, but readers are encouraged to read the previous review.

According to the American Urological Association Guidelines on the Management of Priapism, priapism can be subdivided into three categories: ischemic, stuttering, and nonischemic. Ischemic priapism (veno-occlusive, low flow) is a persistent erection marked by rigidity of the corpora cavernosa (CC) and little or no cavernous arterial inflow. In ischemic priapism, there are time-dependent changes in the corporal metabolic environment with progressive hypoxia, hypercarbia, and acidosis that typically generate penile pain. Penile sinusoids are regions prone to red blood cell sickling in SCD men because of blood stasis and slow flow rates, and ischemic priapism is thought to result from prolonged blockage of venous outflow by the vaso-occlusive process. Clinically, there is congestion and tenderness in the CC, sparing the glans and corpus spongiosum, usually with a prolonged course of over 3 hours, and frequently resulting in fibrosis and erectile dysfunction. Stuttering priapism (acute, intermittent, recurrent ischemic priapism) is characterized by a pattern of recurrence, but an increasing frequency or duration of stuttering episodes may herald a major ischemic priapism. Nonischemic priapism (arterial, high flow) is a persistent erection caused by unregulated cavernous arterial inflow. Typically, the corpora are tumescent but not rigid, the penis is not painful and is most frequently associated with trauma [12–16].

Conventional treatments are largely symptomatic, usually administered after the episode of priapism has already occurred, because the etiology and mechanisms involved in the development of priapism are poorly characterized [17, 18]. Preventive interventions have been proposed but, without a clear idea of the molecular mechanisms involved, they remain largely impractical to be applied in a regular basis in the clinic [17]. Due to the difficulty in exploring these mechanisms in patients, the use of animal models of priapism has become of utmost importance to decipher this devastating clinical challenge [19]. Animal models for priapism include dogs [20, 21], rabbits [22], rats [23–27], and mice [28–41].

Molecular biology and genetic engineering have been widely used in animal models to explore gene function in both human physiology and in the study of pathology of human priapism. Four major priapism animal models have been developed and have yielded greater knowledge on the intrinsic mechanisms underlying priapism: the intracorporal opiorphins gene transfer rat model [42–45], the endothelial nitric oxide synthase (eNOS) with or without neuronal NOS (nNOS) knock-out ($eNOS^{-/-} \pm nNOS^{-/-}$) mouse models [28, 29, 31–33], the adenosine deaminase knock-out ($Ada^{-/-}$) mouse model [35, 36, 40, 41] and the transgenic sickle cell Berkeley mouse model [30, 33, 34, 37–39]. However, the Berkeley mouse is the only well-accepted animal model that displays clinical manifestations similar to those seen in humans with severe forms of SCD, including priapism [30, 34].

Priapism is essentially a derangement of normal erection. Penile erection is a hemodynamic event that is regulated by smooth muscle relaxation/contraction of corpora cavernosa and associated arterioles during sexual stimulation. The penile flaccidity (detumescence state) is mainly maintained by tonic release of norepinephrine through the sympathetic innervations of vascular and cavernosal smooth muscle cells [46]. During penile erection (tumescence state), vascular smooth muscle relaxation decreases vascular resistance, thereby increasing blood flow through cavernous and helicine arteries and filling sinusoids, which are expanded due to the relaxation of smooth muscle cells in the CC [47]. This physiological relaxation of penile smooth muscle is mainly, although not solely, mediated by the neurotransmitter nitric oxide (NO) that is produced by enzymes called NO synthases (NOS). NOSs are subdivided into three isoforms, endothelial NOS (eNOS or NOS3), neural NOS (nNOS or NOS1), and inducible NOS (iNOS or NOS2) [48, 49]. In the penile smooth muscle, NO is released from both nitrergic nerves and the sinusoidal endothelium [46, 50–52]. NO stimulates the soluble guanylyl cyclase (sGC) in the cavernosal smooth muscle, triggering increased synthesis of cyclic GMP (cGMP) that provides the main signal for smooth muscle relaxation [53]. cGMP levels in the CC are regulated by the rate of synthesis determined by sGC and the rate of cGMP hydrolysis mediated by phosphodiesterase type 5 (PDE5) [54, 55]. It has been reported that plasma hemoglobin released by intravascularly hemolysed sickle erythrocytes consumes NO, reducing its bioavailability in the erectile tissue, skewing the normal balance of smooth muscle tone towards vasoconstriction [17, 56, 57]. Champion and collaborators [33] showed that the penile smooth muscle of SCD transgenic mice presents with dysregulated PDE5A expression activity. Moreover, these mice had spontaneous priapism, amplified CC relaxation response mediated by the NO-cGMP signaling pathway, and increased intracavernosal pressure *in vivo* [37, 38].

Recent evidence has shown that another signaling pathway that may also contribute to the pathophysiology of priapism in SCD involves adenosine regulation. Similarly to NO, adenosine is a potent vasodilator produced by adenine nucleotide degradation. Adenosine is predominantly generated by adenosine monophosphate (AMP) dephosphorylation catalyzed by intracellular 5′-nucleotidase. Hydrolysis of s-adenosyl-homocysteine also contributes to intracellular adenosine formation [58, 59]. Extracellular adenosine may be generated by both adenine nucleotide degradation and dephosphorylation by ectonucleotidases [60]. Adenosine is then catabolized by two enzymes: adenosine kinase (ADK), which phosphorylates adenosine to AMP and is an important regulator of intracellular adenosine levels; and adenosine deaminase (ADA), which catalyzes the irreversible conversion of adenosine to inosine [58].

Several physiological processes may be affected by extracellular adenosine and this is mediated by four different receptors, referred to as A_1, A_{2A}, A_{2B}, and A_3. All four subtypes are members of the G protein-coupled receptor (GPCR) superfamily. The activation of the A_1 and A_3 adenosine receptors inhibits adenylyl cyclase activity and

also results in increased activity of phospholipase C, while activation of the A_{2A} and A_{2B} subtypes increases adenylyl cyclase activity [58, 61]. Adenosine-induced vasodilation is mediated by increasing intracellular cyclic adenosine monophosphate (cAMP) levels in vascular smooth muscle cells via A_2 receptor signaling [62, 63]. cAMP activates protein kinase A (PKA) resulting in decreased calcium-calmodulin-dependent MLC phosphorylation and enhanced smooth muscle relaxation [64]. Its role in penile erection has been investigated in studies showing that intracavernous injection of adenosine resulted in tumescence and penile erection [36, 61, 65]. In addition, adenosine induces NO synthesis in endothelial cells through A_2 receptor signaling, and adenosine-mediated CC relaxation is partially dependent on endothelium-derived NO [36, 66–70].

A priapic phenotype in $Ada^{-/-}$ mice was identified and led to further investigation of the impact of adenosine in the pathophysiology of priapism [59]. Previous reports showed that high levels of adenosine caused prolonged corporal smooth muscle relaxation *in vitro*. However, this effect was quickly corrected by intraperitoneal injection of a high dose of polyethylene glycol-ADA (PEG-ADA), which effectively reduces adenosine levels systemically [36, 71]. Moreover, adenosine induced significant increases in cavernosal cAMP levels via A_{2B} receptor activation. This demonstrated that A_{2B} receptor signaling is required for adenosine-mediated stimulation of cAMP production in CC smooth muscle cells [36, 71]. Mi and collaborators [36] have studied adenosine levels in the penis of sickle cell mice and have found a significant increase in adenosine levels, suggesting that overproduction of adenosine may contribute to priapic activity in SCD [71, 72]. Sickle cell mice submitted to PEG-ADA treatment suffered significant reduction of force and duration of relaxation when compared with untreated mice [71]. In addition, increased adenosine levels contributed to the development of penile fibrosis in $Ada^{-/-}$ mice as well as in transgenic sickle cell mice [72]. These findings suggest a general contributory role of elevated adenosine in the pathophysiology of priapism associated with SCD.

Although the penile vascular endothelium and smooth muscle cells are sources of vasodilation factors such as NO and adenosine, there are vasoconstriction pathways important to the penile hemodynamics, such as the Rho-kinase (ROCK) pathway. The RhoA/ROCK signal transduction pathway has been shown to influence erectile function *in vivo* through an array of mechanisms, including vasoconstriction of the penile vasculature via smooth muscle contraction and regulation of eNOS [73–76]. This pathway is involved in the regulation of smooth muscle tone by modulating the sensitivity of contractile proteins to Ca^{2+} [77]. RhoA regulates smooth muscle contraction by cycling between a GDP-bound inactive form (coupled to a guanine dissociation inhibitor, RhoGDI) and a GTP-bound active form [78–80]. Upstream activation of heterotrimeric G proteins leads to the exchange of GDP for GTP, an event carried out by the guanine exchange factors (GEFs) p115RhoGEF [81], PDZ-RhoGEF [82], and LARG (Leukemia-associated RhoGEF) [83], which are able to transduce signals from G protein-coupled receptors to RhoA [84–86]. ROCK is activated by RhoA and inhibits myosin phosphatase through the phosphorylation of its myosin-binding subunit, leading to an increase in Ca^{2+} sensitivity. The RhoA/ROCK Ca^{2+} sensitization pathway has been implicated in the regulation of penile smooth muscle contraction and tone both in humans and animals [77, 87]. ROCK exerts contractile effects in the penis by Ca^{2+}-independent promotion of myosin light chain (MLC) kinase or the attenuation of MLC phosphatase activity and reduction in endothelial-derived NO production [88]. RhoA activation, ROCK2 protein expression, as well as total ROCK activity decline in penile of SCD transgenic mice, highlighting that the molecular mechanism of priapism in SCD is associated with decreased vasoconstrictor activity in the penis [39]. Therefore, should impaired RhoA/ROCK-mediated vasoconstriction contribute to SCD-associated priapism, this pathway may become a novel therapeutic target in the management of this complication.

There has been no definite advance in the management of sickle cell-associated acute, severe priapism. Penile aspiration with or without saline intracavernosal injection and eventually performing surgical shunts remains mainstays of care, with no evident benefit of more common approaches, such as intravenous hydration, blood transfusions, and urinary alkalinization [89, 90]. Pharmacological interventions in such cases have been limited to intracavernosal use of sympathomimetic drugs, such as epinephrine, norepinephrine, and etilefrine, but there are anecdotal reports of acute use of PDE5 inhibitor sildenafil [91].

Nonetheless, most attempts to control SCD priapism have focused on its recurrent, stuttering form. Small case series of hormonal manipulation with diethylstilbestrol [92], gonadotropin-releasing hormone (GnRH) analogues [93], and finasteride [94] have been reported to successfully manage recurrent priapism. Increasing smooth muscle tone with oral α-agonist etilefrine has also yielded only anecdotal evidence of benefit [95]. Unfortunately, a prospective study comparing etilefrine and ephedrine failed to demonstrate superiority or equivalence of both drugs in preventing recurrent priapism due to poor compliance and low recruitment reducing statistical power, but some evidence was obtained reassuring safety of the use of such strategies, and possibly indicating a lower severity of priapism attacks among compliant patients [96]. This favors off-label use of pseudoephedrine at bedtime advocated by some experts [57, 90]. Hydroxyurea has also been effective in preventing priapism recurrence in SCD in a small number of cases [97, 98]. Based on current knowledge of NO-dependent pathways, the use of PDE5 inhibitors has been studied. One clinical trial testing tadalafil in SCD patients has been terminated, but no outcome data have yet been published (ClinicalTrials.gov NCT00538564), and one ongoing trial aims at the effect of sildenafil in the same setting (ClinicalTrials.gov NCT00940901). Despite these efforts, scientists have become less optimistic concerning the tolerability of this approach, ever since the premature termination of the sildenafil trial for pulmonary hypertension in SCD patients, in which subjects on PDE5 inhibitor were more likely to have severe pain crises requiring hospitalization [99]. Therefore, novel therapies for preventing and treating priapism in SCD

are still warranted if the incidence of impotence among these patients is expected to be reduced in the long term.

3. Infertility

Progress in the therapy of SCD, particularly the use of hydroxyurea, has considerably improved the prognosis of patients with SCD [100, 101], with their mean life expectancy reaching much over 40 years [102–104], rendering infertility an important issue. Nevertheless, long before hydroxyurea became a standard of care in SCD, seminal fluid parameters of SCD males had been reported to fall within the subfertile range due to decreased sperm concentration, total count, motility, and altered morphology [105–107], and a more recent study reported over 90% of patients had at least one abnormal sperm parameter [108].

Hydroxyurea (HU) has been reported to impair spermatogenesis, causing testicular atrophy, reversible decrease in sperm count, as well as abnormal sperm morphology and motility [108–114], and its current or previous use should be among the first probable causes to be considered in SCD patients complaining of infertility. Moreover, sperm abnormalities prior to HU have been attributed to variable effects of hypogonadism induced by SCD itself, and lack of appropriate testosterone production seems to be exacerbated by HU use in a mouse SCD model [115].

Considering that male fertility does not rely solely on the quality of the seminal fluid, other causes that may also render male patients with SCD prone to suffer from infertility include sexual problems, such as loss of libido, premature ejaculation, frequent priapism, and priapism-related impotence [105–107, 116–121].

Finding a single main cause for male infertility in a particular SCD patient is highly unlikely and probably will involve some degree of endocrinological impairment. A broader understanding of how hypogonadism takes place in SCD is necessary to explain fertility problems and requires knowledge of the complexity of sex hormone production regulation.

4. Hypogonadism

The etiology of hypogonadism in SCD patients is multifactorial, as several mechanisms have been suggested to contribute to its occurrence, such as primary gonadal failure [117, 122, 123], associated with or caused by repeated testicular infarction [124], zinc deficiency [125, 126], and partial hypothalamic hypogonadism [127].

Physical and sexual development are affected in both male and female SCD patients, with onset of puberty (menarche) and appearance of secondary sexual characteristics (pubic and axillary hair and beard) being usually delayed. The delay is greater in homozygous SCA and S-β^0-thalassemia than in SC disease and S-β^+-thalassemia [128–130]. Moreover, studies in male patients with SCD reported reduction of ejaculate volume, spermatozoa count, motility, and abnormal sperm morphology [106, 116].

Biochemical analyses have demonstrated low levels of testosterone and dihydrotestosterone and variable levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in patients with SCD [105–107, 118, 119, 121, 131]. The comparison between patients and controls matched according to stage of development of secondary sexual characteristics showed higher levels of LH in sickle cell disease, favoring some role for hypergonadotropic hypogonadism.

Leydig cells of the testes and other steroidogenic tissues produce hormones by a multienzymatic process, in which free cholesterol from intracellular stores is transferred to the outer and then to the inner mitochondrial membrane. Leydig cells produce androgens under the control of LH or its placental counterpart human chorionic gonadotropin (hCG), as well as in response to numerous intratesticular factors [114, 132]. LH/hCG receptors belong to the sGC-coupled seven-transmembrane-domain receptor family, whose activation leads to stimulation of adenylyl cyclase [133]. The resulting accumulation of intracellular cyclic adenosine monophosphate (cAMP) levels and the concomitant activation of the cAMP-dependent protein kinase (PKA) lead to the phosphorylation of numerous proteins, including the steroidogenic acute regulatory (StAR) protein [134, 135]. StAR localizes predominantly to steroid hormone-producing tissues and consists of a 37 kDa precursor containing an NH2-terminal mitochondrial targeting sequence and several isoelectric 30 kDa mature protein forms [136–138]. Steroid production in gonadal and adrenal cells requires both *de novo* synthesis and PKA-dependent phosphorylation of StAR-37 protein [139]. The newly synthesized StAR is functional and plays a critical role in the transfer of cholesterol from the outer to the inner mitochondrial membrane, whereas mitochondrial import and processing to 30 kDa StAR protein terminate this action [140–142].

HbS polymerization is mediated by upstream activation of adenosine receptor $A_{2B}R$ by hypoxia, and hemolysis of irreversibly sickled red blood cells increases adenosine bioavailability through conversion of ATP by ectonucleotidases CD39 and CD73, thus predisposing patients with SCD to sustained high levels of cAMP [143, 144]. From this point of view, steroidogenesis could be expected to be increased in these patients.

Although Leydig cell steroidogenesis is predominantly regulated by cAMP/PKA, other pathways also influence this process [145], including the NO-cGMP signaling pathway [146]. NO promotes a biphasic modulation in the androgen production, stimulatory at low concentrations, and inhibitory at high concentrations [49, 147, 148]. SCA causes NO depletion, and in low levels, NO stimulates Leydig cell steroidogenesis by activating sGC [48, 49, 149] and promotes the formation of low levels of cGMP, albeit enough to activate the cGMP-dependent protein kinase (PKG) and phosphorylate StAR [49, 150]. This signaling is controlled by phosphodiesterases (PDEs) [151] and active transport systems that export cyclic nucleotides (multidrug-resistance proteins) from the cell [152]. In zona glomerulosa cells, activation of PKG II by cGMP regulates basal levels of aldosterone production and phosphorylation of StAR

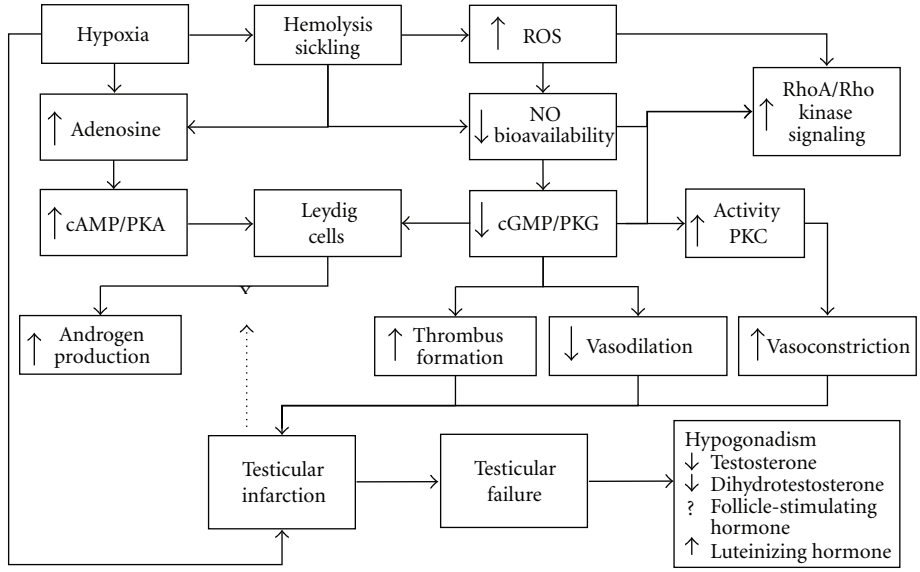

Figure 1: Schematic pathophysiology of hypogonadism and testicular infarction in sickle cell disease. The dashed arrow represents the blocking effect of gonadal failure over cyclic nucleotide-stimulated androgen production.

protein [150], but whether there is a role for cGMP in the zona reticularis, where adrenal androgenesis takes place, is unknown.

Hypogonadism observed in patients with SCD with lower circulating testosterone and higher LH levels suggests that, at least in this setting, despite the reduced cGMP- and elevated cAMP-mediated stimuli on androgen production, gonadal failure with Leydig cell impairment predominates in sex hormone production dysfunction (Figure 1). This further highlights that primary hypogonadism is possibly largely underdiagnosed and elicits more studies on the pathogenesis of testicular infarction.

5. Testicular Infarction

Segmental testicular infarction is an infrequent cause of acute scrotum and is rarely reported, with fewer than 40 cases published at the time of this paper. Its etiology is not always well defined, and it may be, at first, clinically mistaken for a testicular tumour [153, 154]. Common causes for testicular infarction are torsion of the spermatic cord, incarcerated hernia, infection, trauma, and vasculitis [131]. The usual presentation is a painful testicular mass unresponsive to antibiotics [155]. This testicular disorder has been associated with epididymitis, hypersensitivity angiitis, intimal fibroplasia of the spermatic cord arteries, polycythemia, anticoagulant use, benign testicular tumors and, in the interest of this review, sickle cell trait and sickle cell disease [124, 131, 155–158].

Testicular infarction related to sickling has been very rarely reported with only five individual cases found retrospectively, three associated with sickle cell disease and two with sickle cell trait [124, 155–157, 159]. Holmes and Kane reported the first testicular infarction in a patient with SCD who presented with testicular swelling unresponsive to antibiotics. Physical examination revealed that a lesion suspicious for malignancy and ultrasonography demonstrated a hyperechoic mass with an anechoid rim and normal blood flow in the surrounding parenchyma. Radical orchiectomy revealed hemorrhagic infarction with sickle blood red cells. In another case report, SCA patient presented with acute scrotum and history of acute chest syndrome, splenic infarction, osteomyelitis, and hemolysis. Physical examination demonstrated an erythematous, tender, swollen testicle and ultrasound once again revealed normal echotexture and blood flow. Surgical exploration and pathological examination diagnosed segmental testicular infarction with vascular congestion and sickled red blood cells [124]. In the last testicular infarction case report in a patient with SCD presented with increased testicular volume, scrotal ultrasonography showed both echogenic and hypoechogenic regions and Doppler ultrasonography revealed vascular changes compatible with testicular infarction. Radical orchiectomy was performed 10 days after the initial presentation and microscopic evaluation showed necrotic seminiferous tubules devoid of nuclear debris, congestion, or acute inflammatory infiltrate, consistent with coagulative necrosis of ischemic origin [131].

Testicular blood flow is dependent on the internal spermatic, cremasteric, and deferential arteries. Obstruction of venous outflow may create venous thrombosis, testicular engorgement, and subsequent hemorrhagic infarction. In SCD, low oxygen tensions in erythrocytes lead to sickling cells that lose pliability in the microcirculation. Consequently, capillary flow becomes obstructed, worsening local tissue hypoxia, perpetuating the cycle of sickling, and promoting testicular infarction [124, 131, 157].

The cyclic nucleotides and protein kinases may play an important role in the pathophysiology of testicular infarction

in SCD. Enhanced hemolysis and oxidative stress contribute to a reduction in nitric oxide (NO) bioavailability due to NO scavenging by free hemoglobin and reactive oxygen species (ROS) generation [160, 161]. As mentioned before, testicular NO signaling pathway is involved in the regulation of Leydig cell steroidogenesis [48, 49, 147–149, 162–164] but may also influence testicular circulation. We suggest that the reduction of NO bioavailability and consequent reduction of GMPc levels and of activity of PKG may decrease the vasodilation process in the testes. Moreover, reduced NO levels in patients with sickle cell disease contribute to the development of thrombus formation in the vascular system and could further enhance local ischemia [165, 166]. Furthermore, the cGMP-dependent protein kinase signaling pathway would normally inhibit RhoA-induced Ca^{2+} sensitization, RhoA/ROCK signaling, and protein kinase C (PKC) activity that mediate contraction in vascular smooth muscle [167–171]. Thus, reduced NO levels may decrease cGMP-dependent protein kinase activity and promote increasing RhoA-induced Ca^{2+} sensitization and PKC activity, favoring vasoconstriction in the testes. Therefore, tissue hypoxia, sickling of red blood cells, reduced levels of NO, possible thrombus formation, increased RhoA-induced Ca^{2+} sensitization, and PKC activity may all lead to capillary and venous flow obstruction promoting testicular infarction (Figure 1).

Although testicular infarction in SCD has been very rarely reported, it has been speculated that silent testicular infarctions are much more common but generally overlooked clinically. Testicular biopsy in patients is rarely performed and additional studies are necessary to establish the true incidence of testicular infarction in patients with SCD or even sickle cell trait.

6. Urinary Bladder Dysfunction

The urinary bladder has two important functions: urine storage and emptying. Urine storage occurs at low pressure, implying that the bladder relaxes during the filling phase. Disturbances of the storage function may result in lower urinary tract symptoms (LUTSs), such as urgency, increased frequency, and urge incontinence, the components of the hypoactive or overactive bladder syndromes [172, 173]. The passive phase of bladder filling allows an increase in volume at a low intravesical pressure. The bladder neck and urethra remain in a tonic state to prevent leakage, thus maintaining urinary continence. Bladder emptying is accompanied by a reversal of function in which detrusor smooth muscle (DSM) contraction predominates in the bladder body that is accompanied by a concomitant reduction in outlet resistance of the bladder neck and urethra [174–176]. The bladder filling and emptying are regulated by interactions of norepinephrine (sympathetic component released by hypogastric nerve stimulation), acetylcholine and ATP (parasympathetic components released by pelvic nerve stimulation) with activation of adrenergic, muscarinic, and purinergic receptors, respectively [175].

Urinary bladder dysfunction is rarely spontaneously reported by SCD patients to their caregivers. With increasing survival of these patients, physicians may expect that urinary complaints increase in association with classical urological disorders associated with advanced age, such as urinary stress incontinence in multiparous women and benign prostatic hyperplasia in men. Nonetheless, clinical observations of medical complaints involving the urinary bladder start as early as childhood, with enuresis, and continue onto adulthood with nocturia and urinary tract infections, to name a few, although frequently neglected.

Nocturia has long been attributed to constant increased urinary volumes in SCD. As part of the renal complications of sickling, renal medullary infarcts lead to decreased ability to concentrate urine, yielding higher daily urinary volumes [177], compensatory polydipsia, and eventually, the need for nocturnal bladder voiding.

For comparison, the effects of polyuria on bladder function have been better characterized in diabetic bladder dysfunction (DBD). Both SCD and diabetes mellitus cause increased urinary volume and, to some extent, the two diseases involve cellular damage by oxidative stress mediators; so data from previous studies on DBD may help shed some light on preliminary data on bladder function in SCD animal models by understanding a known model of bladder dysfunction.

It has been suggested that DBD comprehends so-called early and late phases of the disease, owing to cumulative effects of initial polyuria secondary to hyperglycemia, complicated by oxidative stress influence on the urothelium and nervous damage in the long term of the natural history of diabetes mellitus. In the early phase of DBD, the bladder is hyperactive, leading to LUTS comprised mainly by nocturia and urge incontinence. Later in the course of the disease, the detrusor smooth muscle becomes atonic, abnormally distended, and incontinence is mainly by overflow associated with a poor control of urethral sphincters, and voiding problems take over [178].

DSM physiology also involves cyclic nucleotides and activation of protein kinases. DSM contractions are a consequence of cholinergic-mediated contractions and decreased β-adrenoceptor-mediated relaxations [179]. DSM contains a heterogeneous population of muscarinic receptor subtypes [180, 181], with a predominance of the M2 subtype and a smaller population of M3 receptors. However, functional studies showed that M3 receptors are responsible for promotion of contraction in the DSM of several animal models [182–185] and in humans [186, 187]. Activation of M3 muscarinic receptors in the DSM promotes stimulation of phospholipase C, activates PKC, and increases formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) to release calcium from intracellular stores, leading to DSM contraction [87]. Moreover, activation of M2 receptors also induces a DSM contraction indirectly by inhibiting the production of cAMP, reducing PKA activity, and reversing the relaxation induced by β-adrenoceptors [179]. Hence, both mechanisms promote urinary bladder emptying.

There is evidence that the Ca^{2+}-independent RhoA/ROCK pathway is involved in the regulation of smooth

muscle tone by altering the sensitivity of contractile proteins to Ca^{2+} [77]. This pathway has been shown to influence erectile function *in vivo* through an array of mechanisms, including phosphorylation of the myosin-binding subunit of MLC phosphatase, resulting in increased myosin phosphorylation. RhoA, a member of the Ras (Rat Sarcoma) low molecular weight of GTP-binding proteins, mediates agonist-induced activation of ROCK. The exchange of GDP for GTP on RhoA and translocation of RhoA from the cytosol to the membrane are markers of its activation and enable the downstream stimulation of various effectors such as ROCK, protein kinase N, phosphatidylinositol 3-kinase, and tyrosine phosphorylation [77]. The RhoA/ROCK Ca^{2+} sensitization pathway has been implicated in the regulation of bladder smooth muscle contraction and tone in humans and animals [77, 188–191]. Thus, alterations in the contraction or relaxation mechanisms of DSM during the filling and emptying phases may contribute to urinary bladder dysfunction. Patients with SCD have not been evaluated for bladder dysfunction in a systematic manner, but preliminary data have shown that Berkeley mice (homozygous SS) exhibit hypocontractile DSM *ex vivo*, due to a significant decrease of contractile responses to muscarinic agonist carbachol and electrical field stimulation [192]. This bladder dysfunction may contribute to the increased risk of urinary tract infections observed in SCD patients.

In an epidemiological study of 321 children with SCD, 7% had a documented urinary tract infection (UTI), one-third had recurrent infections, and two-thirds had had a febrile UTI [193]. As in normal children, there was a strong predominance of females, and gram-negative organisms, particularly *Escherichia coli*, were usually cultured. Most episodes of gram-negative septicemia in SCD are secondary to UTI [194]. Moreover, UTIs are more frequent during pregnancy in women with SCA or sickle cell trait [195–197]. The prevalence of UTI in women with SCA is nearly twofold that of unaffected black American women. This association appears to be directly related to HbS levels, since patients with sickle trait have an increased prevalence of bacteriuria, but to a lesser degree than those with SCA. More recently, a study detected that a group of SCD children and adolescents had more symptoms of overactive bladder than a control group [198]. This could be a first documentation of a clinically evident of an early phase of sickle cell bladder dysfunction, but whether there is a late, hypotonic bladder phase in older sickle cell adults remains to be demonstrated.

The presence of increased intracavernosal pressure associated with the amplified corpus cavernosum relaxation response (priapism) mediated by NO-cGMP signaling pathway, the lack of RhoA/ROCK-mediated vasoconstriction in sickle cell transgenic Berkeley mice, and the association of priapism with genitourinary infections and urinary retention further suggest the possibility that changes in the DSM reactivity may contribute to urogenital complications in SCD [36, 38–40, 192]. Despite advances in the understanding of urogenital disorders in the SCD, further studies should clarify the pathophysiological mechanisms that underlie genitourinary manifestations of SCD.

7. Conclusions

Urogenital disorders in SCD are the result of pleotropic effects of the production of the abnormal sickling hemoglobin S. While priapism still stands out as the most frequently encountered, current knowledge of the effects of cyclic nucleotide production and activation of protein kinases allows to suspect underdiagnosis of bladder dysfunction and hypogonadism secondary to testicular failure. Moreover, despite our growing understanding of these complications, adequate, efficacious, and well-tolerated treatments are still unavailable, and male patients continue to suffer from infertility and erectile dysfunction. Further work in, both clinical assessments and experimental studies in this field are promising and should help increase physicians' awareness of the importance of more accurate diagnoses, design improved therapeutic strategies, and eventually, achieve better quality of life for SCD patients.

Abbreviations

ROS: Reactive oxygen species
NO: Nitric oxide
cAMP: Cyclic adenosine monophosphate
PKA: Cyclic adenosine monophosphate-dependent protein kinase
cGMP: Cyclic Guanosine monophosphate;
PKG: Cyclic Guanosine monophosphate protein kinase;
PKC: Protein kinase C.

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Research Article

Spatiotemporal Dysfunction of the Vascular Permeability Barrier in Transgenic Mice with Sickle Cell Disease

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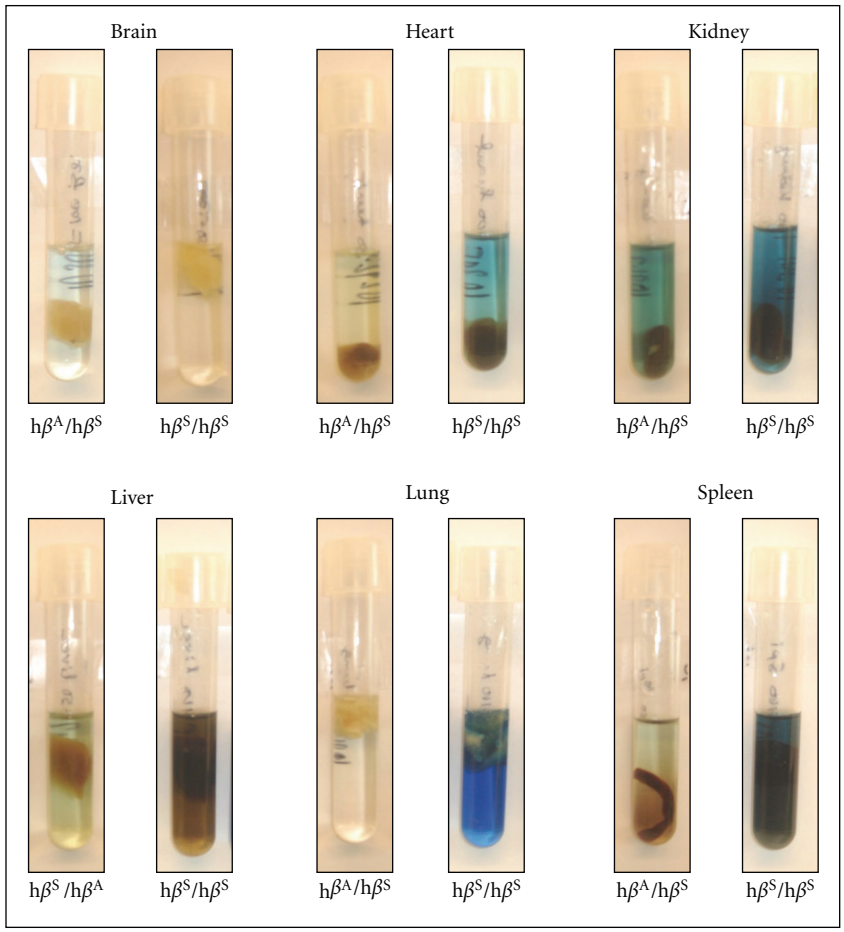
Sickle cell disease (SCD) is characterized by chronic intravascular hemolysis that generates excess cell-free hemoglobin in the blood circulation. Hemoglobin causes multiple endothelial dysfunctions including increased vascular permeability, impaired reactivity to vasoactive agonists, and increased adhesion of leukocytes to the endothelium. While the adhesive and vasomotor defects of SCD associated with cell-free hemoglobin are well defined, the vascular permeability phenotype remains poorly appreciated. We addressed this issue in two widely used and clinically relevant mouse models of SCD. We discovered that the endothelial barrier is normal in most organs in the young but deteriorates with aging particularly in the lung. Indeed, middle-aged sickle mice developed pulmonary edema revealing for the first time similarities in the chronic permeability phenotypes of the lung in mice and humans with SCD. Intravenous administration of lysed red blood cells into the circulation of sickle mice increased vascular permeability significantly in the lung without impacting permeability in other organs. Thus, increased vascular permeability is an endothelial dysfunction of SCD with the barrier in the lung likely the most vulnerable to acute inflammation.

1. Introduction

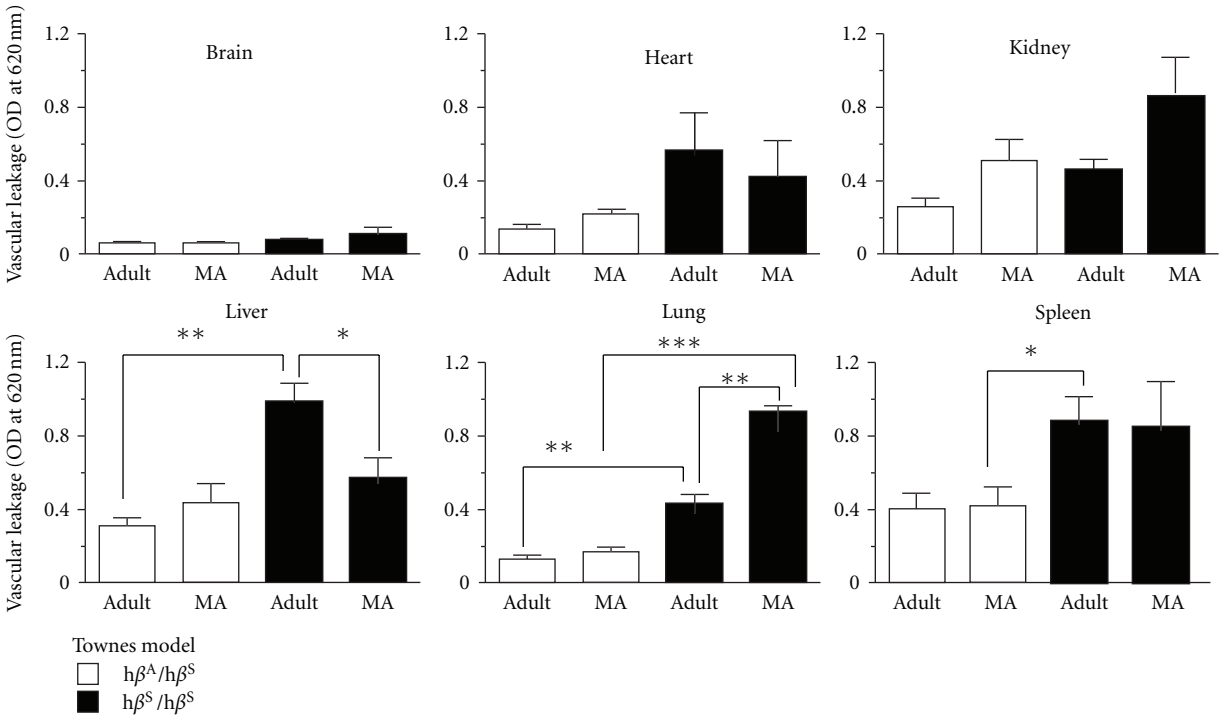
Sickle cell disease (SCD) is characterized by the production of red blood cells with increased propensity for lysis and adhesion [1]. Its clinical manifestations fall broadly into two subphenotypes defined by hyperhemolysis and vasoocclusion [2]. At least 30% of the hemolysis in SCD is intravascular [3], which means that the endothelial wall in this disease is persistently exposed to cell-free hemoglobin. The endothelium is a semipermeable barrier that regulates the response of the vascular wall to inflammatory agonists. This response involves activation of adhesion molecule expression, increased permeability of the endothelium, and extravasations of fluid from the blood into interstitial tissue compartments [4]. Increased vascular permeability results from opening of gaps at sites of endothelial cell-cell contacts. There are multiple indicators of systemic inflammation in SCD [5]. In addition, markers of vascular inflammation have also been documented [6–8]. There is increased expression of adhesion molecules in the pulmonary endothelium of the Berkeley sickle mice [9], although the histology of these same mice shows less severe inflammatory and ischemic changes and no evidence of pneumonia [10]. Nonetheless, they spontaneously develop pulmonary hypertension [11], which is a major problem in SCD [12]. Pulmonary edema and the acute chest syndrome implicate increased vascular permeability in both chronic and acute complications of SCD [13, 14]. Despite this significance, there is currently no knowledge of the vascular permeability phenotypes of major organs that are impacted by SCD.

2. Materials and Methods

2.1. Transgenic Sickle Mice. Experiments were performed using protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. The Berkeley [15] and Townes [16] transgenic SCD mouse models used have previously been described.



(a)



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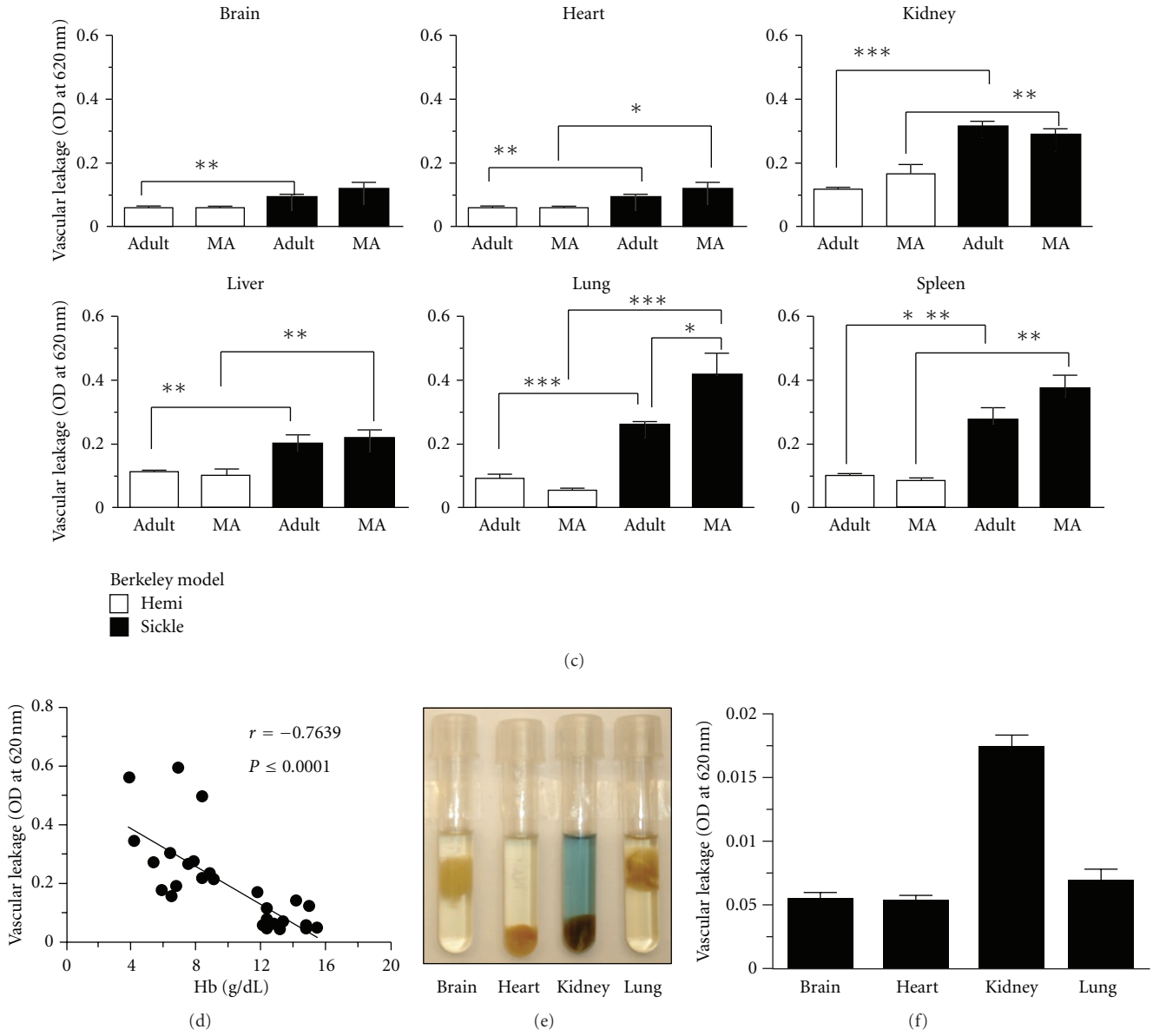

Figure 1: Vascular barrier dysfunction in sickle mice. (a) Representative images of organs isolated from sickle mice after injection with Evans blue dye and incubation in formamide for three days. (b, c) Vascular leakage in the indicated organs in adult (3–6 months) and middle-aged (10–13 months) mice of the Townes (heterozygotes-HbAS ($h\beta^A/h\beta^S$) and homozygote sickle-HbSS ($h\beta^S/h\beta^S$)) and Berkeley (hemizygotes and sickle) models. The number of mice studied was as follows: Townes: $n = 3$ for each genotype and age group; Berkeley: Sickle adult, $n = 9$; sickle middle-aged $n = 6$; hemizygote adult, $n = 10$, hemizygote middle-aged $n = 5$. (d) Vascular leakage correlates with hemoglobin. Data shown is the vascular leakage in the lung for a total of 30 mice (15 sickle and 15 hemizygotes) of the Berkeley model. (e) Typical images for the indicated organs isolated from young (4–6 weeks old) Berkeley mice injected with Evans blue dye and incubated in formamide for 3 days. (f) Histogram showing the quantification of Evans blue extravasation of major organs in young sickle mice ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

2.2. Vascular Leakage and Lung Edema. Vascular leakage was studied by intravenous injection of cell-impermeable Evan's Blue dye as widely described by several investigators. Mice were injected with 100 μL of 1% cell-impermeable Evans Blue dye (Sigma-Aldrich, St. Louis, MO) in PBS intravenously through tail vein. After 40 min, mice were anesthetized by i.p. injection of avertin (300 mg/kg body weight). To remove the dye from circulation, mice were perfused by injecting 40 mL of PBS containing 2 mM EDTA through left ventricle of the heart allowing the blood to flow out by puncturing renal artery. Organs were harvested and incubated in formamide for 3 days to extract the dye and OD determined at 620 nm. For edema analysis mice were euthanized and the right lobe removed and weighed immediately using an isometric

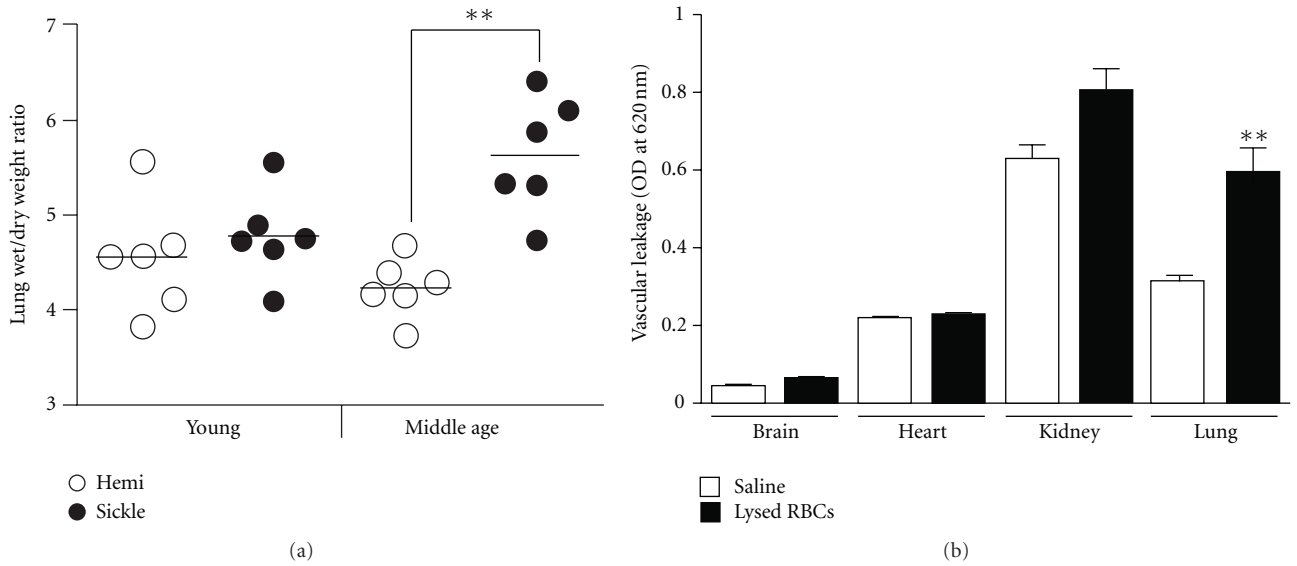

FIGURE 2: Chronic and acute changes in vascular permeability in sickle mouse lungs. (a) Lung edema in middle-aged Berkeley sickle mice as determined by wet/dry weight ratios ($n = 6$). Control groups include young sickle mice ($n = 6$) and young ($n = 6$) and middle-aged ($n = 6$) hemizygotes. (b) Vascular leakage in the indicated organs in Berkeley sickle mice intravenously injected with lysed red blood cells. Note that permeability is significantly increased by lysed red blood cells in the lung but not in other organs. $^{**}P < 0.01$.

transducer (Harvard Apparatus, Holliston, MA). Lungs were dried in an oven at 80°C containing desiccant crystals for 24 h, dry weight determined, and ratios calculated.

2.3. Statistical Analyses. GraphPad software version 5.0 was used. Differences in vascular leakage and weights were analyzed using *t*-test and correlation studies performed using Pearson's test.

3. Results and Discussion

In SCD, the adhesive and vasomotor defects of the vasculature are well defined [17–20], while the vascular permeability remains poorly appreciated. To address this knowledge gap, adult (3–6 months) and middle-aged (10–13 months) mice were injected with 1% Evans blue via the tail vein and the amount of dye that leaked from the circulation into the parenchyma of individual organs examined. Figure 1(a) shows virtually no leakage in the brain contrary to the clear evidence of endothelial barrier breakdown in the other organs. Quantification of vascular leakage revealed that the endothelial barrier is generally more permeable in the sickle mice than in control littermates (Figures 1(b) and 1(c)), despite some differences in the two transgenic models. Indeed, there was a significant correlation between steady-state hemoglobin concentration and lung permeability (Figure 1(d)) ($r = -0.7639$, $P < 0.0001$), indicating that endothelial barrier dysfunction is related to an aspect of SCD. Unlike most organs, vascular permeability in the lung in middle-aged sickle mice was significantly higher than in adult mice, highlighting a role for age in this disease process. This was investigated by extending our study to include younger mice aged 5-6 weeks. Remarkably, permeability in the heart and lung at this early stage of the disease was identical to that of the brain, which is widely known to have a highly restrictive barrier (Figures 1(e) and 1(f)). Thus, the endothelial barrier in SCD is normal in most organs in the young, becomes abnormal during adulthood, and deteriorates further with aging particularly in the lung.

Pulmonary edema is a common postmortem finding in SCD and yet it has not been appreciated as a chronic lung complication of SCD, probably because of the confounding effect of death [21, 22]. While histology does not reveal evidence of edema in the Berkeley sickle mice [10], we have used a more sensitive approach to clearly demonstrate that vascular permeability in the lungs of both the Berkeley and Townes sickle mice is increased. Our result is in agreement with a recent study that investigated permeability exclusively in the lung of 12–16-weeks-old NY1DD sickle mouse using the same approach [23]. In agreement with these permeability findings, we discovered that the average wet lung weight of middle-aged sickle mice is significantly heavier than that of age-matched hemizygotes (0.64 mg ± 0.04 versus 0.54 mg ± 0.02; $P = 0.03$), and, accordingly, the wet/dry weight ratio, widely used to confirm edema in lungs, was significantly higher ($P = 0.002$) (Figure 2(a)). Taken together, these results show for the first time that middle-aged sickle mice develop pulmonary edema. We cannot exclude the possibility that chronic heart failure contributed to the lung edema reported here; however, the Berkeley mice used in the lung weight measurements were at least three months younger than those reported to have heart failure [11]. Importantly, we show that lung edema correlates with higher vascular permeability in sickle mouse lungs. This concordance advances both conceptual and practical research objectives. It suggests

that the permeability phenotypes of the lung in mice and humans with SCD are similar, and it validates, for the first time, the use of the Evans blue extravasation approach to study vascular permeability in transgenic sickle mice.

The permeability phenotypes identified here likely reflect the intrinsic properties of individual organs, as well as of endothelial cell types. For instance, it is well established that heterogeneity of endothelial cell junction contributes to unique permeability attributes [24], and this may account for some of the dramatic differences in permeability phenotypes reported here (e.g., brain and lung). However, endothelial cells in individual organs may also respond differently to barrier disrupting factors found in SCD. Among these factors, cell-free hemoglobin is unique because it is released in abundance during acute intravascular hemolysis, which is a daily event in SCD. We assessed the response of major vascular beds in the sickle mice to acute hemolysis. Leukodepleted packed red blood cells were lysed by repeated freeze-thaw cycles and intravenously administered to sickle mice via tail vein. Compared to saline, the lysed red blood cells increased vascular permeability by 2-fold in the lungs of adult sickle mice but had a modest or negligible impact on the kidney, brain, and heart (Figure 2(b)). That lysed red blood cells caused morbidity and significantly altered barrier function in the Berkeley sickle mice indicates this and other severe models of SCD can be used to unlock mechanisms of acute hemolysis in SCD. In particular the vulnerability of the lung endothelial barrier to lysed red blood cells highlights acute intravascular hemolysis as a potential trigger of the acute chest syndrome since a decreasing concentration of hemoglobin is invariably associated with this condition [14].

In conclusion, SCD appears to be characterized by weakening of the endothelial barrier, which predisposes some organs, such as the lung to acute loss of barrier function, reminiscent of the acute chest syndrome. Ongoing studies are focused on unraveling the relationship between acute hemolysis and the endothelial barrier in SCD.

Authors' Contribution

S. Ghosh designed and performed most of the experiments. F. Tan characterized the transgenic mice and performed research. S. F. Ofori-Acquah designed the study and provided overall oversight of the projects.

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Research Article

Hematopoietic Stem Cell Function in a Murine Model of Sickle Cell Disease

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Previous studies have shown that the sickle environment is highly enriched for reactive oxygen species (ROS). We examined the oxidative effects of sickle cell disease on hematopoietic stem cell function in a sickle mouse model. *In vitro* colony-forming assays showed a significant decrease in progenitor colony formation derived from sickle compared to control bone marrow (BM). Sickle BM possessed a significant decrease in the KSL (c-kit$^+$, Sca-$^{1+}$, Lineage$^-$) progenitor population, and cell cycle analysis showed that there were fewer KSL cells in the G_0 phase of the cell cycle compared to controls. We found a significant increase in both lipid peroxidation and ROS in sickle-derived KSL cells. *In vivo* analysis demonstrated that normal bone marrow cells engraft with increased frequency into sickle mice compared to control mice. Hematopoietic progenitor cells derived from sickle mice, however, demonstrated significant impairment in engraftment potential. We observed partial restoration of engraftment by n-acetyl cysteine (NAC) treatment of KSL cells prior to transplantation. Increased intracellular ROS and lipid peroxidation combined with improvement in engraftment following NAC treatment suggests that an altered redox environment in sickle mice affects hematopoietic progenitor and stem cell function.

1. Introduction

Sickle cell disease (SCD) is one of the most common inherited hemoglobinopathies in the world. In the United States, approximately 1 in 600 African Americans have been diagnosed with SCD [1]. SCD is an autosomal recessive genetic disorder caused by a substitution of glutamic acid by valine in the beta subunit of the hemoglobin gene. This substitution results in the production of abnormal hemoglobin (HbS). Deoxygenated HbS polymerizes, resulting in intravascular hemolysis of the red blood cell and release of hemoglobin and other compounds into the plasma [2]. Repeating cycles of polymerization and hemolysis lead to vaso-occlusion and ischemia-reperfusion injury. Inherent in these processes are inflammatory responses and oxidant stress which result in pathological outcomes such as acute chest syndrome, pulmonary hypertension, and stroke in patients with SCD [3].

Oxidative stress is a result of increased production of reactive oxygen species (ROS) combined with decreased production or availability of antioxidants. Cellular metabolism of oxygen can lead to the production of ROS such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. ROS can impair the proper function of DNA, lipids, proteins, and carbohydrates [4]. An imbalance in the amount of pro-oxidants and antioxidants leads to an environment of oxidative stress, cell dysfunction, or cell death. Antioxidants

such as nitric oxide (NO), superoxide dismutase (SOD), and reduced glutathione (GSH) function to neutralize excess ROS.

There is increasing evidence that oxidative stress and ROS play a pivotal role in the pathophysiology of numerous diseases including neurodegenerative diseases, cardiovascular diseases, cancers, and arthritis [5–10]. Oxidative stress has been linked to vascular defects leading to hypertension and atherosclerosis as well as cardiac defects leading to contractile dysfunction and dysrhythmias [11]. In addition to the ability to induce mutations in DNA, ROS play a key role in cell signaling and cell regulatory pathways and thus play a pivotal role in the development of tumors and malignancies [12–16].

In SCD, under low-oxygen conditions, HbS polymerizes leading to hemolysis and a significantly shortened lifespan [17]. Hemolysed RBCs release hemoglobin, iron, and arginase into the plasma resulting in decreased nitric oxide availability and leading to imbalanced vascular homeostasis and oxidative stress [17–19]. Sickle RBCs generate more superoxide, hydrogen peroxide, and lipid oxidation products compared to normal RBCs [20]. Increased ROS in platelets and polymorphonuclear neutrophils along with decreased glutathione levels has also been documented in patients with SCD [21]. In addition, endothelium exposed to sickled RBCs become activated, causing sickled RBCs and leukocytes to adhere to the activated endothelium, resulting in a release of cytokines and ROS [6, 22, 23]. In this study, we studied the effects of this pro-oxidant and proinflammatory environment on hematopoietic progenitor and stem cells in the Berkeley model of sickle cell disease.

2. Materials and Methods

2.1. Animals. Sickle mice, originally supplied by Dr. Pászty [24], express exclusively human α-, β^{sickle}, and γ-globin and exist on a mixed genetic background (FVB/N, 129, DBA/2, C57BL/6, and Black Swiss). Breeding and pregnant sickle mice were fed TestDiet no. 0007573 (Purina). The colony is maintained by breeding female hemizygous mice with 2 copies of the transgene to homozygous male mice. The resulting pups are hemizygous, expressing one (H-1) or two (H-2) copies of the transgene, one copy of murine beta globin, but no expression of murine alpha globin. Homozygous "sickle" mice that only express human gamma, alpha, and beta^{sickle} globins are easily distinguishable from wild type and the H-1 and H-2 hemizygotes by hemoglobin electrophoresis (Figure 1) [24, 25]. H-1 mice have ~28% sickle hemoglobin and H-2 mice have ~45%. Despite having only one copy of the sickle hemoglobin transgene, H-1 mice are hematologically more severe (Figure 1). H-2 and C57BL/6 were primarily used as control mice for the homozygous mice. All breeding and experimental procedures were performed at Emory University in accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC).

2.2. Flow Cytometry. Bone marrow was harvested and stained with the following fluorochrome-conjugated antibodies: CD45.1-PE, CD45.2-APC, c-kit-FITC, Sca1-PE, and Lineage-APC (B220, CD3, CD11b, GR-1, and Ter119; BD Pharmingen, San Diego, CA), and KSL cells were sorted on a FACSAria. Cells from the peripheral blood were analyzed at various time points on an LSR II flow cytometer (BD Biosciences, San Jose, CA). Propidium iodide was used to exclude dead cells. For ROS analysis, cells were incubated in 160 μM Dichlorodihydrofluorescein diacetate (H_2DCFDA) (St. Louis, MO, Sigma-Aldrich) or 5 μM *N*-(fluorescein-5thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (DHPE) (Carlsbad, CA, Invitrogen) in HBSS for 15 min or 60 min, respectively, at 37°C shaking in the dark. Cells were washed and analyzed by flow cytometry. Absolute cell counts were performed using Trucount tubes (BD Biosciences).

2.3. HPP Assay. Twenty thousand freshly isolated bone marrow mononuclear cells were plated in methylcellulose medium (StemCell Technologies Inc., Vancouver) containing recombinant cytokines (100 ng rat steel factor, 1600U m-CSF, 75U IL-3, 5000U IL-1α, 30 ng/mL EGF, and 30 ng/mL FGF) to analyze the colony-forming potential of stem cells derived from the bone marrow of sickle and control mice. Cells were cultured for 10 days, and colonies were counted using an inverted microscope.

2.4. Hemoglobin Analysis. Differential hemoglobin electrophoresis of peripheral blood from Berkeley sickle mice was performed to determine hetero- and homozygosity of the murine and human globin genes (Helena Titan III electrophoresis system, Helena Laboratories, Beaumont, TX) [26]. As shown in Figure 1, C57BL/6 mice express the murine beta globin "single" allele, the H-1 and H-2 hemizygotes express increasing amounts of human beta^{sickle} globin in the presence of the murine allele expressing "diffuse" beta globin, and the mice homozygous for the deletions of murine alpha and beta globins express only human beta^{sickle} globin.

2.5. Transplants

2.5.1. The Effect of the Recipient Environment. To test the effects of transplanting into the sickle microenvironment, 1×10^7 bone marrow cells from C57BL/6 mice (expressing CD45.2) were transplanted into homozygous sickle ($n = 8$) and B6.SJL-Ptprc^a Pepc^b/BoyJ mice ($n = 10$; both expressing CD45.1) without ablation. While sickle and C57BL/6 share the MCH class I allele H-2K^b, their genetic backgrounds are not identical and therefore are unlikely to share the full haplotype. We have previously shown that T-cell costimulation blockade promotes engraftment across allo-barriers in this model, and it was therefore included in this protocol. Specifically, 500 μg each of hamster antimouse-CD40L (MR1; BioExpress, Lebanon, NH) and human CTLA4-immunoglobulin (generous gift from Dr. C. Larsen) was given intraperitoneally on days 0, 2, 4, and 7 relative to BMT.

2.5.2. Competitive Repopulation Study. To test the repopulation capacity of individual stem cell populations, three

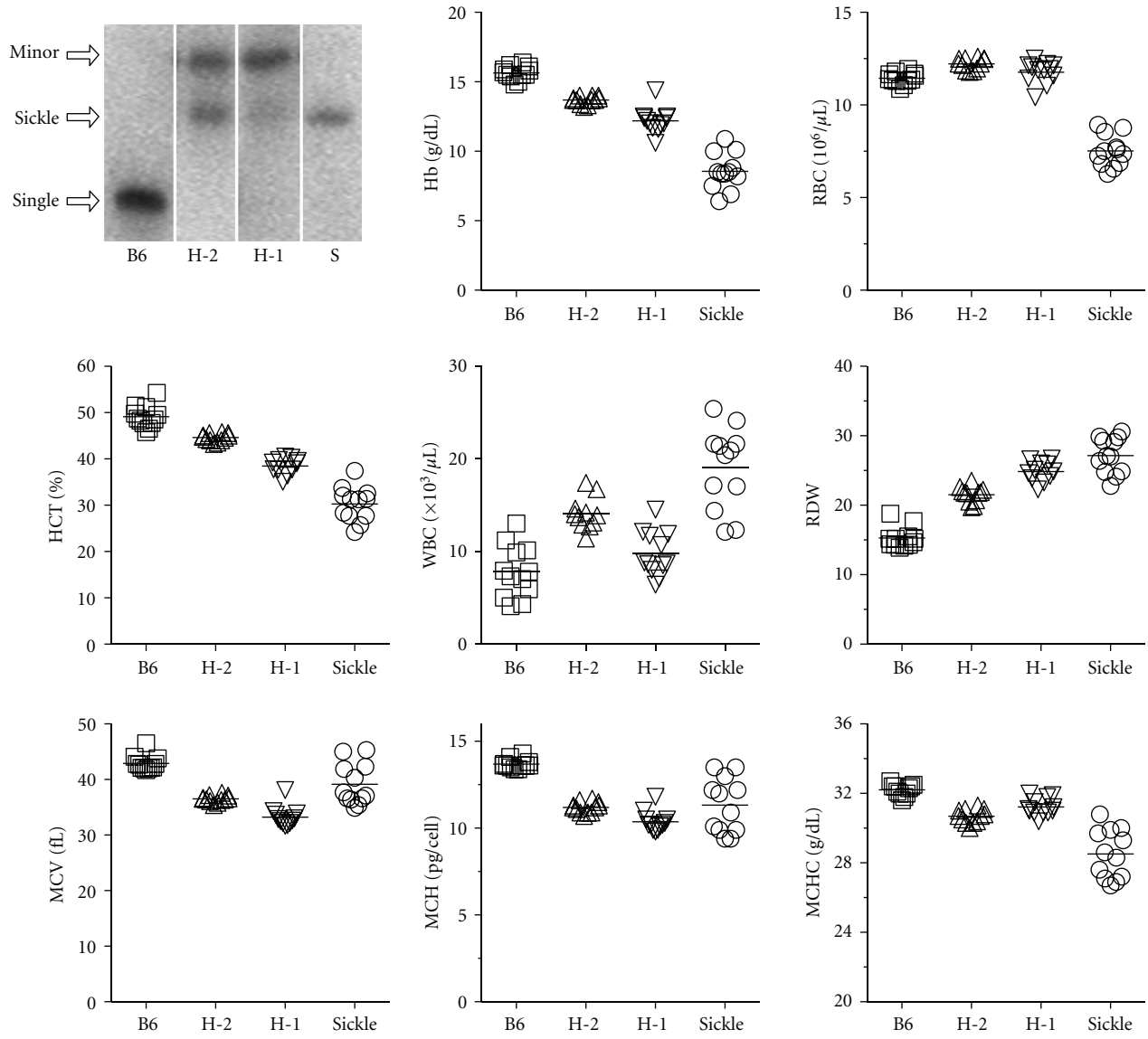

Figure 1: Hemoglobin and hematology profile of Berkeley sickle mice. Hemoglobin electrophoresis of RBC from C57BL/6, H-2, H-1, and homozygous sickle mice. C57BL/6 mice show a "single" hemoglobin band, whereas the H-2 and H-1 hemizygotes show beta-sickle globin resulting from two or one copy of the transgene, respectively, in combination with the minor band of diffuse beta-globins. Homozygous sickle mice only have the characteristic band of beta-sickle globin. The complete blood counts of C57BL/6 mice, H-2, H-1, and homozygous sickle mice ($n = 10$ per genotype) are also shown with the hemizygous mice having intermediate values between C57BL/6 mice and homozygous sickle mice.

thousand KSL cells derived from homozygous H-2 sickle, or B6.SJL-Ptprc^a Pepc^b/BoyJ, were sorted using a FACSAria (BD Biosciences, San Jose, CA) and were cotransplanted with 2×10^6 competitive bone marrow cells derived from C57BL/6 mice. Due to the potential differences in background, F1 recipient mice were generated by breeding H-2 sickle mice (expressing CD45.1) to C57BL/6 mice (expressing GFP under the beta globin promoter and CD45.2). These recipient mice expressed GFP+, CD45.1, and CD45.2 double positive cells (F1 mice). All recipients ($n = 4$ each group) were conditioned with two doses of 550 cGy total body irradiation on the day of BMT. To test the ability of the glutathione precursor to alleviate oxidant stress in the selected stem cell populations, groups of donor mice were also treated for four weeks with 0.163 g/L n-acetyl cysteine (NAC) in their drinking water (fresh NAC drinking water was made every two days). Mice were 12 weeks of age at the time of bone marrow harvest and transplantation.

3. Statistics

All statistical comparisons were performed using GraphPad Prism software utilizing one-way ANOVA with a Tukey posttest analysis unless otherwise stated ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

4. Results

4.1. Stem Cell Number and Function. Absolute cell counts were performed for stem/progenitor cells that were negative for markers of mature hematopoietic cells (T-cells, CD3; B-cells, B220; RBC, Ter-119; Myeloid cells, GR-1/Mac-1) while being positive for c-kit and Sca-1 (KSL, Figure 2(a)). Sickle mice had significantly fewer stem cells than control mice. We further quantified the number of a defined hematopoietic stem cell population (HSC; KSL/CD150+/CD48−) [27]. These cells were also significantly reduced in homozygous mice compared to controls (t-test, Figure 2(b)). As there were less stem cells in the bone marrow, we investigated the cell cycle status of HSC as a reduction in the number of quiescent cells (G_0 of the cell cycle) has been associated with mobilization of HSC in normal tissues, and HSCs are reported to be mobilized in sickle cell patients [28]. Figure 2(c) shows a reduced number of cycling KSL cells suggesting a reduced number of quiescent stem cells in the bone marrow. We then tested the functional capacity of KSL cells using the *in vitro* high proliferative potential (HPP) assay. Sickle-derived stem cells formed significantly fewer colonies compared to control ($P < 0.001$, Figure 2(d)).

4.2. Oxidant Damage to Bone Marrow-Derived Cells. Using flow cytometric analysis and DCF and DHPE dyes, we measured the intracellular content of ROS and lipid peroxidation, respectively, in KSL cells. H_2DCFDA becomes deacetylated by intracellular esterases as it crosses the membrane and becomes brightly fluorescent once oxidized by ROS producing DCF [29]. DHPE loses its fluorescence upon reaction with peroxy radicals [29]. Sickle-derived KSL cells demonstrated significantly increased lipid peroxidation and ROS compared to those derived from normal and hemizygous mice ($P < 0.05$, Figures 2(e)–2(f)). Importantly, in all of the above assays, the hemizygous mice showed an intermediate phenotype that correlated to their hematological defect.

4.3. Engraftment in the Sickle Microenvironment. We then addressed the issue of whether the sickle bone marrow environment was more conducive to engraftment by donor cells. To compare the engraftment efficiency of bone marrow cells into control (C57BL/6) and homozygous sickle mice (sickle), we transplanted 1×10^7 control male bone marrow cells into mice receiving only costimulation blockade. As early as four weeks after transplantation, there was a significant increase in engrafted donor cells in the sickle mice compared to the control mice ($P > 0.001$, Figure 3(a)). This level of engraftment is remarkable considering the nonablative protocol, and the continued increase with time suggests that donor cells have a survival or proliferation advantage in the sickle environment. Peripheral RBC markers also showed correction of sickle hematology towards control levels indicating the survival advantage of normal over sickle RBC (Figures 3(b)–3(d)) [30].

4.4. Engraftment Capacity of Sickle Hematopoietic Cells. We then compared the engraftment potential of KSL cells derived from control, hemizygous, and homozygous sickle mice in a competitive repopulation assay to determine if sickle-derived KSL cells were functionally impaired. First, we bred male hemizygous sickle mice to female C57BL/6 mice to generate mice that expressed both CD45.1+ and CD45.2+ antigens on the surface of their cells (F1) allowing us to distinguish donor and host KSL as well as donor BM competitor cells. By HPLC, we confirmed that host F1 mice carried one copy of the human β sickle globin gene, as well as murine α and β globins (data not shown). For each experiment, KSL cells were derived from untreated, as well as NAC treated, control, hemizygous (H-2), and homozygous mice and transplanted into lethally irradiated F1 mice. Peripheral blood was analyzed at 4, 8, 12, 16, and 24 weeks after transplantation (Figure 4(a)). In two separate experiments, homozygous sickle-derived KSL cells demonstrated significantly reduced engraftment capabilities compared to control mice. NAC treatment improved engraftment of homozygous sickle KSL cells but did not fully correct the defect (Figure 4(b)).

5. Discussion

Despite the benefits of hydroxyurea, people with SCD have limited treatment options with the only curative treatment remaining hematopoietic stem cell transplant (HSCT). While the numbers of children receiving transplant have continued to grow and now total in the hundreds, the majority are still performed with myeloablative conditioning (recently reviewed in [31]). The outcomes of these transplants in children are generally good with high levels of disease-free survival. However [32], there are significant concerns regarding long-term toxicities and complications from the preparative regimen, especially with regard to continued CNS complications and gonadal toxicities [33].

The continued desire to be able to offer transplant to a much larger selection of patients, including adults, has driven a number of trials utilizing a variety of nonmyeloablative protocols [34–36]. Most recipients have not had sustained engraftment, but Hsieh et al. successfully transplanted a small group of adults with mobilized peripheral blood from matched sibling donors [37]. These limited successes, continued issues with posttransplantation immunosuppression, and the limited number of HLA-matched donors will drive alternative transplant protocols and techniques for therapy with genetically corrected autologous cells.

These are similar findings to those in the murine model of Ataxia telangiectasia (ATM), which also demonstrated significant impairment of stem cell populations at least partially due to ROS and oxidative stress [38, 39]. Ataxia telangiectasia is a rare, recessive genetic disorder that causes neurological degeneration. The *Atm* gene controls DNA repair, cell cycle, and redox homeostasis. Ito et al. found that bone marrow-derived stem cells from *Atm* knock-out mice possess increased ROS and activated p38 MAPK that resulted in a reduction in KSL number, decreased colony-forming potential, loss of quiescence, and defective self-renewal capacity [38]. Similarly, sickle bone marrow has reduced colony-forming potential, and fewer sickle HSCs are in G_0 suggesting

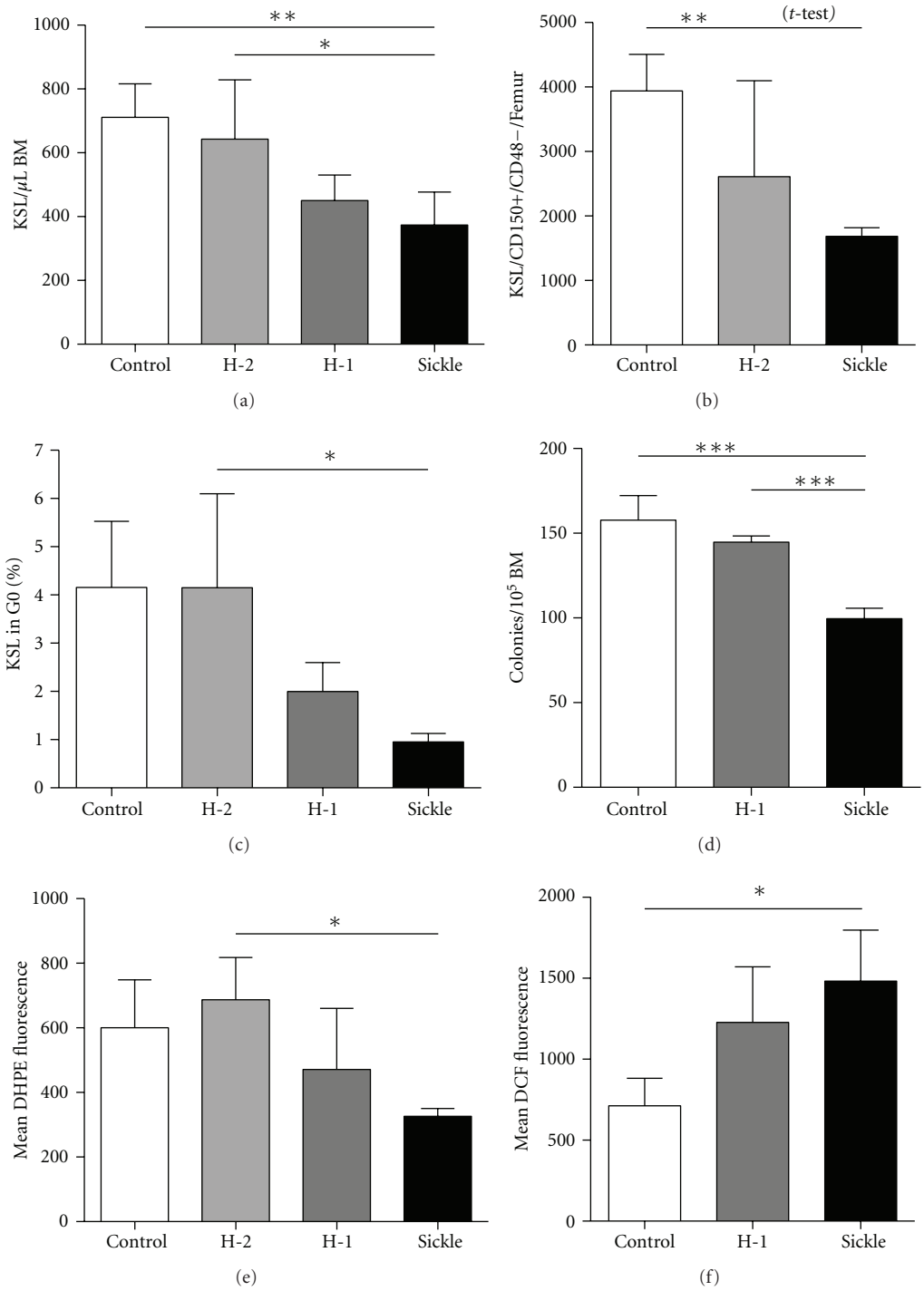

Figure 2: Stem cell number and oxidant state in Berkeley sickle mice. (a) Quantification of KSL progenitor cells in the bone marrow of control, hemizygous, and homozygous sickle mice showing a significant reduction in number in homozygous sickle mice. (b) Further examination of a phenotypically defined HSC population (KSL/CD150+/CD48−) also shows a reduction of HSC in sickle BM. (c) HSCs in the stem cell niche are known to be quiescent, and homozygous mice again show significant reduction in KSL cells in the G_0 phase of the cell cycle possibly indicating the mobilization of sickle progenitor cells. (d) The *in vitro* colony-forming, high proliferative potential assay (HPP) of stem/progenitor cells further indicates a significant reduction in colony-forming cells in homozygous mice. (e) To examine the effect of oxidant stress on hematopoietic progenitors, we measured lipid peroxidation using the fluorescent indicator DHPE. A reduction in fluorescence indicates increased activity of hydroxyl radicals. (f) Further quantification of reactive oxygen species was shown in sickle mice by the increased production of DCF (all graphs are presented as mean ± SD, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

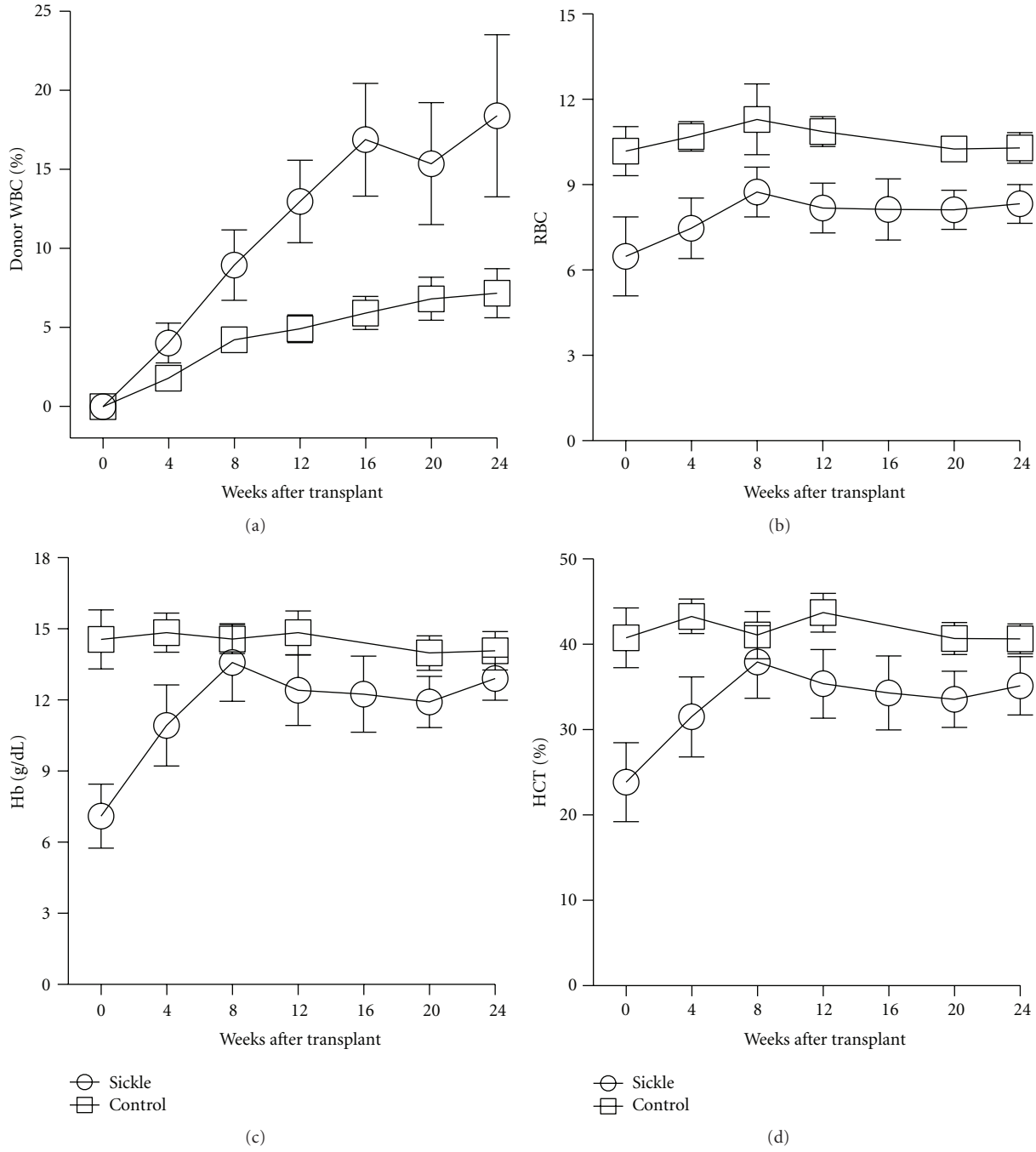

Figure 3: Preferential engraftment in sickle mice. (a) Peripheral WBC populations from normal mice engraft in homozygous sickle mice at a significantly ($P < 0.01$) faster rate than control mice when transplanted without ablation but in the presence of costimulation blockade. The levels of WBC engraftment coincide with (b)–(d) corresponding correction of RBC, hemoglobin, and hematocrit in recipient mice.

that stem cells are mobilized. This is consistent with the high peripheral WBC count in both mice and humans and previous reports of mobilization in SCD.

The oxidant damage of murine sickle HSC further translates into a functional defect in a reduced activity in a competitive repopulation assay. Interestingly, both hemizygote and homozygotes were affected. Antioxidant therapy in the form of NAC provided partial correction of the phenotype. Similar results were found in the ATM model where six weeks of treatment with either NAC or catalase antioxidants restored $Atm^{-/-}$ HSC CFU ability to near normal levels [38]. *In vivo* full HSC engraftment was only achieved when recipient mice were also treated with NAC. Oxidant-mediated HSC dysfunction is commonly seen in number of other model systems including Fanconi anemia ($Fancc^{-/-}$) where ROS lead to increased apoptosis of $Fancc^{-/-}$ cells [40]. A lack

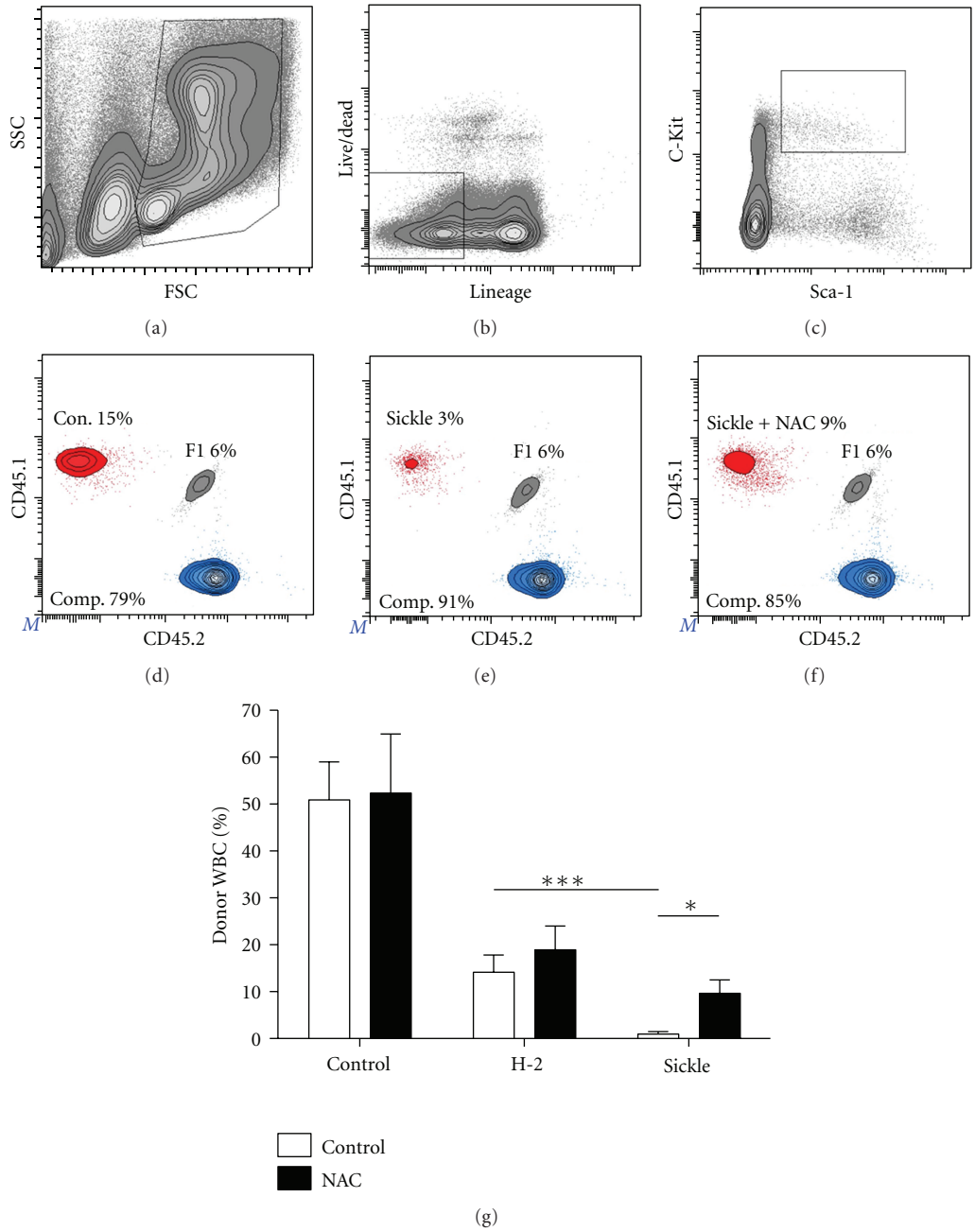

Figure 4: Functional defect of sickle hematopoietic stem cells in a competitive repopulation assay. Strategy for sorting KSL cells from whole bone marrow: mononuclear cells gated on FSC and SSC of whole BM (a), gated lineage and propidium iodide negative on mononuclear cells (b), and c-kit+, Sca1+, Lin− (KSL) cells (c). Peripheral blood engraftment after transplantation. Representative flow cytometry plots showing KSL cells from control mice engrafted at 15% (d), sickle KSL cells engrafted at 3% (e), and NAC- treated sickle KSL cells engrafted at 9% (f). Composite data showing a reduced capacity for engraftment between hemizygous and control mice and a further defect in KSL cells from homozygous mice 24 weeks after transplant (g). Graphical representation of the data from all mice: pretreatment of donor cells with NAC partially restored engraftment of KSL cells from homozygous sickle cell mice.

of FoxO family members also leads to an increase in ROS and a reduction in both HSC number and reconstitution ability [41]. In both of these models, antioxidant therapy with NAC is able to reverse or ameliorate the defects. Oxidant mediated stem cell damage is not limited to hematopoietic cells, for example, Kim and Wong demonstrated an oxidant-mediated defect in $atm^{-/-}$ neural stem cells that was responsive to NAC [42]. It is interesting to speculate that the high levels of oxidant stress could affect other organ-specific stem cell populations, and that this might be an important factor in the ongoing repair of sickle-related organ pathology. The HSC defects should also be considered when designing gene therapy

protocols first, as there may be a reduced number of HSCs available for collection and secondly as the HSCs have a reduced engraftment potential prior to *ex-vivo* manipulation.

Clinically, patients with severe sickle disease, who would be a desirable target population for HSCT, are likely to encounter more complications during transplant due to ongoing disease-related pathology and inflammation. However, with successful immunomodulatory strategies our data would suggest that engraftment of HSC into the sickle environment itself should be successful, and donor cells may have a comparative advantage. This could be especially important if designing approaches based on mixed hematopoietic chimerism that have been successfully used in murine model systems [30, 43].

All of the major pathologic consequences of SCD such RBC lysis, endothelial activation, and vaso-occlusion either induce or exacerbate the production of ROS with subsequent effects being likely to contribute to further pathologic processes (recently reviewed in [3, 4]). Consequently, a number of investigators have studied the use of antioxidants in SCD, mostly focusing on RBC effects. Vitamins C and E and NAC have all been shown to reduce ROS and increase the levels of glutathione in sickle RBC and PMN *in vitro* [21]. Treatment of NAC reduced the formation of dense RBC and increased the levels of intracellular glutathione in RBC of patients with SCD; importantly this correlated with a reduction in the number of vaso-occlusive crises during the treatment period [44]. Similarly, treatment with NAC for 6 weeks reduced phosphatidyl serine exposure on the membrane of sickle RBC and the levels of cell-free hemoglobin [45] indicating a cellular effect for an oral antioxidant.

In summary, we describe the effects of sickle-mediated oxidant stress on the bone marrow environment and hematopoietic stem and progenitors and detail defects in HSC function that raise important concerns when designing future stem cell therapies for sickle cell disease.

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Clinical Study

Integrating Interactive Web-Based Technology to Assess Adherence and Clinical Outcomes in Pediatric Sickle Cell Disease

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Research indicates that the quality of the adherence assessment is one of the best predictors for improving clinical outcomes. Newer technologies represent an opportunity for developing high quality standardized assessments to assess clinical outcomes such as patient experience of care but have not been tested systematically in pediatric sickle cell disease (SCD). The goal of the current study was to pilot an interactive web-based tool, the Take-Charge Program, to assess adherence to clinic visits and hydroxyurea (HU), barriers to adherence, solutions to overcome these barriers, and clinical outcomes in 43 patients with SCD age 6–21 years. Results indicate that the web-based tool was successfully integrated into the clinical setting while maintaining high patient satisfaction (>90%). The tool provided data consistent with the medical record, staff report, and/or clinical lab data. Participants reported that forgetting and transportation were major barriers for adherence to both clinic attendance and HU. A greater number of self-reported barriers ($P < .01$) and older age ($P < .05$) were associated with poorer clinic attendance and HU adherence. In summary, the tool represents an innovative approach to integrate newer technology to assess adherence and clinical outcomes for pediatric patients with SCD.

1. Introduction

Sickle cell disease (SCD) is a genetic red blood cell disorder characterized by the sickling of red blood cells resulting in pain episodes, organ damage, risk for infections, and decreased life expectancy [1]. Care guidelines for SCD recommend that patients attend routine clinic appointments one to two times per year and more frequently if there are complications or if clinical monitoring is needed to assess tolerance to medications and other treatments [1, 2]. Preventative care such as flu shots, immunizations, and monitoring labs is also essential to effectively manage sickle cell disease [3]. Hydroxyurea (HU), which is used to increase fetal hemoglobin (Hb F), has been shown to decrease morbidity and mortality in patients with SCD [4]. Studies have indicated that daily oral HU use is associated with reduced pain crises, hospitalizations, acute chest syndrome, and transfusions and improved growth and health-related quality of life [5–9]. Thus, the consequences of nonadherence to clinic attendance and HU treatments result in increased morbidity [10], healthcare costs [11], and decreased quality of life [12].

There is limited research available examining treatment nonadherence in pediatric SCD. A meta-analytic review found a nonadherence rate for clinic appointments in pediatric populations of approximately 40% [13]. Similarly, nonadherence rates for clinic appointments in SCD range between 36%–44% [14, 15]. On the surface, it would seem that patients who attend their appointments would demonstrate higher levels of adherence to their treatment regimen; however, the relationship between clinic attendance and

treatment adherence appears to be complex. As an example, Finney et al. [16] found that the 48% of patients who kept their follow-up appointment had been nonadherent to their prescribed regimen. Thus, it is important to assess barriers to the treatment regimen even with patients who attend appointments regularly.

Research on medication adherence in SCD suggests higher rates for acute medications than daily medications. Dampier et al. [17] found that 85% of adolescents with SCD took analgesic medications on days when they experienced sickle-cell related pain. In contrast, studies found that 38%–60% of pediatric and adult patients were adherent to their prescribed days of home chelation therapy or deferoxamine usage [18, 19] and rates ranged from 12%–67% for young children with SCD taking prophylactic penicillin [20–22]. Studies of HU adherence in SCD have typically utilized small sample sizes or single measures of adherence (e.g., pill counts) [23]. Overall, these studies indicate variable rates of adherence and lower rates for long-term trials [23, 24]. For example, Zimmerman and colleagues discontinued HU in 12% of their participants due to nonadherence [24]. Data from our own clinic indicate that 30% of patients who were prescribed HU were discontinued due to nonadherence. Poor adherence with HU may have unintended consequences as the medication can be discontinued on the assumption that the patient is a nonresponder, while other patients may have their dose increased to a level that is toxic when they actually begin taking it. Standardized and multidimensional approaches to measuring adherence are needed to ensure that patients receive optimal benefits from the medication while also minimizing risks to patients.

Overall, research indicates low and variable adherence rates to different components of the SCD treatment regimen. Studies identifying barriers to adherence have indicated a clear link between adherence and poor clinical outcomes in pediatric populations [25]. In pediatric SCD samples, studies have identified the following as key barriers: competing activities, health status, patient-provider relationships, adverse clinical experiences, and forgetting [26]. Other studies have identified sociocultural barriers to adherence including developmental factors, transportation, and health literacy [27–29]. Multicomponent, behavioral, and educational interventions to promote adherence have been found to be well-established pediatric treatments, [30, 31]; however, a recent study indicates that the quality of the adherence assessment is one of the best predictors for improving clinical outcomes [25]. Newer technologies (e.g., computer-based, text messaging) represent an opportunity for developing high quality, standardized and cost-effective assessments of treatment adherence and clinical outcomes such as patient experience of care (e.g., communication with and responsiveness of staff, quality of information received, wait time, satisfaction, etc.), disease-specific outcomes (e.g., labs) and health-related quality of life [32]. These types of approaches have been used effectively in chronic illness populations including adults with hypertension, diabetes [33, 34], and pediatric asthma [35].

The current study represents a first step in integrating interactive web-based technologies in SCD clinical care. The aims of the current study were to pilot an interactive family-based web-based tool, the Take-Charge Program, to assess adherence and clinical outcomes including: (1) patient HU adherence, barriers to HU adherence, and potential solutions to improve HU adherence; (2) patient clinic attendance adherence, barriers to clinic attendance adherence, and potential solutions to improve clinic attendance; and (3) clinical outcomes (patient experience of care, sickle-related outcomes hemoglobin level, ANC, MCV, and percent fetal hemoglobin level for HU patients).

2. Participants and Methods

Data presented in this paper are from the baseline assessment of a larger longitudinal study being conducted at a tertiary urban pediatric medical center in the Midwest. Eligible participants were patients of a comprehensive SCD clinic, age 6 to 21 years (and their caregivers), and prescribed hydroxyurea (HU) therapy or referred by clinic staff for attendance problems. Patients who had significant health complications (e.g., acute illness, recent stroke) that would interfere with the completion of the study or significant cognitive or developmental disabilities were excluded due to the demand on participants to understand questions in the assessment. Of the 182 patients in the clinic, 98 were eligible based on the above criteria. To date, 47 patients have been enrolled in the study with 4 being withdrawn because they no longer met criteria; thus, data will be presented on the 43 participants in the sample. Potential participants were identified by the clinical staff and the research team confirmed eligibility criteria. All participants were approached during a scheduled clinic visit. After obtaining consent, data collection proceeded at that visit.

2.1. Measures. Patients and caregivers completed the following measures.

2.1.1. Background Information Form. This form summarized personal/family demographic information, including participant school/vocational history, parent education, family income, family transitions, and life events. In addition, self-report of pain frequency and intensity, and hospital and emergency room visits over the past year was collected for comparison with data collected from the medical record review.

2.1.2. Barriers to Care Questionnaire [36]. This validated and reliable 40-item questionnaire measures parents' report of encounters or situations that may interfere with access to care, use of care, the patient-physician experience, or adherence with medical instructions. Barriers are conceptualized as multidimensional and include pragmatics (logistics, cost), expectations about care, health knowledge and beliefs, marginalization and health care navigation skills.

2.1.3. The Take Charge Program (Web-Based Tool). A voice-automated interactive web-based assessment tool was developed based on questions from the Disease Management

Interview [37] and consultation from clinic staff. Caregiver and child dyads complete the tool which takes approximately 15–20 minutes. The measure included questions and prompts that enabled patients and caregivers to identify barriers to adherence to clinic attendance and HU from a standard list [26, 37] and at least one strategy for improving adherence. The voice-automation increased the validity as literacy was not required to complete the measure. The initial development of the Take-Charge Program has been described elsewhere [38]. During the second phase of development, patients and caregivers matched barriers and potential solutions for HU. Cognitive interviewing was used to ensure that questions were being understood as intended [36, 38]. The web-based tool assessed clinic attendance, hydroxyurea adherence, and patient experience of care. The Clinic Attendance Module assessed self-reported adherence to clinic appointments on a 10-point Likert-type scale with 10 representing perfect adherence. The hydroxyurea module assessed self-reported HU adherence on a 10-point Likert-type scale with 10 representing perfect adherence. Both modules also asked participants to select applicable barriers and a potential solution to address these barriers. The Patient Experience of Care Module, an adapted version of Krahn et al.'s [39] questionnaire using a 4-point Likert scale and 2 open-ended questions assessed (1) wait time in clinic, (2) understanding of treatment recommendations by healthcare team, (3) time spent with healthcare team, and (4) helpfulness of web-based tool. Once participants identified a potential solution to try, clinic staff or a member of the research team utilized a standardized problem-solving intervention adapted from Behavioral Family Systems Therapy [40] to help participants develop a specific plan to implement the solution.

2.1.4. Medical Record Review. Electronic medical records were reviewed to confirm participant's type of SCD and collect the following data: hemoglobin level, ANC, MCV, percent fetal hemoglobin, clinic attendance, ER visits, and hospitalizations. Information on the participant's prescribed treatment plan was also collected and verified with clinic staff.

2.2. Data Analysis. Data collected from the web-based tool and electronic medical record (EMR) were integrated into a single database. Descriptive statistics and frequencies were utilized to summarize demographics, health characteristics, health care utilization, self-reported adherence, barriers, potential solutions, and patient experience of care. Pearson product-moment correlation coefficients were computed to conduct exploratory analyses to assess the relationship between adherence to clinic visits (e.g., self-reported barriers on the Take-Charge Program, number of barriers), adherence to HU (e.g., self-reported barriers on the Take-Charge Program, number of barriers), demographics, patient experience of care and sickle cell related outcomes. All analyses were conducted in IBM SPSS Statistics 19 (SPSS: An IBM Company).

3. Results

3.1. Participants. Study participants included 43 youth with SCD ($M = 12.81+3.98$ years; 39.5% Male; 79.0% HbSS; 9.3% HbSC; 4.7% Hβ^+Thal; 7.0% other) and their primary caregivers (83.7% mothers; 7.0% fathers; 9.3% other). Additional demographic characteristics are reported in Table 1. This sample is representative of the total SCD clinic population, with the exception of hemoglobin type (fewer participants with HbSC) but is consistent with the fact that the majority of participants in the study were on HU therapy. With respect to clinical characteristics, 62.2% of the sample reported having six or less pain days in the past 12 months, and 61.5% reported missing 6 days of school for pain in the past 12 months. Emergency room (ER) visit and hospitalization data indicated that most participants had three or fewer ER visits (95.1%) or hospitalizations (90.5%) in the past 12 months (see Table 2).

3.2. Clinic Attendance. According to the data, approximately half of participants (47.5%) indicate that they "always come" when describing their clinic attendance over the previous 12 months. This self-reported adherence was similar to data obtained from the EMR which indicated that 55% of patients never missed an appointment during this same period. Although about half of patients missed at least one appointment, a higher percent (75%) understood that their SCD providers recommended clinic appointments at least twice a year, and participants attended 84.5% of all scheduled appointments. Top-rated barriers on the Barriers to Care Questionnaire (BCQ) were related to pragmatics (i.e., logistics, cost) ($M = 76.1$; SD = 15.7) and healthcare navigation skills ($M = 79.4$, SD = 22.4). When asked specifically about barriers to clinic attendance on the Take-Charge Program, participants reported the following barriers: transportation difficulties (22.5%), inability to take off from work/school (17.5%), forgetting (10%), waiting too long (7.5%), competing activities (e.g., sports; 5%), feeling tired (5%); dislike of treatments (2.5%), feeling it is unnecessary (2.5%), and other (10%). Other barriers included getting appointment dates and times confused, not having transportation vouchers/setup, and not having appointments available at a time that works with the family's schedule. For potential solutions, 47.1% chose an individualized solution; 29.4% reported that they would try scheduling their appointment at a better/different time; and 5.9% reported that they would try setting an alarm (e.g., phone).

Participants were asked to rate their adherence on a scale of 0 to 10 with 10 being no problems with adherence. The mean rating for the sample was 8.9/10 ($N = 40$; SD : 2.1). They were also asked to rate how many visits they miss per year on average. The mean for missed visits was 1.0 ($N = 40$; SD = 1.4). Exploratory analyses showed that a greater percentage of no shows over the 12 month period prior to study enrollment was positively related to (1) more self-reported barriers to clinic attendance on the Take Charge Program $r = .550$, $n = 40$, $P < .001$; (2) the number of visits required $r = .455$, $n = 39$, $P < .01$; (3) age $r = .317$, $n = 41$,

Table 1: Patient demographics.

	N (%)	Mean (SD)
Hemoglobin type		
HbSS	34 (79.0)	
HbSC	4 (9.3)	
Hb^{+}Thal	2 (4.7)	
Other	3 (7.0)	
Gender		
Male	17 (39.5)	
Female	26 (60.5)	
Age		12.81 (3.98)
Race		
African-American	43 (100)	
Grade in school, median		6th
Primary caregiver		
Mother	36 (83.7)	
Father	3 (7.0)	
Other relative	4 (9.3)	
Highest grade completed by caregiver		
High school	15 (34.9)	
Some college	14 (32.6)	
College degree	13 (30.2)	
Grad school	1 (2.3)	
Family income		
< $10,000	14 (33.3)	
$10,000–20,000	4 (9.5)	
$21,000–30,000	6 (14.3)	
$31,000–50,000	5 (11.9)	
> $51,000	13 (31.0)	

$P < .05$; and (4) marginally related to satisfaction with clinic visits $r = .323$, $n = 40$, $P < .052$.

3.3. Hydroxyurea Adherence. While less than a third of participants (26.7% saying "yes") reported that they "always took their HU," overall, participants rated themselves as an 8.8 on a 10-point scale for "how often do they take their medicines?" which converts to 88%. When asked about missing doses, participants reported that they missed an average of 1.3 doses of HU per week. Participants reported the following barriers to HU on the Take-Charge Program: forgetting (56.7%), not having the medication with me (26.7%), the medications running out (23.3%), yucky taste or smell (12.5%), upset stomach (12.5%), and being not sure why I take it (3.1%). Other barriers reported included not wanting to stop what the patient is doing to take medication and taking too many medications. For potential solutions, 20% chose to try putting the medication next to something they do every day (e.g., toothbrush, breakfast table); 15% chose to use an alarm clock or cell phone alarm; 15% chose to use a pill box; and 15% chose to use a calendar. Additional solutions selected were reminder calls, and coordinating better with child/caregiver. Also, clinic staff rated that approximately 40% of participants were adherent to medications based on clinical data but staff felt that the clinical data of the other 60% indicated nonadherence or that further monitoring was needed. Number of missed doses of HU during the previous two weeks was related to age, with older age being related to greater nonadherence $r = .372$, $n = 31$, $P = .036$ and greater number of barriers reported on the Take-Charge Program $r = .421$, $n = 31$, $P = .023$.

3.4. Clinic Integration. Nearly all participants and their parents (41/43) completed the web-based assessment tool while waiting to see the care team for their appointment. The tool collected accurate and complete data with minimal missing data (3 participants due to technical errors). The majority of participants (64.9%) rated the web-based tool as very helpful and another twenty-five percent (24.3%) rated it as a little helpful.

3.5. Clinical Outcomes

Patient Experience of Care. The majority of patients (82%) reported a reasonable wait time (43% not at all; 29% short time) and only 18% reported that their wait time for the visit

Table 2: Patient clinical characteristics.

	N (%)	Range
Pain days in past 12 months*		0–45
0 days	5 (11.6)	
1-2 days	9 (20.9)	
3–5 days	6 (14.0)	
6–10 days	7 (16.3)	
>10 days	16 (37.2)	
Missed school days due to pain in past 12 months+*		0–45
0 days	8 (19.0)	
1-2 days	6 (14.3)	
3–5 days	7 (16.7)	
6–10 days	9 (21.4)	
>10 days	12 (28.6)	
Hospitalizations in past 12 months+*		0–20
0	15 (35.7)	
1-2	19 (45.2)	
3	5 (11.9)	
4	1 (2.4)	
5	1 (2.4)	
20	1 (2.4)	
ER visits in past 12 months+*		0–20
0	8 (19.0)	
1-2	27 (64.3)	
3	5 (11.9)	
8	1 (2.4)	
20	1 (2.4)	

*$n = 37$
+*$n = 42$

was too long. With respect to the visit itself, approximately 79.5% of participants reported that they were satisfied with the amount of time the medical team spent with them during their visit. In addition, the majority of participants (82.1%) reported that what the medical team shared with them was very helpful. Specifically, participants reported that the medical information was helpful (16%), and they found discussions about the treatment plan (e.g., discussion around medicine dosage and test results; about steps she needs to take to stay healthy) very beneficial (12%). Some participants (38%) also reported that other things made the visit positive (e.g., toys, movies).

Sickle Cell-Related Lab Values. Lab values from the date of enrollment into the study (or within 30 days of enrollment) were obtained from an electronic portal that pulls data from the electronic medical record system. The mean hemoglobin level for participants was 9.7 ($N = 39$; SD = 1.28). For those participants on HU therapy, the mean percent fetal hemoglobin was 23.1 ($N = 26$; SD = 15.6), the MCV was 98.0 ($N = 28$; SD = 13.9), and the ANC was 4.2 ($N = 27$; SD = 2.44). There was not a significant relationship between lab values and patient satisfaction with the amount of time spent with the medical team or the helpfulness of the medical information shared during the visit.

4. Discussion

This study highlights the potential to efficiently integrate interactive web-based technology in a clinic-based setting to assess treatment adherence, patient experience of care, and disease-specific outcomes in pediatric SCD. This study is significant as it piloted an innovative and high quality assessment process for capturing adherence data, including the barriers to adherence and potential solutions for addressing these barriers. The data from the tool showed a number of interesting trends. First, the tool proved to be a useful means for collecting data to understand adherence to clinic visits. Self-reported adherence to clinic visits was consistent with data from patients' EMR as both sources revealed a 12-month clinic attendance adherence rate ("always coming" and "attending all visits") of approximately 50%. It should be noted that the number of missed visits and adherence may be relative given that patients had 2 to 17 visits scheduled over the course of the year based on the complexity of treatments and disease-related complications (e.g., hospital discharge followup). Taken together, these findings suggest that nearly

85% of all scheduled clinic visits were attended by participants in this study. Understanding barriers to nonadherence was also important, especially given that adherence to clinic visits does not take into account cancellations, same day cancellations or rescheduled visits as a nonattended visit. Finally, data from the Take-Charge Program was integrated with patients' EMR data providing a wealth of data to inform clinical practice in "real time."

The barriers to clinic attendance endorsed by participants were multifaceted and included logistical (transportation, getting off work), health care navigation skills (using calendars to manage multiple appointments and medications), socioeconomic (lack of insurance), and disease-related barriers (did not feel well). BCQ mean scores for this sample were consistent with mean scores for other pediatric populations with similar challenges (e.g., asthma, children with special health care needs) [36, 41]. Participants also identified potential solutions for improving adherence and attendance (which is the basis for a larger longitudinal intervention study). Additional potential risk factors for no-shows emerged from exploratory analysis of the data which found that nonadherence to clinic visits increased with age, more required visits, and self-reported barriers to attendance. These findings, though preliminary, further highlight the richness of the data and provide meaningful trends and a basis for prioritizing patients who may be in need of additional clinical supports to ensure patient engagement and attendance.

The Take-Charge tool was also piloted to better understand the subset of patients on HU (74% of the sample). Anecdotally, nonadherence to HU is cited as a problem for many children and adolescents with SCD although developmentally-appropriate approaches for children and adolescents are very limited. The findings support that the Take-Charge tool was useful for assessing relevant information from participants on HU related to their perceptions of how they are to be taking their medications, concerns about side-effects and other barriers. Data from the tool highlighted that approaches to working with patients around medication management will need to address organizational issues [18, 19] such as helping patients and parents use calendars, phone alarms, and emerging innovative technologies (e.g., pill cases that glow in the dark and beep) to reduce the potential for "forgetting" to take medications, getting them refilled, and packing them when away from home.

Qualitative data from participants further highlight the importance of implementing family-based strategies and the need to tailor them appropriately to individual needs. Medication side-effects (e.g., taste, smell, upset stomach) and lack of awareness (i.e., to address patients who are not sure why they are taking medication) are other important barriers to address. Interestingly, some of the reasons that are commonly considered for nonadherence (e.g., the probability for loss of hair, stigma, fear of blood draws, and that the medicine would not make a difference) were not endorsed by these participants but still may be important for patients who do not agree to try the medication or who show early signs of nonadherence. Several participants noted that transportation and lack of insurance were barriers not only to clinic attendance, but also to HU adherence, highlighting the pervasiveness of income and access to health care on adherence. The barriers that are assessed in the tool appear to have some clinical utility as more barriers were related to a higher number of missed doses of medicine during the previous two weeks prior to the study. This study also supports previous research which suggests that age should also be considered as a target in the clinical evaluation, given the potential for nonadherence to medication increased with age [10, 34]. Data support the potential for the tool to reliably assess adherence and other health utilization outcomes while fostering individualized and family-based solutions for addressing barriers to adherence to clinic visits and medication. Patient experience of care data on the clinical integration of the tool showed positive trends with at least 90% of participants endorsing satisfaction/helpfulness of the tool. It was positive that satisfaction with the clinic visit was also not compromised with >90% also endorsing satisfaction with the clinic visit.

The limitations of the study should be noted. First, given the pilot/feasibility nature of the study, only patients who were engaged in our clinic were included in the study. It will be important in future clinical research to understand and address the barriers of patients (perhaps via the web or other engaging methods) who have lost contact or who are unable to attend clinic because of barriers and risks to determine if more significant intervention is needed. Second, the study included a range of participants across a broad developmental level. In addition, since this was a family-based assessment and intervention study, individual reports of barriers for patients and caregivers were not collected. This limitation should be overcome in future studies by insuring that older patients identify individual barriers to treatment adherence. Third, patients were diverse in income, disease severity, and other factors. Replicating this study with a larger sample to better understand adherence within developmental and disease-related subgroups will be important. Fourth, data collection from the EMR was a challenge in this study as corresponding lab values were not available for all study visits. It will be essential to better coordinate lab draws and study visits and to ensure study funds to pay for corresponding labs so that data can be tracked over time. Finally, the data reported here are cross-sectional and some are self-reported in nature. Future research is needed to monitor clinical outcome data longitudinally and to assess the relationship between adherence and clinical outcomes over time.

A next step in advancing our research is to refine the tool. There were a number of "other" responses that received a high endorsement which justify some additional revision of existing screens to include additional response options. While this program has the potential to be used as a tool to help improve adherence and clinical outcomes during follow-up visits, a goal and a challenge will be to maintain high patient satisfaction with the program. As the program becomes further standardized, another goal will be to streamline the clinic integration process and to pilot it in other SCD clinic settings. In spite of these limitations the study's overall goal was met which was to integrate the

web-based tool within a clinic-based setting (rather than in a nonclinical research setting) and to assess adherence and clinical outcomes for pediatric patients with SCD.

Conflict of Interests

All the authors declare that they have no conflicts of interests.

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Research Article

Elevated Circulating Angiogenic Progenitors and White Blood Cells Are Associated with Hypoxia-Inducible Angiogenic Growth Factors in Children with Sickle Cell Disease

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We studied the number and function of angiogenic progenitor cells and growth factors in children aged 5–18 years without acute illness, 43 with Hemoglobin SS and 68 with normal hemoglobin. Hemoglobin SS subjects had at least twice as many mononuclear cell colonies and more circulating progenitor cell than Control subjects. Plasma concentrations of erythropoietin, angiopoietin-2, and stromal-derived growth factor (SDF)-1α were significantly higher in children with Hemoglobin SS compared to Control subjects. In a multivariate analysis model, SDF-1α concentration was found to be associated with both CPC number and total white blood cell count in the Hemoglobin SS group, suggesting that SDF-1α produced by ischemic tissues plays a role in mobilizing these cells in children with Hemoglobin SS. Despite having a higher number of angiogenic progenitor cells, children with Hemoglobin SS had slower migration of cultured mononuclear cells.

1. Introduction

Sickle cell anemia (Hemoglobin SS) is characterized by hemoglobin polymerization and the formation of inflexible sickled erythrocytes. Accumulation of sickled erythrocytes in the microcirculation causes acute vaso-occlusive events that lead to pain and acute organ injury. Chronic arterial vasculopathy, with intimal proliferation and arterial stenosis, can lead to complications such as stroke and pulmonary hypertension. The etiology of arterial stenosis in sickle cell anemia is poorly understood. We hypothesize that intimal proliferation in sickle cell anemia is due to abnormal reparative responses to ongoing vessel injury. Hemolytic anemia, vaso-occlusion, and abnormal flow dynamics in sickle cell anemia may contribute to vessel injury. Chronic intravascular hemolysis releases free heme, which binds avidly to nitric oxide (NO), causing NO depletion, and subsequent vasoconstriction and inflammation [1]. Erythrocyte-derived reactive iron and oxygen species are also directly injurious to endothelium [2]. Repetitive episodes of acute vaso-occlusion cause tissue ischemia and reperfusion, which also lead to inflammation and increased oxidative stress [3]. Evidence of ongoing inflammation and vascular injury is present in people with sickle cell anemia even when asymptomatic, with elevated levels of high sensitivity C-reactive protein (hsCRP) [4] and circulating endothelial cells [5].

Reendothelization after vascular injury is a critically important process to restoring and maintaining vascular homeostasis. Endothelial progenitor cells (EPCs) are recruited from the bone marrow and home to sites of vascular injury. Recruitment and homing of EPCs are intimately regulated by cytokines and growth factors released at the sites of vascular insult. Reduced numbers of endothelial progenitor colonies have been found in adults with cardiovascular risk factors [6], diabetes [7], and those with established cerebrovascular disease [8]. Cardiovascular disorders are also associated with functional impairments in EPC migration or angiogenesis [9]. Endothelial progenitor cells are elevated during acute myocardial infarction [10], stimulated by hematopoietic growth factors such as erythropoietin [11], granulocyte colony-stimulating factor (G-CSF), or granulocyte-macrophage colony stimulating factor (GM-CSF), and by treatment with HMG-CoA reductase inhibitors (statins) [12] or angiotensin-2 receptor antagonists [13].

To date, there is limited information about the number and function of EPCs or the growth factors involved in EPC recruitment and homing in people who have sickle cell disease. Van Beem reported elevated numbers of circulating EPCs (expressing CD34 and VEGFR2) in adults with Hemoglobin SS or Sβ^0-thalassemia during painful crisis, but there was no difference between asymptomatic adults with sickle cell disease and healthy controls [14]. The higher number of circulating EPCs during painful crisis was associated with increased serum levels of erythropoietin, soluble VCAM-1 (sVCAM-1), and vascular endothelial growth factor (VEGF).

Several angiogenic growth factors have been found to be elevated in Hemoglobin SS. Angiopoietin (Ang)-2 and erythropoietin were higher in adults with Hemgoglobin SS compared to healthy controls and further elevated during acute painful crisis [15]. Higher levels of vascular endothelial growth factor (VEGF) were found in subjects with Hemoglobin SS compared to controls in some studies [16, 17], but not in others [15]. When present, higher VEGF levels were found to be associated with reduced odds of elevated tricuspid valve regurgitant velocity by echocardiography in children with sickle cell disease, a noninvasive measure suggesting pulmonary artery hypertension [16]. Conversely, children with sickle cell disease with elevated tricuspid regurgitant velocity had higher concentrations of platelet-derived growth factor (PDGF)-BB. Higher levels of SDF-1 have been found in adults with Hemoglobin SS than controls, particularly in those who had pulmonary hypertension [18].

There is ongoing debate about the *in vitro* phenotype of endothelial progenitor cells. Circulating cells expressing hematopoietic stem cell marker CD34, vascular endothelial growth factor receptor (VEGFR)-2, and early progenitor marker CD133 have been considered to represent EPCs, though recent studies show that these cells were immature hematopoietic cells that did not differentiate into EPCs or form vessels [19]. In a study of the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on vascular function in adults with peripheral arterial disease, treatment-induced increase in the number of circulating CD34-expressing cells correlated with clinical improvements in flow-mediated dilation and pain-free walking time [20], suggesting that undifferentiated hematopoietic cells have angiogenic potential or are a surrogate marker of vascular repair cells. In this paper, we refer to the cultured cells as mononuclear cells and the cells measured from the peripheral blood as circulating progenitors cells (CPCs) with angiogenic potential.

Taken together, there is evidence that people with sickle cell disease have vessel injury and proangiogenic growth factor responses, but limited information about vascular reparative function in sickle cell disease. We hypothesize that vascular complications in people with sickle cell disease arise from altered repair mechanisms, most likely due to abnormal angiogenic cell functions. We expect CPC numbers to be normal or elevated, stimulated by high levels of erythropoietin that is seen with chronic anemia. We report here our findings of cultured mononuclear colony and CPC number in children with Hemoglobin SS versus healthy Controls, their relationship to plasma levels of angiogenic growth factors, and the migration of cultured mononuclear cells.

2. Materials and Methods

2.1. Blood Sample Collection. The study protocol was approved by the Institutional Review Boards of Morehouse School of Medicine and Children's Healthcare of Atlanta, and the Grady Hospital Research Oversight Committee. Written informed consent was obtained from each participant's parent or guardian and verbal assent from the volunteer before sample collection. Venous blood was collected from African American children aged 5–18 years old without symptoms of acute illness. *Controls* had Hemoglobin AA or AC, and sickle cell anemia subjects had *Hemoglobin SS* or Hb S-β^0 thalassemia. Children treated with hydroxyurea, recent red blood cell transfusion within the previous 90 days, or who had cardiovascular risk factors, such as overweight or obesity, cigarette smoking, diabetes, or hypertension, were excluded. Complete blood counts were performed by standard methods by the clinical laboratories used by the clinic sites.

2.2. Circulating Progenitor Cell Quantitation. Whole blood samples were labeled with monoclonal antibodies for FITC-conjugated anti-human CD34 (clone 8G12, 0.6 μg/mL final concentration, Becton Dickinson, Franklin Lakes, NJ), PE-conjugated anti-human VEGFR2 (clone 89106, 1.2 μg/mL, R&D Systems, Minneapolis, MN), PERCP-conjugated anti-human CD45 (clone 2D1, 0.6 μg/mL, Becton Dickinson), APC-conjugated anti-human CD133/1 (clone AC133, 0.85 μg/mL, Miltenyi Biotec Inc., Auburn, CA), and PE-Cy7 conjugated anti-human CXCR4 (clone 12G5, 0.5 μg/mL, eBioscience Inc., San Diego, CA), or their isotype controls at the same concentrations, and analyzed by FACS. Thirty microliters of antibody cocktail was added to 300 μL of whole blood, or 15 μL of the isotype control cocktail was added to 150 μL of blood. Samples were incubated in the dark for 15 minutes. Red blood cells were lysed by adding 1.5 mL of lysing solution (NH_4Cl 0.15 M, $KHCO_3$ 10 mM, EDTA 0.1 mM) into each tube. Lysis was stopped by adding 1.5 mL of staining media (phosphate-buffered saline [PBS]

without Mg^{++} or Ca^{++}, heat-inactivated fetal calf serum 3%, and NaN_3 0.1%) and mixing gently. Immediately before acquisition on a flow cytometer, 100 μL of Accucheck Counting Beads (PCB100, Invitrogen) were added and mixed gently. Cells were washed twice and resuspended in 500 μL of staining media. Samples were kept in the dark until run on the flow cytometer, within 4 hours of initial processing. Data are reported for cells with low (dim) CD45 expression (which excludes mature leukocytes).

2.3. Mononuclear Cell Colony Assay. Mononuclear cell culture was performed according to a modification of the protocol of Hill et al. [6]. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density fractionation and cultured on fibronectin-coated plates (Becton Dickinson) in M199 medium (Invitrogen, Carlsbad, CA) with fetal calf serum 20% (Invitrogen) and penicillin 100 U/mL and streptomycin 100 μg/mL (Invitrogen), at 37°C in 5% CO_2. The same lot of fetal calf serum was used throughout the study for all samples. After 48 hours, nonadherent cells were replated at a concentration of 10^6 per well in fibronectin-coated plates. Media were replenished every two days. Colony-forming units (>200 micron diameter) in each well were counted 7 days after replating. The reported number of colonies per well is the average of 3 wells per subject.

2.4. Immunofluorescent Staining. Mononuclear cells were seeded onto fibronectin-coated four-chamber slides (Becton Dickinson) at a concentration of 2×10^6 cells per chamber. Cells were grown to confluence over 14 days with media replenished every two days. Confluent monolayers were fixed with 4% paraformaldehyde in PBS. Cells were permeabilized with ice cold methanol for five minutes and blocked with goat serum 10% (Dako, Carpinteria, CA) for one hour. Cells were incubated overnight with murine primary antibodies directed against human CD31 (clone JC70A, 10 μg/mL final concentration, Dako), human endothelial nitric oxide synthase (-eNOS, clone 3, 1.25 μg/mL, Becton Dickinson), human CD14 (clone TUK4, 0.5 μg/mL, Dako) or isotype controls at the same concentrations (mouse IgG_1 or mouse IgG_{2a}, Dako), in PBS with goat serum 2%. Cells were washed with PBS and incubated for one hour with secondary antibodies (Alexafluor (AF)-488 goat anti-mouse IgG_1 or AF-594 goat anti-mouse IgG_{2a}, Invitrogen) at 1 : 500 dilution in PBS with goat serum 2%. Slides were counterstained with DAPI nuclear stain (Invitrogen).

2.5. Wound Migration Assay. Mononuclear cells were grown until confluent on fibronectin-coated plates for 14 days, as described above. An aseptic linear wound was made across the mononuclear cell monolayer using sterile 20 μL pipette tip. Digital photographs were taken at 0 and 24 hours after the wound was made. The wound area was measured for each time point using image analysis software (Image-Pro Plus v. 6.2 for Windows, Bethesda, MD). The difference in wound area between hours 0 and 24 was expressed as

$$\%\text{Area migrated} = \frac{\text{Area}_{\text{Hr}\,0} - \text{Area}_{\text{Hr}\,24}}{\text{Area}_{\text{Hr}\,0}} \times 100. \quad (1)$$

The average wound area of 3 wells was reported for each subject.

2.6. Angiogenic Growth Factor Assays. Quantitative enzyme-linked immuno-sorbent assay (ELISA) for human erythropoietin, angiopoietin-2, and SDF-1α (R&D Systems, Minneapolis, MN), and a Human Angiogenesis Assay Panel (BioRad, Hercules, CA) were used to measure plasma concentrations of growth factors. Results for erythropoietin were expressed as mIU/mL or pg/mL of plasma for the other growth factors. Each ELISA sample was run in duplicate.

2.7. Oxidative Stress Markers. Plasma concentrations of the redox pairs cysteine and cystine (Cys, CySS) and reduced and oxidized glutathione (GSH, GSSG) were measured by high pressure liquid chromatography (HPLC), and the redox potentials for the Cys/CySS and GSH/GSSG couples (E_hCySS, E_hGSSG, resp.) were calculated using the method of Jones [21]. This method includes sample preparation and storage procedures to reduce artifacts that can be caused by hemolysis or GSH thiol-disulfide exchange. Urinary 8-isoprostanes (8-iso-Prostaglandin-$F_{2\alpha}$) were measured by ELISA (Enzo LifeLife Sciences, Plymouth Meeting, PA).

2.8. Classification of Variables and Data

2.8.1. Outcome Variables. The main outcome variables were blood-derived mononuclear cell colonies and CPC populations. Total white blood cell (WBC) count was analyzed as an intermediate outcome variable.

2.8.2. Exposure Variables. The main exposure variable was Hemoglobin Group, defined as a categorical variable with two levels, Hemoglobin SS and Control. The other exposure variables were the clinical characteristics (age, weight, sex), peripheral blood counts, angiogenic growth factors, and oxidative stress markers.

2.9. Statistical Analysis. Data were summarized using frequencies and percentages for categorical variables and means (and standard deviation) for continuous variables. Data that were not normally distributed were transformed using natural logarithm transformation. The antilogarithms of the means of the transformed data are reported as geometric means.

2.9.1. Univariate Analyses. Simple linear regression models were fitted to determine the relationships between the number of each CPC type (outcome variables) and individual clinical variables or biomarkers (exposure variables).

2.9.2. Multivariate Analyses. Bivariate linear regression models were fitted to examine the relationship between hemoglobin group SS and number of CPCs, as well as the confounding effects of the other exposure variables. Confounders were defined as those variables causing at least 25% reduction in the β-coefficient compared to the main exposure variable alone. Multiple linear regression analyses were then performed to examine the relationship between

Table 1: Subject characteristics and hematologic parameters. There was no difference in age and sex of the participants in each group. Hemoglobin SS subjects had significantly lower hemoglobin concentration and higher WBC and platelet counts (indicated by asterisks (*)).

	Control			Hemoglobin SS			P value
	n	Arithmetic mean (95th% CI)	Geometric mean (95th% CI)	n	Arithmetic mean (95th% CI)	Geometric mean (95th% CI)	
Age (yrs)	68	12.8 (12–13.8)	12.3 (11.4–13.3)	43	12 (11–13)	11.5 (10.4–12.6)	0.26
Sex (males)	68	30 (44%)		43	20 (47%)		
Hemoglobin (gm/dL)	39	13.6 (12.1–15.2)	13.2 (12.4–14.1)	19	8.4 (7.8–9)	8.3* (7.7–8.9)	<0.001
WBC ($\times 10^3$/mL)	39	5.4 (4.9–6)	5.2 (4.7–5.7)	18	12.1 (9.6–14.4)	11.4* (9.7–13.4)	<0.001
Platelets ($\times 10^3$/mL)	39	280 (261–298)	274 (257–293)	18	413 (373–454)	406* (368–448)	<0.001

Table 2: Number of mononuclear cell colonies and CPCs for each Hemoglobin Group. Children with Hemoglobin SS had more mononuclear cell colonies and circulating progenitor cells than Controls. Mononuclear cell colonies are reported per well, and CPCs in cells/μL. Significant differences in geometric means between Control and Hemoglobin SS groups are indicated by asterisks (*).

Outcome variables	Main exposure variables (Hemoglobin Group)						Fold difference	P value
	Control			Hemoglobin SS				
	n	Arithmetic mean (95th% CI)	Geometric mean (95th% CI)	n	Arithmetic mean (95th% CI)	Geometric mean (95th% CI)		
Colonies	63	13.5 (8.5–18.5)	8.1 (5.8–11.5)	39	28.8 (16.1–41.6)	16.5* (11–24.6)	2	0.01
CD34	43	1.7 (1.3–2.1)	1.4 (1.2–1.7)	17	5.4 (2.3–8.5)	3.6* (2.3–5.6)	2.6	<0.001
CD34/CD133	43	1.0 (0.7–1.2)	0.8 (0.7–1.0)	17	2.0 (1.0–2.9)	1.3 (0.8–2.2)		0.1
CD34/CXCR4	43	0.6 (0.5–0.7)	0.48 (0.4–0.6)	17	3.2 (1.2–5.2)	1.66* (0.9–3.2)	3.5	<0.001
CD34/VEGFR2	43	0.12 (0.09–0.15)	0.08 (0.06–0.1)	17	0.68 (0.2–1.1)	0.36* (0.2–0.6)	2.6	<0.001
CD34/CXCR4/VEGFR2	43	0.2 (0.1–0.3)	0.1 (0.07–01)	17	0.6 (0.2–1)	0.29* (0.15–0.5)	2.9	0.002

hemoglobin group and number of CPCs, controlling for all confounding variables.

Similar univariate and multivariate analyses were performed using WBC as an intermediate outcome. All analyses were performed using STATA Data Analysis and Statistical software (College Station, TX), and level of statistical significance was set at 0.05.

3. Results

A total of 111 children were studied, 68 Controls and 43 with Hemoglobin SS. Clinical features are shown in Table 1. As expected, children with Hemoglobin SS had significantly lower hemoglobin concentration, and higher white blood cell and platelet counts than Controls. There were no significant differences in age or sex between the two groups.

3.1. Angiogenic Progenitor Cells. On average, twice as many mononuclear cell colonies were grown from the blood of children with Hemoglobin SS than healthy Control children (geometric mean 16.5 versus 8.1 colonies/well, $P < 0.05$) (Table 2). A subset of the cultured cells expressed platelet endothelial cell adhesion molecule (PECAM or CD31) and endothelial nitric oxide synthase (eNOS), two markers of mature endothelial cells (Figure 1). There was no significant difference in percentage of cultured cell expressing CD31 or eNOS between Hemoglobin SS and Control groups, and the cells did not express the monocytic marker, CD14 (data not shown).

Similarly, there were at least twice as many CPCs expressing CD34, CD34/VEGFR2, CD34/CXCR4, or CD34/CXCR4/VEGFR2 in the children with Hemoglobin SS compared to Controls (Table 2), but no difference in the number of cells expressing CD34/CD133. Circulating progenitor cells expressing CD34/CD133/VEGFR2 are not reported due to very low numbers. The differences between Hemoglobin SS and Controls were highest in CPC expressing CXCR4 (SDF-1α receptor). Thus, using two different assay methods, we found that asymptomatic children with Hemoglobin SS have a higher number of circulating angiogenic progenitor cells.

3.2. Angiogenic Growth Factors. Plasma concentrations of three angiogenic growth factors were significantly higher in children with Hemoglobin SS compared to Control subjects: erythropoietin (13.5-fold), angiopoietin-2 (4-fold), and stromal derived growth factor (1.7-fold) (Table 3). In the angiogenesis multiplex assay, there were no significant differences between Hemoglobin SS and Control subjects in plasma concentrations of vascular endothelial growth factor (VEGF), hepatic growth factor (HGF), interleukin (IL)-8, follistatin, platelet endothelial cell adhesion molecule (PECAM)-1, or platelet-derived growth factor (PDGF-BB) (20 subjects tested in each group) (data not shown). The concentrations of the three elevated angiogenic growth factors were found to be collinear, which is consistent with their common regulation by hypoxia inducible factor (HIF) (data not shown).

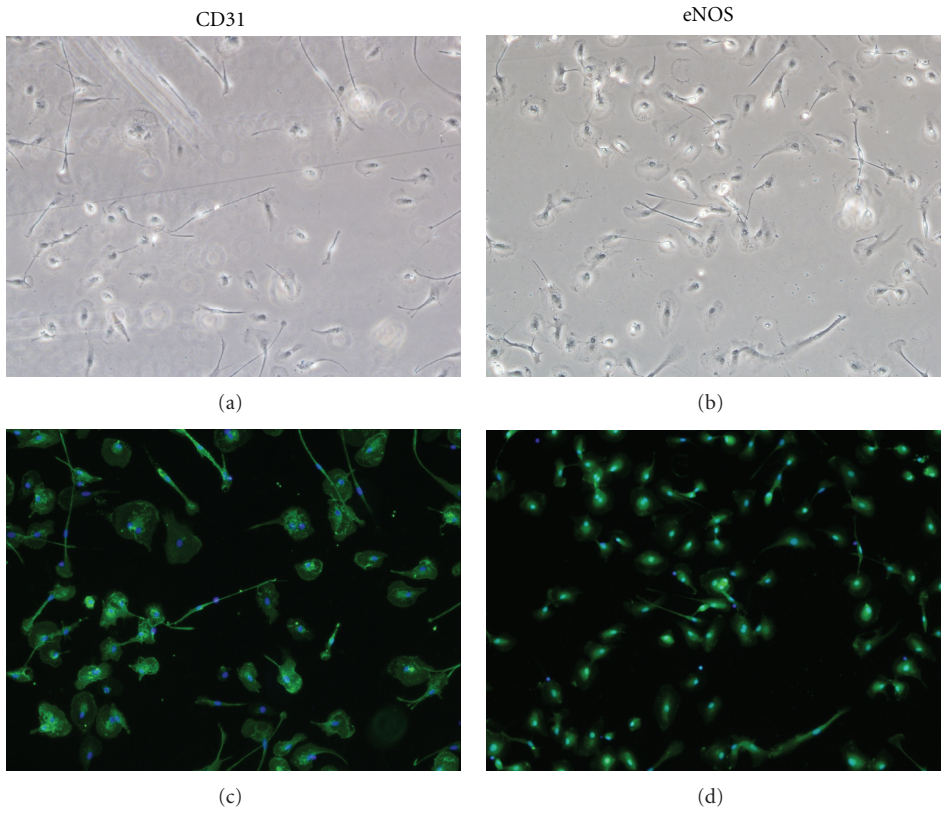

Figure 1: Immunofluorescent staining of cultured mononculear cells for endothelial antigens CD31 and endothelial nitric oxide synthase (eNOS). CD31 and eNOS staining was observed in a subset of cells. A representative sample from a subject with Hemoglobin SS is shown. (a) shows phase contrast image and (c) shows CD31 staining (green) with nuclei stained with DAPI (blue) in the same field. (b) Shows phase contrast and (d) shows eNOS staining (green) for the same sample.

3.3. Oxidative Markers. Both cysteine and cystine were significantly higher (1.5-fold) in the children with Hemoglobin SS compared to Controls. There was no difference in levels of reduced glutathione (GSH) between groups, whereas the oxidized form of glutathione (GSSG) was 2-fold lower in the children with Hemoglobin SS (Table 3). The calculated redox potential for CyS/CySS (E_hCySS) was significantly lower (more reduced) in the Hemoglobin SS group than Controls, but there was no difference in E_hGSSG between groups. Urinary isoprostane levels were also not different between groups.

3.4. Wound Migration. Migration across a linear wound was measured in a subset of mononuclear cell samples. Mononuclear cells from Hemoglobin SS subjects ($n = 5$) migrated over a significantly smaller percentage of the original wound area in 24 hours than cells from healthy Controls ($n = 8$) (28 versus 59%, respectively, $P < 0.01$) (Figure 2). The assay was not performed if samples had not grown to confluence within the 14–16-day culture period.

3.5. Multivariate Analyses. Supplementary Table 1 (available online at doi:10.1155/2012/156598) shows the results of univariate analyses. Circulating progenitor cell number was significantly associated with erythropoietin, angiopoietin, SDF-1α, hemoglobin concentration, WBC and platelet counts, and CySS and CySH levels. The strongest associations were found with total WBC, SDF-1α, or erythropoietin.

A multivariate model was then used to test the role of the exposure variables as possible determinants of the elevated number of CPCs in children with Hemoglobin SS. Total white blood cell count was strongly associated with all CPC types (Table 4). When CPC number was corrected per 100 WBC, CD34/VEGFR2-expressing cells remained significantly higher in the Hemoglobin SS group than Controls (mean 0.61 versus 0.25 per 100 WBC/μL, $P = 0.04$), but there were no differences for the other CPC types. To test for the effects of the angiogenic growth factors alone, WBC was excluded from the initial model.

Erythropoietin was associated with CD34 and CD34/CXCR4 numbers, and angiopoietin-2 was associated with CD34/CXCR4/VEGFR2 number. However, SDF-1α was consistently associated with the number of all CPC types in the Hemoglobin SS group (Table 5). SDF-1α in combination with either erythropoietin or angiopoietin-2 had slightly stronger associative effects. None of the other exposure variables or biomarkers, including total hemoglobin, reticulocytes, or platelet count were found to be associated with CPC number in the Hemoglobin SS group.

White blood cells were then analyzed as a possible intermediate outcome. In univariate analysis, total WBC was

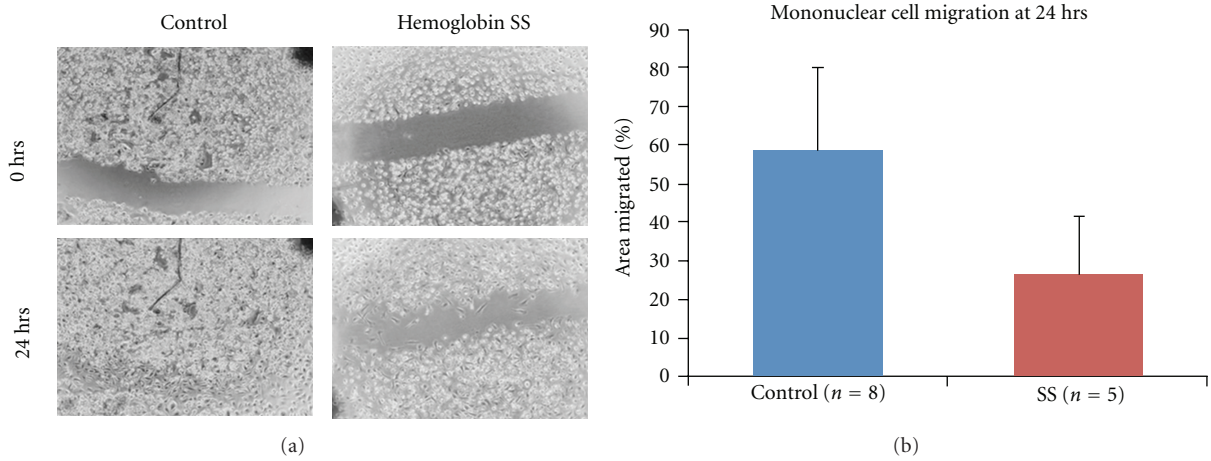

Figure 2: Mononuclear cell migration. Migration across a wound over 24 hours *was significantly less* in children with Hemoglobin SS than Controls. (a) shows a representative pair of Control and Hemoglobin SS wound migration assays. The freshly made wound was photographed at 0 (zero) hours, and the area migrated is measured after 24 hours. (b) shows the cumulative data for 8 Control and 5 Hemoglobin SS samples. The mean area migrated was less in children with Hemoglobin SS (28% versus 59% of the original wound area, $P < 0.01$).

Table 3: Angiogenic growth factors and oxidant stress markers for each Hemoglobin Group. Children with Hemoglobin SS had higher levels of three angiogenic growth factors, higher cysteine and cystine, and lower oxidized glutathione than Controls. Significant differences in the geometric means between Control and Hemoglobin SS groups are indicated by asterisks (*).

Other exposure variables	Main exposure variables (Hemoglobin Group)						Fold difference	*P* value
	Control			Hemoglobin SS				
	n	Arithmetic mean (95th% CI)	Geometric mean (95th% CI)	*n*	Arithmetic mean (95th% CI)	Geometric mean (95th% CI)		
Erythropoietin (IU/mL)	68	6.6 (4.8, 8.4)	4.2 (3.2, 5.5)	42	72.4 (55.6, 89)	56.6* (43.9, 73)	13.5	<0.001
Angiopoietin-2 (pg/mL)	45	1922 (1343, 2501)	1144 (809, 1616)	27	5946 (4661, 7231)	4968* (3820, 6461)	4.3	<0.001
SDF-1α (pg/mL)	64	2376 (2192, 2561)	2270 (2104, 2449)	38	4065 (3660, 4471)	3877* (3491, 4305)	1.7	<0.001
CyS (μM)	44	8.0 (7, 9)	7.4 (6.5, 8.4)	19	11.5 (9.5, 13.5)	10.8* (9, 13)	1.5	0.001
CySS (μM)	44	23.9 (22.3, 25.5)	23.4 (21.9, 24.9)	19	34.6 (30.4, 38.8)	33.7* (30, 37.8)	1.5	<0.001
E_hCySS (mV)	44	−81.1 (−84,–78)		18	− 87.1* (−92, −83)			0.026
GSH (μM)	44	1.1 (1, 1.2)	1.0 (0.9, 1.2)	19	1.1 (0.7, 1.5)	0.89 (0.7, 1.2)		0.38
GSSG (μM)	44	0.1 (0.08, 0.1)	0.08 (0.07, 0.1)	19	0.08 (0, 0.17)	0.04* (0.02, 0.07)	0.5	0.002
E_hGSSG (mV)	44	−117.2 (−121, −113)		18	124.1 (−132, −117)			0.11
Urinary 8-isoprostanes (ng/mL)	40	15 (9.7, 20.2)	8.88 (6.2, 12.7)	16	8.9 (7.2, 10.5)	8.4 (7, 10.1)		0.83

Table 4: Bivariate linear regression showing relationship between Hemoglobin SS group, WBC, and CPC types. WBC was found to be a strongly associated with CPC number in the Hemoglobin SS group. Significant reductions in the beta-coefficient (>25%) compared to the main exposure variable are marked with an asterisk (*).

Exposure variables	Outcome variables							
	CD34		CD34/CXCR4		CD34/VEGFR2		CD34/CXCR4/VEGFR2	
	β	Adj r^2	β	Adj r^2	β	Adj r^2	β	Adj r^2
Group = Hb SS	0.94	0.19	1.24	0.28	1.45	0.29	1.06	0.14
Hb SS + WBC	−0.11*	0.36	0.22*	0.39	1.06*	0.29	0.32*	0.17

Table 5: Multivariate linear regression showing relationship between Hemoglobin SS group, angiogenic growth factors, and CPC populations. SDF-1α was consistently associated with the relationship between Hemoglobin SS status and all CPC types (highlighted). Significant reductions in the beta-coefficient (>25%) compared to the main exposure variable are marked with an asterisk (*).

Exposure variables	Outcome variables							
	CD34		CD34/CXCR4		CD34/VEGFR2		CD34/CXCR4/VEGFR2	
	β	Adj r^2	β	Adj r^2	β	Adj r^2	β	Adj r^2
Group = Hb SS	0.94	0.19	1.24	0.28	1.45	0.29	1.06	0.14
Hb SS + Erythropoietin	0.57*	0.16	0.51*	0.29	1.44	0.25	1.02	0.1
Hb SS + Angiopoietin-2	1.0	0.17	1.12	0.26	1.24	0.25	0.76*	0.11
Hb SS + SDF-1α	0.54*	0.18	0.68*	0.3	1.0*	0.27	0.37*	0.15
Hb SS + SDF-1α + Erythropoietin	0.42*	0.17	0.3*	0.31	1.24	0.26	0.54*	0.14
Hb SS + SDF-1α + Angiopoietin-2	0.71	0.19	0.73*	0.29	0.98*	0.26	0.3*	0.14

Table 6: Multivariate linear regression showing relationship between Hemoglobin SS group, angiogenic growth factors, and WBC. Each angiogenic growth factor was strongly associated with the relationship between Hemoglobin SS and WBC. Significant reductions in the beta-coefficient (>25%) compared to the main exposure are marked with an asterisk (*).

Exposure variables	Intermediate outcome variable WBC	
	β	Adj r^2
Group = Hb SS	0.79	0.73
Hb SS + Erythropoietin	0.007	0.26
Hb SS + Angiopoietin-2	<0.001	0.17
Hb SS + SDF-1α	0.004	0.32

found to have a similar pattern of relationships to the exposure variables as the CPCs (Supplementary Table 2). Multivariate analysis showed that each of the hypoxia-inducible angiogenic growth factors was strongly associated with the relationship between Hemoglobin SS group and elevated WBC (Table 6).

4. Discussion

Vascular complications of sickle cell anemia, such as stroke and pulmonary hypertension, begin in childhood and are characterized by early development of intimal proliferation in cerebral and pulmonary arteries in the absence of cardiovascular risk factors, such as hypertension or hyperlipidemia. The mechanisms linking the primary genetic mutation in β-globin structure to the development of intimal proliferation and arterial stenosis are unknown. We hypothesize that sickle cell anemia is associated with abnormal vascular repair.

We have found that children with sickle cell anemia have a pro-angiogenic phenotype, with a higher numbers of cultured mononuclear cells that express mature endothelial markers, and CPCs with angiogenic potential, and higher angiogenic growth factor levels. The higher number of CPCs in Hemoglobin SS was associated with hypoxia-inducible angiogenic growth factors, either individually or in combination. Stromal derived factor-1α was found to be associated with the number of all CPC types in children with Hemoglobin SS. In contrast, the severity of anemia (hemoglobin level) was not associated with CPC number. White blood cell count was found to be an intermediate outcome, responding in a similar way to the hypoxia-inducible angiogenic growth factors. When corrected for WBC, the number of CD34/VEGFR2 cells was higher in the Hemoglobin SS group, while there were no differences in the other CPC populations. This implies that the elevation in most of the CPC populations in Hemoglobin SS was a secondary effect of WBC mobilization, but that the number of circulating CD34/VEGFR2 cells was independent of elevated WBC.

Despite a higher number of CPCs in children with Hemoglobin SS, cultured mononuclear cells in this group migrated over a smaller area in a 24-hour period, suggesting abnormal reparative function. The wound migration assay is a well-established functional assay for endothelial progenitor cells. A limitation of our study is the small number of subjects whose cells were tested in the wound migration assay. The wound assay was not performed when the cells did not form a confluent monolayer within the two-week culture period. If the cells were tested after becoming confluent over a longer culture period, we were concerned that cell senescence would contribute to variability in the results. Therefore, our results represent only those samples with better *in vitro* cell growth.

Our findings suggest that bone marrow-derived CD34/VEGFR2 cells in asymptomatic children with Hemoglobin SS are mobilized by hypoxia-inducible angiogenic growth factors from ongoing tissue ischemia, probably due to subclinical sickle cell vaso-occlusion. We predict that numbers will be elevated during acute sickle cell complications, that there will be progressive decline in both CPC number and function with increasing age, and that those individuals with the most severe vascular complications may have impaired function. If validated as a consistent finding, impaired mononuclear cell migration may be due to alterations in SDF-1α-mediated CXCR4 signaling. Endothelial progenitor cells from people with coronary artery disease were found to have slower migration, reduced vascular tube formation, and less effect in restoring circulation in a rodent ischemic limb model, in association with lower SDF-1α-induced phosphorylation of JAK-2, a downstream target of CXCR4 [22].

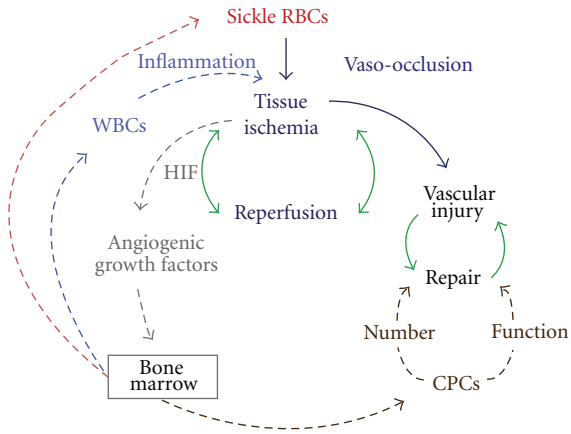

Figure 3: Diagram of CPC and WBC mobilization by vaso-occlusion induced tissue ischemia. Vaso-occlusion by sickled red blood cells (RBCs, red) results in tissue ischemia and reperfusion (navy blue). Repeated episodes of vascular ischemia are likely to promote vessel injury (black). Green arrows depict balanced physiologic processes that aim to restore equilibrium. Hypoxia-inducible factor (HIF, gray) produced by ischemic tissues stimulates angiogenic growth factors and bone marrow mobilization of angiogenic progenitor cells (CPCs, brown) that participate in vascular repair, white blood cells (light blue) that promote inflammation, and more sickle red blood cells.

If functional abnormalities in EPCs can be identified and corrected, EPCs in people with sickle cell disease can possibly be harnessed as cellular therapy to prevent or treat vasculopathy.

In addition, our data suggest that elevated WBC count in children with Hemoglobin SS is also due to mobilization by hypoxia-inducible angiogenic growth factors produced by tissue ischemia. This finding provides a mechanistic link between vaso-occlusion and the well-described elevations of white blood cells and inflammatory mediators in sickle cell disease (Figure 3). This relationship is consistent with findings from the Cooperative Study of Sickle Cell Disease (CSSCD) and a more recent cohort study, which describe elevated baseline leukocytosis as a risk factor for adverse sickle cell disease complications [23, 24]. Total WBC in the asymptomatic state, when not modified by transfusion or hydroxyurea therapy, may be a biomarker for tissue ischemia in sickle cell disease.

5. Conclusions

We found angiogenic CPC number to be elevated in this group of asymptomatic children with Hemoglobin SS, while mononuclear cell migration was slower than in healthy Control children. Stromal-derived factor-1α, a hypoxia-inducible angiogenic growth factor, is strongly associated with the elevated numbers of CPCs and total WBC in children with Hemoglobin SS. Tissue ischemia resulting from vaso-occlusion may promote both proangiogenic and proinflammatory states in sickle cell disease.

Acknowledgments

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Research Article

FK228 Analogues Induce Fetal Hemoglobin in Human Erythroid Progenitors

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Fetal hemoglobin (HbF) improves the clinical severity of sickle cell disease (SCD), therefore, research to identify HbF-inducing agents for treatment purposes is desirable. The focus of our study is to investigate the ability of FK228 analogues to induce HbF using a novel KU812 dual-luciferase reporter system. Molecular modeling studies showed that the structure of twenty FK228 analogues with isosteric substitutions did not disturb the global structure of the molecule. Using the dual-luciferase system, a subgroup of FK228 analogues was shown to be inducers of HbF at nanomolar concentrations. To determine the physiological relevance of these compounds, studies in primary erythroid progenitors confirmed that JMA26 and JMA33 activated HbF synthesis at levels comparable to FK228 with low cellular toxicity. These data support our lead compounds as potential therapeutic agents for further development in the treatment of SCD.

1. Introduction

Several classes of pharmacological compounds that reactivate γ-globin gene transcription have been identified. They include cytotoxic agents, DNA methyl transferase, and histone deacetylase (HDAC) inhibitors. Cytotoxic compounds terminate actively cycling progenitors and perturb cellular growth to trigger rapid erythroid regeneration and γ-globin gene activation. S-stage cytotoxic drugs, such as cytosine arabinoside [1], myleran [2], vinblastine [3], and hydroxyurea [4, 5], induce HbF production in primates and humans [4, 6, 7]. The Multicenter Study of Hydroxyurea established this agent as the first FDA-approved treatment for SCD [7]. Hydroxyurea was shown to reduce vaso-occlusive episodes in the majority of sickle-cell patients treated. However, limitations to using hydroxyurea such as bone marrow suppression [8], concerns over long-term carcinogenic complications, and a 30% non-response rate [7, 9], make the development of alternative therapies desirable.

The HDAC inhibitors have also been shown to be potent HbF inducers. These agents target HDACs, which play a dynamic role in regulating cell cycle progression and chromatin conformation by changes in histone acetylation status. Aberrant transcriptional repression mediated by Class I and II HDACs has been demonstrated in many cancers [10]. Thus, HDAC inhibitors have been developed as promising anticancer therapeutics [11]. Structurally diverse classes of natural and synthetic HDAC inhibitors bind target HDACs to block histone deacetylation [12] and produce an open chromatin confirmation and gene activation [13].

There has been great interest in HDAC inhibitors as HbF inducers to treat SCD. They include (1) short-chain

fatty acids such as sodium butyrate (NaB), the first HDAC inhibitor reported [14, 15]; (2) the benzamides (i.e., MS-275); (3) non cyclic and cyclic hydroxamates, like SAHA (suberoylanilide hydroxamic acid) and TSA (Trichostatin A); (4) cyclic peptides including FK228 (depsipeptide). NaB induces differentiation in mouse erythroleukemia cells via Stat5 phosphorylation and HbF synthesis through p38 mitogen-activated protein kinase signaling [16–18]. Other fatty acids including phenylacetate and propionate [19–21], induce HbF in erythroid progenitors, however, these agents are rapidly metabolized and oral preparations are not available. These published studies serve as the basis for research efforts to develop HDAC inhibitors as therapeutic agents for SCD.

Of the hydroxamic acid derivatives, the prototype TSA is a potent HDAC inhibitor [22, 23]. It interacts with a divalent zinc-binding motif in the binding pocket of Class I and II HDACs [24]. Other HDAC inhibitors in the hydroxamic acid class include the second-generation analogues of TSA, identified from a library screen of 600 synthesized compounds [25]. The most widely studied TSA analogues are SAHA and Scriptaid. SAHA targets HDAC1, 3, and 4 and inhibits prostate cancer cell growth *in vitro* and *in vivo* [26, 27]. Recently, it was demonstrated by Pace and colleagues that SAHA and Scriptaid induce HbF synthesis comparable to NaB and TSA in erythroid cells and β-YAC transgenic mice respectively [28]. However, limitations to the further development of these agents included toxicity in primary cells.

Another potent HDAC inhibitor is FK228, also known as depsipeptide, isolated from *Chromobacterium violaceum* [29]. This compound has a unique bicyclic structure and is a stable pro-drug activated by the reduction of the disulfide bond by glutathione to produce an active form (redFK) after uptake into cells [30]. The reduced sulfhydryl group interacts strongly with the zinc ion at the active site of the enzyme and has been shown to inhibit tumor proliferation *in vitro* and *in vivo* at nanomolar concentrations [27, 31, 32]. Recently, FK228 was tested in the μLCR$\beta_{pr}R_{luc}{}^{A}\gamma_{pr}F_{luc}$ GM979 stable cell line and erythroid progenitors grown in methylcellulose colonies produced from peripheral blood mononuclear cells [33]. FK228 was shown to induce HbF in both systems. The level of γ-globin and β-globin promoter activity was quantified indirectly using firefly (γ) and renilla (β) luciferase activity.

Drug-mediated HbF induction remains the best approach to ameliorate the symptoms and complications of SCD. Among many compounds, FK228 showed efficacy in inducing γ-globin transcription at low concentrations, however, cell toxicity was observed and the drug is difficult to synthesize. It is a bicyclic depsipeptide almost exclusively comprised of unnatural amino acids, D-valine, D-cysteine and, (Z)-dehydrobutyrine (Dhb) as well as a (3S, 4E)-3-hydroxy-7-mercapto-4-heptenoic acid, which is a key component to form the highly constrained bicyclic structure. The high content of the unnatural amino acids and the constrained bicyclic structure make it extremely stable in physiological condition. Simon and coworkers first reported its total synthesis in 1996 [34], and suggested a laborious synthetic route with moderate yield (18% overall yield with over 16 steps).

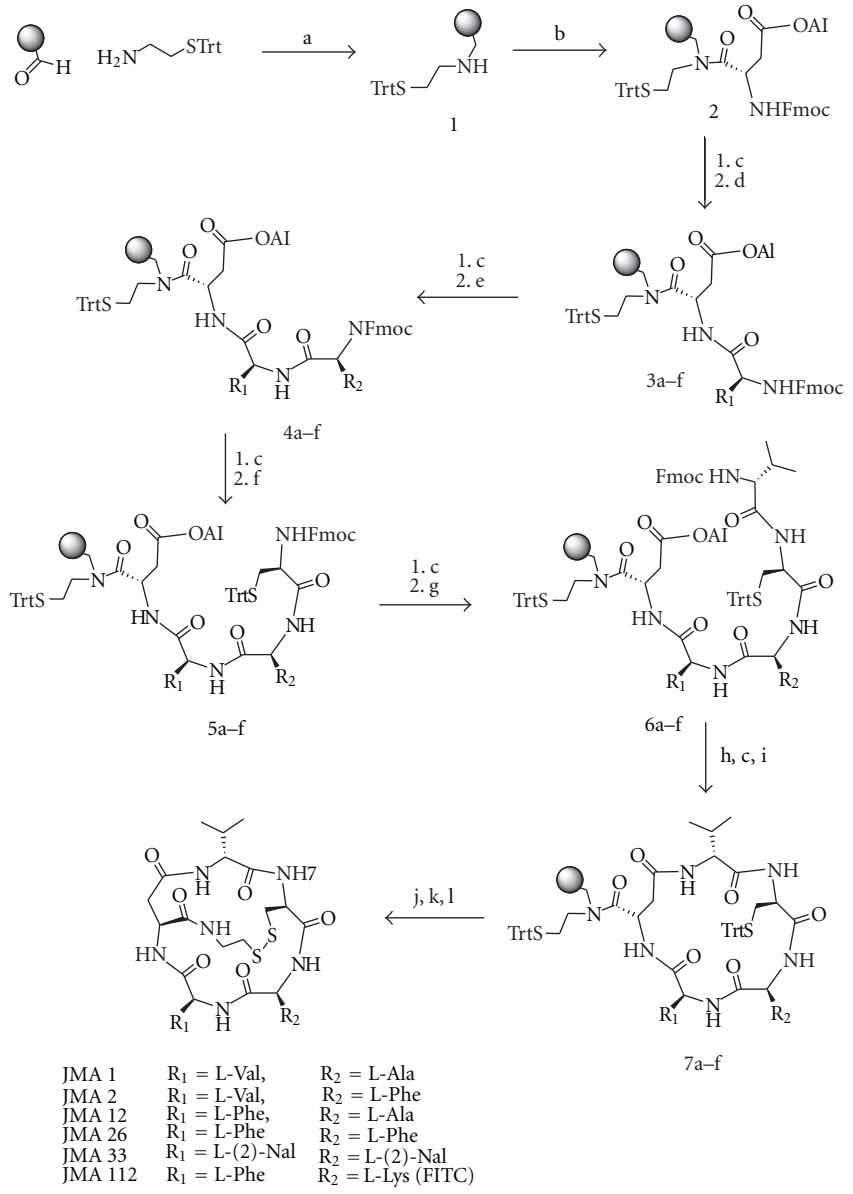
Despite its exceptionally high *in vitro* and *in vivo* activity, FK228 has not been explored due to its non-trivial and challenging synthesis, which hampered its production and the design of analogues. The latter would aide our understanding of the molecular mechanism of FK228 and to achieve higher potency and selectivity for HbF induction. In fact, only a few FK228 analogues have been created to date even after intensive synthetic efforts were made [34–36]. To circumvent this problem, we used *in silico* structure analysis and molecular modeling to design twenty FK228 structural analogues that can be easily synthesized [37]. Furthermore, two isosteric substitutions were made without altering its global conformation.

The objective of our study was to investigate the ability of the newly synthesized FK228 analogues to induce γ-globin gene transcription using a dual luciferase-based assay system. We identified two lead compounds, JMA26 and JMA33, which induce HbF expression in primary erythroid progenitors. The potential of HDAC enzymes as druggable targets in the treatment of SCD is discussed.

2. Materials and Methods

2.1. Synthesis of FK228 Analogues. The synthesis of all FK228 analogues described in this study was accomplished by following the previously reported solid-phase synthetic procedure outlined in Scheme 1 [37]. Briefly, S-trityl cysteamine was loaded on aminomethylated polystyrene (AM-PS) resin that was previously functionalized with a backbone amide linker (BAL) [38]. The resulting secondary amine 1 was then coupled with the first amino acid, Fmoc-L-Asp(OAl) to give the aspartylcysteamine 2 with high yield (98%). Thus, the aspartylcysteamine moiety designed to replace the heptenoic acid in the native FK228, was constructed in a single reaction step. The remaining four amino acids were introduced sequentially using the standard N-Fmoc/^{t}Bu solid-phase peptide synthesis strategy to build the linear pentapeptides 6a-f. After the allyl and N-Fmoc protecting groups were removed, the macrolactams 7a-f were formed with HBTU (O-benzotriazole-N,N,N′,N′-tetramethyluronium hexafluorophosphate) as a coupling reagent with high yields and purity (>95%). S-Trityl protecting groups were removed with dilute 1% trifluoroacetic acid and the resulting free thiols were oxidized with iodine to produce the bicyclic FK228 analogues. The analogues were cleaved from the resin with TFA (>95%) and characterized by reverse phase—high performance liquid chromatography and electrospray ionization mass spectrometry.

2.2. Cell Culture. Human KU812 leukemia cells were grown in Iscove's Modified Dulbecco's medium (IMDM) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), penicillin (100 U/mL), and streptomycin (0.1 mg/mL). The cells were incubated at 37°C with 5% CO_2. Cell count and viability were determined using a hemocytometer and 2% Trypan blue exclusion. Inductions



Scheme 1: Synthesis of FK228 analogues. Shown in the schematic are the steps, reagents, and conditions used for FK228 analogue synthesis. Compounds 1–7 are the intermediates during the synthesis. For steps 7a–f, the different R_1 and R_2 group substitutions were made to generate the various JMA analogues shown. Symbols: (a) $NaBH_3CN$; (b) Fmoc-Asp(OAl), DIC; (c) Piperidine; (d) Fmoc-AA_1, HBTU; (e) Fmoc-AA_2, HBTU; (f) Fmoc-D-Cys(Trt), HBTU; (g) Fmoc-D-Val, HBTU; (h) $Pd(PPh_3)_4$, DMBA; (i) HBTU; (j) 1% TFA; (k) I_2; (l) TFA (>95%).

were performed with one million cells treated for 48 hr with the following drugs purchased from Sigma (St Louis, MO): 50 μM Hem (hemin), 2 mM NaB (sodium buytrate), 0.5 μM TSA, 10 mM Cys (cysteine), 1.5 nM FK228, and 100 μM HU (hydroxyurea). We also tested 5 μM SAHA, a gift from Merck & Co. Inc. (Whitehouse Station, NJ).

2.3. KU812 Stable Lines. KU812 stable cell lines were created by co-transfecting wild-type KU812 cells with p*EGFP-NI* (G418 selectable marker) *and the* μLCR$\beta_{pr}R_{luc}{}^{A}\gamma_{pr}F_{luc}$ dual-reporter a kind gifts from Dr. George Stamatoyannopoulos (University of Washington). Briefly, the 315-bp human β-globin gene promoter sequence was inserted upstream of the Renilla along with a polyadenylation signal downstream to create $P\beta_{pr}R_{luc}$. Likewise, 1.4 kb of human Aγ-globin promoter was inserted upstream of firefly luciferase to create ${}^{A}\gamma_{pr}F_{luc}$. The μLCR (locus control region), $P\beta_{pr}R_{luc}$, and ${}^{A}\gamma_{pr}F_{luc}$ fragments were subsequently cloned into the mammalian vector, pRL-null [39].

The dual-luciferase reporter lines were produced using 10 μg each of linearized μLCR$\beta_{pr}R_{luc}{}^{A}\gamma_{pr}F_{luc}$ and p*EGFP-NI* plasmids co-transfected into KU812 cells by electroporation

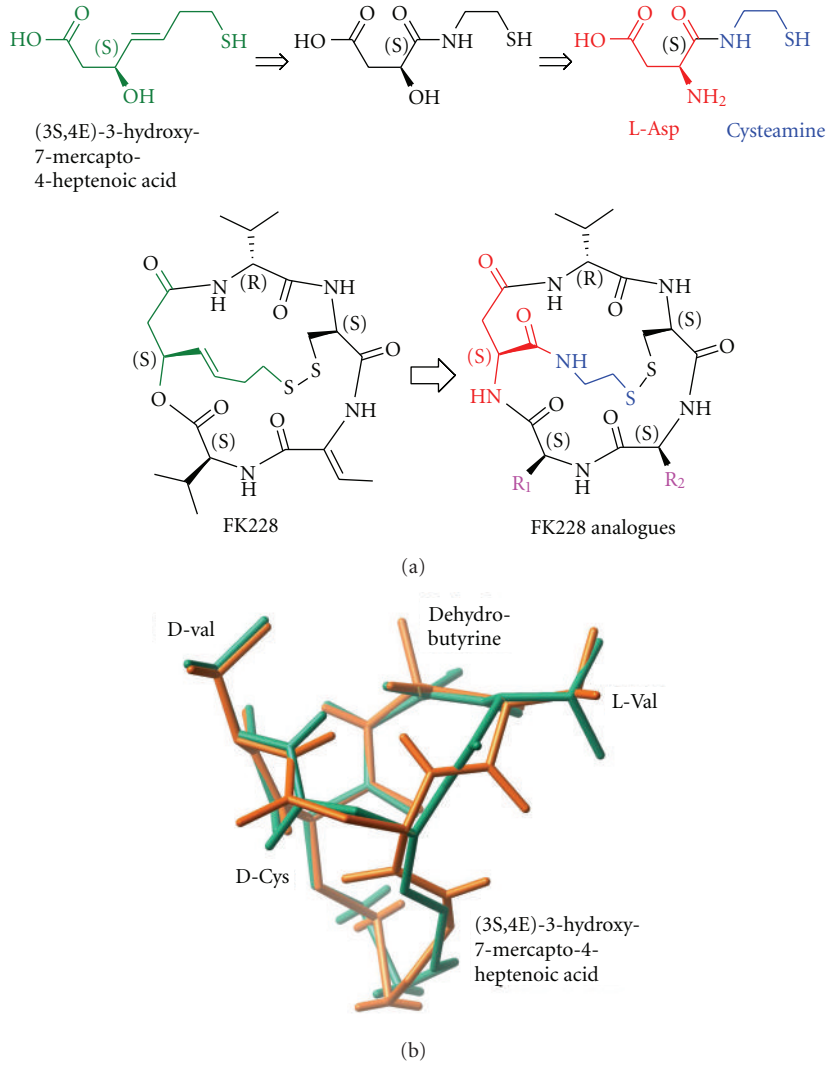


Figure 1: Structures of FK228 analogues. (a) The parent compound FK228 was transformed into novel structural analogues by two isosteric substitutions. The modification of the trans-double bond and ester linkage in the native FK228 with two isosteric amide functional groups allows facile synthesis of analogues as well as retention of the same backbone structure. Various amino acids such as Val, Ala, Phe, 2-Nal, and Lys were introduced to investigate potency of the analogues. (b) Superimposed structures of FK228 (green) and a modified FK228 analogue (orange).

at 260 V, 975 μF (Bio-Rad, Hercules CA). After 72 hr, G418 was added at a concentration of 900 μg/μl for 3 days then maintained under selection pressure indefinitely at a concentration of 400 μg/μl. KU812 stable lines were treated with the various drugs at the same concentrations described above. FK228 and analogues were screened at concentrations between 1–1000 nM for 48 hr and cell toxicity was monitored by 2% Trypan blue exclusion. The effect of drug treatments on γ-globin and β-globin promoter activity was monitored by luciferase assay.

2.4. Dual Luciferase Assay. Luciferase activity was monitored under the different experimental conditions using the Dual Luciferase Assay Reporter System (Promega, Madison, WI). The activity of firefly luciferase represents γ-globin promoter activity (γF), while the renilla luciferase is the read-out for β-globin promoter activity (βR). The β-globin promoter was strategically cloned between the LCR and γ-globin promoter to increase β expression, while simultaneously increasing the sensitivity of detection of γ-globin gene inducers [40].

After drug treatments, KU812 stable cells were washed with 1X phosphate buffered saline and lysed in 1X Passive Lysis Buffer for 15 min, then protein extracts were added to the Luciferase Assay Reagent II and firefly luciferase activity quantified in a Turner Designs TD-20/20 luminometer (Sunnyvale, CA). To measure βR activity, Stop & Glo Reagents was added to measure the renilla luciferase activity. Total protein was determined by Bradford assay on a Beckman DU 640 spectrophotometer (Chaska, MN) and luciferase activity was corrected for total protein.

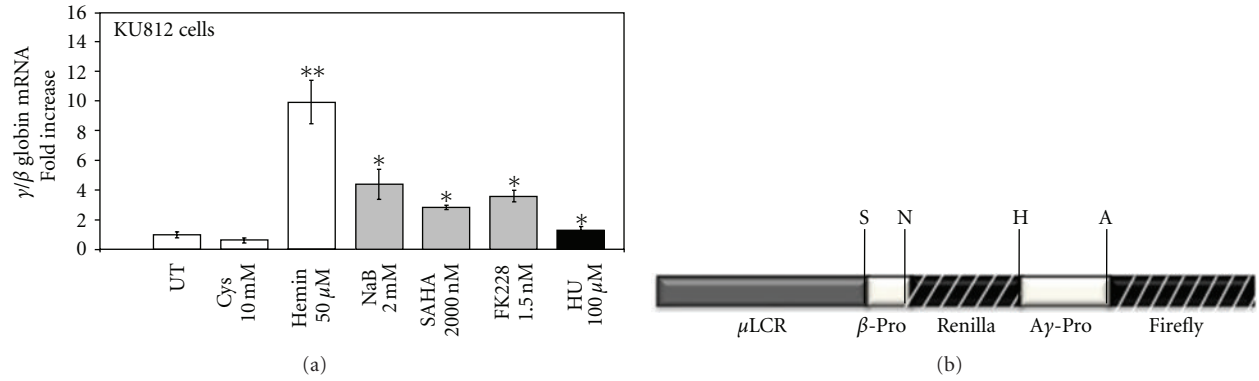


Figure 2: Known HbF inducers activate γ-globin expression in KU812 cells. (a) Induction of γ-globin transcription by known HbF inducers. Cells were treated for 48 hr with the different drug inducers and γ-globin and β-globin gene transcription were measured by RT-qPCR (see Section 2). The relative mRNA levels were plotted as fold increase. Untreated cells (UT) were used as a control and normalized to one. Data were calculated as the means ± standard error of the mean (SEM); $^{*}P < 0.05$ and $^{**}P < 0.01$. (b) Shown is a schematic of the dual luciferase reporter construct $\mu\text{LCR}\beta_{\text{pr}}\text{R}_{\text{luc}}\gamma_{\text{pr}}\text{F}_{\text{luc}}$ which contains a 3.1-kb μLCR cassette linked to a 315-bp human β-globin gene promoter driving the renilla luciferase gene and a 1.4-kb Aγ promoter driving the firefly luciferase gene [33, 39].

2.5. Two-Phase Erythroid Liquid Culture System. The two-phase liquid culture system was established as previously published by Fibach et al. [41] using buffy coat mononuclear cells, purchased from Carter Blood Care (Fort Worth, TX) in accordance with the guidelines of the Institutional Review Board at the University of Texas at Dallas. During phase 1, cells were grown in IMDM medium with 30% fetal bovine serum and 50 ng/mL each of the granulocyte-monocyte colony-stimulating factor, Interleukin-3, and stem cell factor. Subsequently, phase 2 was initiated on day 7 with the addition of erythropoietin (2 U/mL) and stem cell factor (50 ng/mL). The cells were treated on Day 11 with the different test compounds and harvested on Day 14 (72 hr incubation).

2.6. Reverse Transcription—Quantitative PCR (RT-qPCR) Analysis. Total RNA was isolated from samples using RNA Stat-60 (TEL-TEST "B" Inc., Friendswood, TX) and used for RT-qPCR analysis as previously published [42]. Briefly, cDNA was prepared using the Improm-II reverse transcriptase system and oligo $(dT)_{15}$ primers (Promega). qPCR was performed on an iCycler iQ machine (Bio-Rad) using a master mix containing Sybergreen iQ Supermix (BioRad) and 100 pM of each gene-specific primer pairs for γ-globin, β-globin and the internal control GAPDH. Standard curves were generated using serial 10-fold dilutions of Topo7 base plasmids carrying a γ-globin cDNA sequence (Topo7-γ-globin), Topo7-β-globin, and Topo7-GAPDH. The globin mRNA levels were calculated as a ratio of GAPDH (γ/GAPDH, β/GAPDH), and the γ/β-globin mRNA ratio was calculated by dividing γ/GAPDH by β/GAPDH.

2.7. Immunohistochemistry. Primary erythroid progenitor cytospin cell preparations were fixed with 4% paraformaldehyde in phosphate buffered saline for 15 min, washed, and then permeabilized with 0.3% Trition-100 solution for 10 min. Cells were then blocked in a 5% bovine serum albumin solution and immunostaining performed at 4°C overnight with anti-HbF fluorescein isothiocyanate (FITC) conjugated antibody (Bethyl Laboratories Inc., TX). Cell nuclei were stained with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, CA). Primary cells were photographed with an Olympus BX 51 phase contrast epifluorescent microscope equipped with Hoffman Modulation optics. Phase-contrast images were recorded with a CCD camera (1/100 sec exposure) and fluorescence images were photographed through 485/520 nm emission filter. The percent of HbF positive cells was calculated by dividing the number of FITC positive cells by total cells (DAPI positive).

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). Total hemoglobin was quantified using 20 μL of protein extract from one million KU812 cells mixed with 5 mL of Drabkin's reagent (Sigma); then cyanmethemoglobin was measured at the 540 nm wavelength. HbF levels were quantified using the human Hemoglobin F ELISA Quantitation Kit (Bethyl Laboratory, Montgomery, TX). Briefly, 96-well plates were coated with sheep anti-human HbF antibody (1 mg/mL). After blocking with 1% bovine serum albumin, horse radish peroxidase-conjugated secondary antibody (1 mg/mL) was added. Raw data were analyzed using GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA) and HbF levels were calculated as a ratio of total hemoglobin corrected for total protein (HbF/total Hb/total protein).

2.9. HDAC Inhibition Assay. The HDAC Fluorescent Activity Assay (Enzo Life Science, Farmingdale, NY) was used to measure HDAC inhibition in HeLa cells in a 96-well format using Trichostatin A as the positive control per the manufacturer's protocol. This assay is based on the Fluor de Lys (Fluorogenic Histone Deacetylase Lysyl (FDL)) Substrate/Developer. The procedure was as follows: the test compounds FK228, JMA26, and JMA33 were added to HeLa cells along

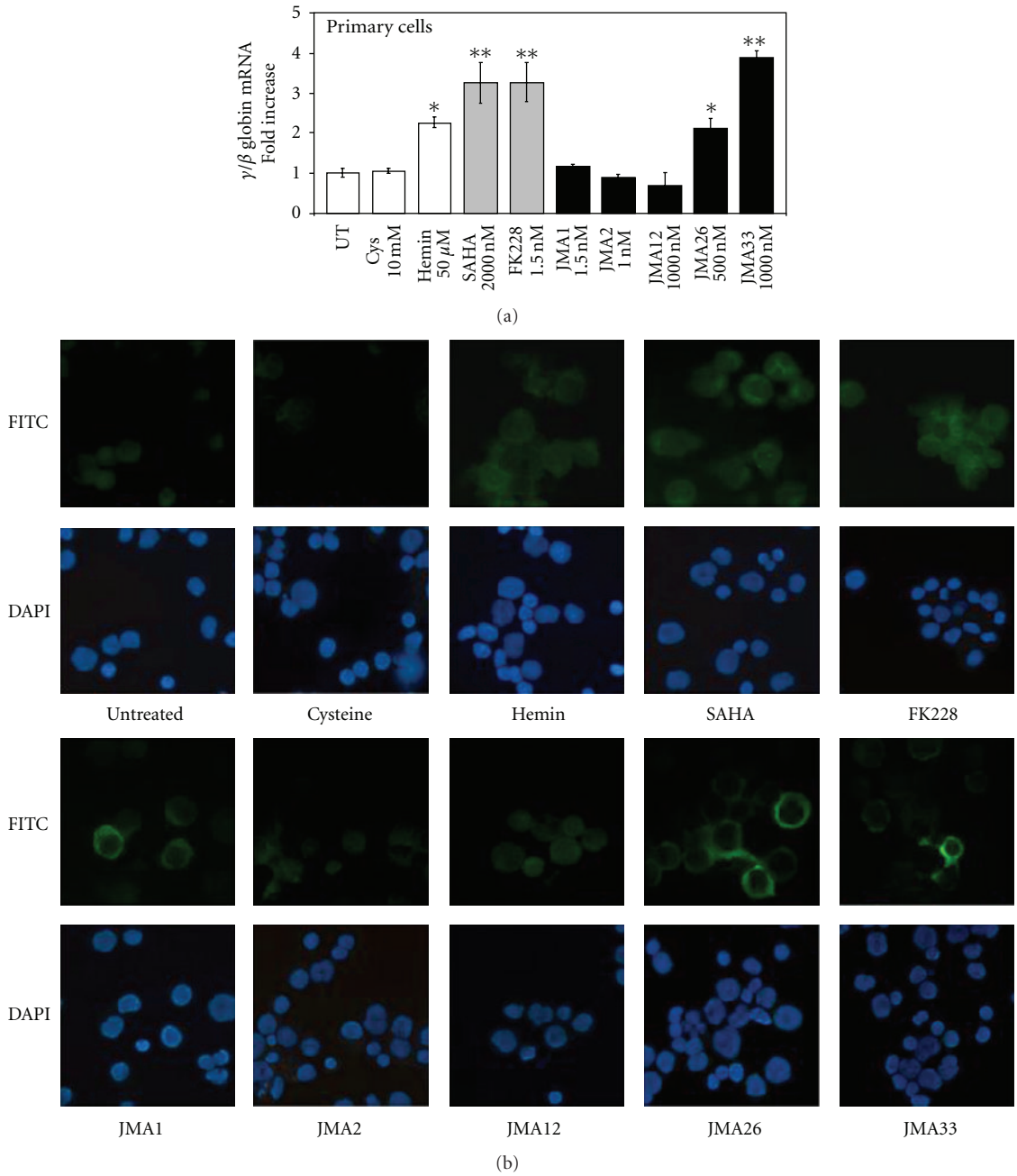


Figure 3: JMA33 and JMA26 induce HbF in primary erythroid cells. (a) Day 11 erythroid progenitors were treated for 48 hr with the controls agents and the FK228 analogues shown and then analyzed by RT-qPCR. Untreated cells (UT) were used as a control and normalized to one. Data were calculated as the means ± SEM. (b) Progenitors were stained with anti-γ-globin FITC conjugated antibody overnight and HbF positive cells for were visualized. DAPI staining was performed to identify cell nuclei and to determine cell counts. Images were photographed at 40X power. The images generated were used to count 500 DAPI positive cells and the %FITC positive cells were calculated accordingly.

with the FDL substrate to allow intracellular drug activation then fluorescence levels were read on the fluorometer at 440 nm (CytoFluor II, PerSeptive Biosystems, Farmingdale, NY). The drug concentrations tested were based on the amount required for HbF induction in primary cells. Data was reported as the mean ± standard deviation (SD) for at least five replicates.

2.10. Statistical Analysis. The data are reported as the mean ± standard error of the mean (SEM) from at least five data points generated from independent drug treatments. Data were analyzed by a two-tailed student's t-test, and values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using Microsoft Excel (Redmond, WA, USA).

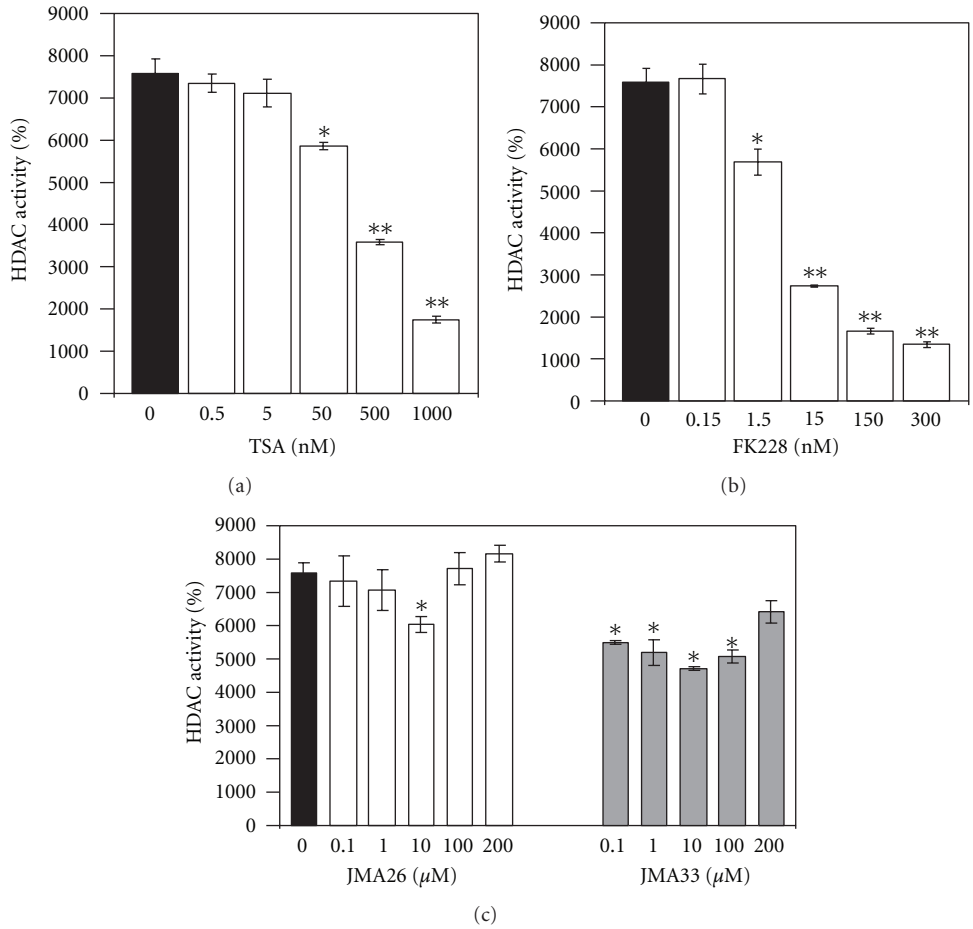


Figure 4: HDAC inhibition assay. The HDAC Fluorescent Activity Assay was used to measure HDAC inhibition in HeLa cells (See Section 2). Compounds and FDL substrate were added to HeLa cells to allow drug activation *in vivo* then fluorescence levels were quantified on the fluorometer at 440 nm. (a) Trichostatin A (TSA) was used as the control. (b) Inhibition studies were conducted for FK228 showing maximal HDAC inhibition at the 300 nM concentration. (c) Similar studies were performed for JMA33 and JMA26. The data are reported as the means ± SEM.

3. Results

3.1. Isosteric Substitutions Do Not Alter the Global Structure of FK228. For the facile synthesis of FK228 analogues, the most synthetically challenging moiety, hydroxy-mercapto-heptenoic acid was modified to a structure that can be easily constructed but has the capability of retaining the structure required for biological activity. We used *in silico* structure analysis and molecular modeling to design structural analogues of FK228 that met these requirements and could be easily synthesized [37]. The design of a novel FK228 analogue is summarized in Figure 1. These compounds were synthesized by modifying the most synthetically challenging unit, (3*S*,4*E*)-3-hydroxy-7-mercaptoheptenoic acid, with two isosteric substitutions without altering its global conformation compared to native FK228. First, the trans-double bond in the heptenoic acid was replaced by an isosteric amide bond. Second, the ester bond required to form the depsipeptide was replaced by another amide bond for facile ring closure that provided higher synthetic yield and increased *in vivo* stability. As shown in Figure 1(b), the structure of the FK228 analogue was found to be almost identical in structure (RMSD = 0.20 Å), indicating that the two isosteric changes neither disturbed the global structure nor altered the backbone structure compared to FK228. However, these changes enabled facile and rapid synthesis using readily available starting materials and high-yielding reactions. While retaining the original stereochemical configurations, the functional groups R_1 and R_2 (Figure 1(b)) were substituted with a variety of amino acids such as Ala, Leu, Phe, 2-Nal, Thr, Asp, and Lys (Scheme 1) to examine the side chain consisting of small, large, aromatic, hydrophilic, and charged alterations.

Twenty FK228 analogues were prepared with high overall yield (75–90%) and purity (80–94%) using the solid-phase synthetic strategy. To further characterize the compounds, selected FK228 analogues were examined by 2D-NMR spectroscopy in (dimethyl sulfoxide) $DMSOd_6$ using Double-quantum filtered, total correlation, and rotating frame Overhauser effect spectroscopy to confirm structures and stereochemistry (data not shown). The FK228 analogues were shown to have outstanding solubility (10 mM) in the

Table 1: γ-globin induction in KU812-γF/βR stable lines by FK228 derivatives[1].

Line 1	Drug concentration	$\gamma/\gamma + 2\beta$ Mean	SEM	P value	Fold change
Untreated	none	0.0402	0.0091	n/a	1
Cys	10 mM	0.0388	0.0092	0.8528	0.950
Hemin	50 μM	0.1732	0.0399	0.0050	4.325
SAHA	2000 nM	0.2157	0.0142	0.0001	5.400
FK228	1.5 nM	0.1197	0.0325	0.0150	2.975
1127Ox	1000 nM	0.0363	0.0022	0.8167	0.900
JMA1	1.5 nM	0.0473	0.0149	0.6720	1.175
JMA2	1.0 nM	0.0157	0.0003	0.1629	0.400
JMA12	1000 nM	0.0187	0.0009	0.2156	0.475
JMA26	500 nM	0.1460	0.0234	0.0004	3.650
JMA33	1000 nM	0.1373	0.0117	0.0002	3.425
JMA112	100 nM	0.0187	0.0007	0.2156	2.453
Line 2	Drug concentration	$\gamma/\gamma + 2\beta$ Mean	SEM	P value	Fold change
Untreated	none	0.1573	0.0150	n/a	1
Cys	10 mM	0.1200	0.0056	0.1977	0.764
Hemin	50 μM	0.6593	0.0839	0.0001	4.261
SAHA	2000 nM	0.6500	0.0208	0.0001	4.140
FK228	1.5 nM	0.6167	0.0338	0.0001	3.929
1127Ox	1000 nM	0.6133	0.0371	0.0001	3.904
JMA1	1.5 nM	0.1260	0.0152	0.2896	0.803
JMA2	1.0 nM	0.1183	0.0071	0.1814	0.752
JMA12	1000 nM	0.1470	0.0027	0.7093	0.936
JMA26	500 nM	0.2487	0.0308	0.0153	1.579
JMA33	1000 nM	0.3313	0.0256	0.0002	2.108
JMA112	100 nM	0.1070	0.0076	0.0938	0.682
Line 3	Drug concentration	$\gamma/\gamma + 2\beta$ Mean	SEM	P value	Fold change
Untreated	none	0.0721	0.0052	n/a	1
Cys	10 mM	0.0860	0.0050	0.1814	1.194
Hemin	50 μM	0.2508	0.0153	0.0001	3.486
SAHA	2000 nM	0.2520	0.0066	0.0001	3.500
FK228	1.5 nM	0.1583	0.0198	0.0007	2.194
1127Ox	1000 nM	0.0370	0.0095	0.0076	0.514
JMA1	1.5 nM	0.0770	0.0036	0.6179	1.069
JMA2	1.0 nM	0.0740	0.0036	0.8463	1.027
JMA12	1000 nM	0.0537	0.0023	0.0779	0.750
JMA26	500 nM	0.2560	0.0192	0.0001	3.555
JMA33	1000 nM	0.5120	0.1765	0.0007	7.111
JMA112	100 nM	0.0467	0.0019	0.0216	0.653

[1] Using three independent KU812-γF/βR stable cell lines, the FK228 analogues were examined for their ability to induce γ-globin. After 48 hr drug treatments, cells were harvested and dual luciferase assay performed. Untreated cells were used as a control and normalized to one. Data were calculated as the means ± standard error of the mean (SEM).

organic solvents ethanol and DMSO and were stable for over one year.

3.2. FK228 Analogues Are Potent Inducers of γ-Globin Expression. We first performed drug induction studies in wild type KU812 cells to determine the ability of analogues to induce endogenous γ-globin gene transcription. KU812 cells have been classified as a multipotential leukemia cell line with the ability to differentiate down the basophilic [43, 44], eosinophilic [45] and erythroid/megakaryocytic

lineages [46]. Previous studies from our laboratory demonstrated that KU812 cells express γ-globin, β-globin and the erythroid markers CD36, and erythropoietin receptor [47]. Therefore, we used these cells to perform initial drug screens to determine the suitability of KU812 cells for our dual luciferase reporter stable lines. We observed a 1.5- to-10-fold increase in the γ/β-globin mRNA levels after Hem (50 μM), NaB (2 mM), SAHA (2 μM)), and FK228 (1.5 nM) treatment (Figure 2(a)). In the untreated and negative controls, cysteine-treated cells, γ-globin gene expression was not induced. These data demonstrated that the intracellular environment in KU812 is conducive to identifying γ-globin gene activators in our FK228 analogue drug screen.

Subsequently, three independent dual-luciferase reporter KU812 stable cell lines were established to analyze the ability FK228 analogues to induce γ-globin promoter activity without an effect on β-globin transcription. The stable cell lines were created with the $\mu\text{LCR}\beta_{\text{pr}}\text{R}_{\text{luc}}\gamma_{\text{pr}}\text{F}_{\text{luc}}$ construct (Figure 2(b)) containing a 3.1-kb μLCR cassette linked to a 315-bp human β-globin promoter driving the renilla (R) and a 1.4-kb Aγ-globin promoter driving the firefly (F) luciferase genes [33, 39]. Since the firefly luciferase gene (γF) has approximately 50% greater luminescence than the renilla gene (βR), the renilla activity was multiplied by two to adjust for the difference in luminescence [33] yielding the $\gamma/\gamma+2\beta$ final measurement. The FK228 analogues were examined at concentrations ranging from 1–1000 nM in the three stable lines. After 48-hour treatments, cells were harvested and protein isolated for luciferase activity using the Dual Luciferase Reporter Assay. Of the twenty compounds tested, five induced γ-promoter activity. The remaining agents were either toxic at the concentrations tested or did not induce γ-globin (data not shown). Table 1 summarizes the γ-promoter activity for FK228 analogues that were tested further in primary erythroid cells. Cell viability by Trypan blue exclusion remained at 90–95% for the concentrations shown. Of note are the FK228 analogues, JMA26 and JMA33 (Table 2) containing aromatic side chains in the functional R_1 and R_2 groups which produced statistically significant γ-promoter activation comparable to FK228. Additional analogues can be designed based on these observations to increase potency, while sparing toxicity.

3.3. FK228 Analogues Activate HbF Synthesis in Primary Erythroid Progenitors. Next, we examined the ability of the lead FK228 analogues to induce HbF expression in primary erythroid progenitors grown from peripheral blood mononuclear cells in the two-phase liquid culture system. As shown in Figure 3(a), JMA26 and JMA33 induced γ-globin transcription at the mRNA level 2.1-fold and 3.9-fold, respectively, compared to a maximal 3.2-fold, induction by SAHA and FK228. However, FK228 derivatives induce γ-promoter activity at significantly lower drug concentrations compared to SAHA and Hem. At the concentrations tested, greater than 90% cell viability was observed in primary cells at all concentrations tested for the synthesized compounds. The similarity of these results to those acquired with the KU812 dual-luciferase reporter cell lines also validates the system for drug screening.

Table 2: FK228 structural analogues.

FK228 analogues	R_1	R_2
JMA1	Val	Ala
JMA2	Val	Phe
JMA12	Phe	Ala
JMA26	Phe	Phe
JMA33	2Nal	2Nal
JMA112	Phe	Lys(FITC)

Val: valine; Phe: phenylalanini; Ala: alanine; 2Nal: 2-naphthylmethyl; Lys: lysine; FITC: fluorescein isothiocyanate.

The next set of studies was performed to determine the ability of JMA26 and JMA33 to induce HbF in primary erythroid progenitors. Using anti-HbF fluorescein isothiocyanate (FITC) antibody, we observed 15.5% HbF-positive progenitors at baseline in untreated cells (Figure 3(b)). Treatment with JMA26 and JMA33 produced 3.0-fold, and 2.5-fold increase in HbF-positive cells, respectively. A similar increase in HbF-positive cells, were produced by hemin, SAHA and FK228 (3.0-fold, 3.2-fold and 3.5-fold). Complementary ELISA data (Table 3) showed a 1.9-fold and 2.5-fold increase in HbF levels produced by JMA26 and JMA33, respectively, compared to a 2.4-fold HbF induction by FK228. We concluded that these lead compounds have the capability to induce HbF in physiologically normal primary erythroid progenitors.

3.4. JMA26 and JMA33 Exhibit HDAC Inhibition Activity. To ascertain the mechanism of HbF induction by the lead compounds, we performed an *in vivo* assay to investigate the ability of JMA26 and JMA33 to act as HDAC inhibitors. The HDAC Fluorescent Activity Assay designed to measure HDAC activity in HeLa cells was completed in a 96-well format. The assay is based on the fact that the Fluor de Lys substrate is deacetylated by HDACs to generate a fluorescent readout. TSA (0.5 to 1000 nM) was used to establish the assay in HeLa cells, showing about 80% HDAC inhibition in our system (Figure 4(a)). By contrast, FK228 produced about 85% inhibition at the 300 nM concentration, which produces marked cell toxicity (Figure 4(b)). Similar studies performed for JMA26 and JMA33 showed 20% and 37% HDAC inhibition, respectively (Figure 4(c)), suggesting HbF induction in erythroid cells occurs by other mechanisms.

4. Discussion

Drug-mediated HbF induction remains the best treatment approach to ameliorate the symptoms and complications of SCD due to its ability to inhibit hemoglobin S polymerization. In addition, HbF provides an effective treatment for β-thalassemia by correcting globin chain imbalance [48]. Other therapies aimed at the underlying molecular causes of the β-hemoglobinopathies include hematopoietic stem cell transplantation [49] and gene therapy involving the transfer of normal γ- or β-globin genes into hematopoietic stem cells. Despite promising results and ongoing research, the option for stem cell transplantation is limited by the lack of suitable

Table 3: Fetal hemoglobin quantification in primary erythroid cells.

	Drug concentration	Mean	SEM	*P* value	Fold change
Untreated	none	0.683	0.0291	n/a	1
Cys	10 mM	0.737	0.1201	0.7773	1.079
Hemin	50 μM	1.515	0.0405	0.0001	2.218
SAHA	2000 nM	1.094	0.1049	0.0197	1.602
FK228	1.5 nM	1.676	0.0506	0.0001	2.454
JMA1	1.5 nM	0.7953	0.0849	0.2803	1.164
JMA2	1.0 nM	0.8120	0.0165	0.0183	1.188
JMA26	500 nM	1.3086	0.0535	0.0005	1.916
JMA33	1000 nM	1.7223	0.0725	0.0002	2.521

donors for the majority of SCD patients. On the other hand, gene therapy offers a universal cure but there are concerns about mutagenesis of target genes due to random vector integration and the effects of viral sequences on nearby gene expression [50]. Therefore, pharmacologic HbF induction remains a viable choice for the development of additional therapeutic options for treating SCD.

Hydroxyurea is the only drug approved by the Food and Drug Administration for the treatment of SCD [7, 51], however, it is not effective in all patients [7] and of minimal benefit in β-thalassemia [52]. Moreover, there are concerns about undesirable side effects including long-term carcinogenesis [53]. Clinical trials with other compounds, such as arginine butyrate [54] and decitabine [55] have shown considerable promise, however, orally active preparations need to be developed to make these agents viable treatment alternatives.

For many years, K562 cells have been used to screen pharmacological agents as potential HbF inducers. For example, NaB, decitabine, and hydroxyurea, among others, stimulate erythroid differentiation in K562 cells and induce γ-globin gene transcription [56]. Many HDAC inhibitors including FK228 are also known to induce HbF. However, synthetic difficulties associated with FK228 production have severely deterred structure-activity studies to aid understanding of its mechanism of action and to improve efficacy. Our data shows that JMA26 and JMA33 increased HbF levels by a mechanism independent of HDAC inhibition.

Many published studies have shown that primary erythroid cells remain the best system to confirm HbF-inducing agents and to serve as a predictor of efficacy *in vivo*. Human burst forming units—erythroid cells in clonogenic assays [57] or erythroid progenitors grown in liquid culture [39, 41]—have been used to evaluate putative HbF inducers. However, these assays are not easily adaptable to large-scale drug screening, thus immortalized cell lines have been investigated for this purpose. Previously, FK228 was tested in the μLCR$\beta_{pr}R_{luc}{}^{A}\gamma_{pr}F_{luc}$ GM979 stable line [33] and was shown to induce γ-promoter activity at the 1 nM concentration. We expanded on these studies to establish a dual-luciferase reporter system. Thus, we used KU812 cells derived from an individual with chronic myeloid leukemia [58] because both γ-globin and β-globin are actively transcribed [47]. Moreover, gene profiling data generated by our laboratory showed that KU812 cells express CD36 and the erythropoietin receptor at levels comparable to day-14 human erythroid progenitors [47].

In this study, when wild-type KU812 cells were treated with Hem, NaB, SAHA, and FK228, we observed a 3- to 10-fold increase in the γ/β-globin ratio. We next tested the FK228 analogues in the KU812 dual-luciferase reporter system created with the μLCR$\beta_{pr}R_{luc}{}^{A}\gamma_{pr}F_{luc}$ construct. Two FK228 analogues identified in the reporter assay, JMA26 and JMA33, showed efficacy as HbF inducer in primary erythroid progenitors suggesting these compounds have the potential for further development.

Our last set of experiments was aimed at understanding the mechanism by which JMA26 and JMA33 induce γ-globin. Histone acetylation is a highly dynamic reversible modification that contributes to gene expression through changes in chromatin conformations. The parent compound FK228 is a class IV cyclic peptide capable of inhibiting Class I HDAC enzymes (HDAC1, 2, 3, and 8) after intracellular reduction of its disulfide bond by glutathione to produce the active reduced form of FK228. The functional sulfhydryl group fits inside the catalytic pocket producing zinc chelation and inhibition of enzymatic activity [59].

The role of HDAC inhibition in HbF induction has been investigated by several laboratories. NaB was the first agent shown to mediate histone H3 and H4 hyperacetylation as a mechanism of HbF induction [60]. Subsequently, many other HDAC inhibitors such as TSA [42], scriptaid [28], SBHA (suberohydroxamic acid), and SAHA (suberoylanilide hydroxamic acid) [59] were shown to be HbF inducers based on the central role of histone hyperacetylation. Subsequently, Perrine and colleagues showed the ability of short-chain fatty acids to induce γ-globin by displacement of an HDAC3-NcoR repressor complex [61]. More recently, there exist chemical genetic screen-identified HDAC1 and HDAC2 as molecular targets facilitating drug-mediated HbF induction [62]. Therefore, to determine the mechanism of action of JMA26 and JMA33, we completed the HDAC inhibition assay.

Using the Fluor de Lys system, FK228 produced strong HDAC inhibition but at a higher concentration (300 nM) than required for HbF induction. Similar studies performed for JMA26 and JMA33 showed 20% and 37% maximal inhibition, respectively. These findings suggest that the alterations in FK228 structure may have uncoupled HDAC inhibition activity as the primary mechanism of HbF induction since higher test drug concentrations did not produce more HDAC inhibition. These data suggests JMA26 and JMA33 may induce γ-globin by mechanisms other than targeting HDACs. Since the FK228 analogues were developed from a structural library designed by molecular modeling, additional compounds can be synthesized with greater HbF inducing potency and selectivity to Class I HDACs. Additional studies will also be conducted to determine other mechanisms by JMA26 and JMA33 that induce HbF such as activation of the p38 mitogen-activated protein kinase or other signaling pathways [17, 63, 64].

5. Conclusions

The current drug treatment options for SCD are limited with hydroxyurea being the only FDA-approved drug. The key finding of this study is the high-efficiency synthesis of FK228 analogues with structural modifications which did not disturb the global chemical structure of the parent compound. The analogues exhibited HbF induction at nanomolar concentrations in primary erythroid progenitors demonstrating physiological relevance. These data support the FK228 analogues as potential therapeutic agents and also validates the KU812 dual-luciferase stable cell lines as an efficacious screening system to identify γ-globin activators. Long-term our goal is to establish a group of HbF inducers that selectively inhibit Class I HDACs to expand our understanding of epigenetic mechanisms of γ-globin gene regulation and to facilitate the development of drug therapy for SCD.

Acknowledgment

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Research Article

Association of Oxidative Stress Markers with Atherogenic Index of Plasma in Adult Sickle Cell Nephropathy

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This paper evaluates the association of oxidative stress and atherogenic index of plasma in order to assess the cardiovascular risk in Sickle cell nephropathy especially as lipoprotein levels are lower in SCD than non-SCD patients. Antioxidant enzymes, malondialdehyde(MDA), urea, creatinine, and glomerular filtration rate were evaluated in 110 confirmed sickle cell disease patients: 65 males in steady state, aged 21.1 ± 6.0 years, 30 males with macroalbuminuria, aged 24.5 ± 7.0, years and 15 with chronic kidney disease (CKD), aged 31.8 ± 2.0 years. The mean activity levels of glutathione peroxidase (GPx), superoxide dismutase (Cu/ZnSOD), and catalase (CAT) were significantly lower ($P < 0.001$) in SCD with macroalbuminuria and CKD while MDA was higher ($P < 0.001$) in SCD with macroalbuminuria and CKD compared with controls. There was negative correlation between GPx ($P < 0.001$), Cu/ZnSOD ($P < 0.02$), and Atherogenic index of plasma in SCD with CKD, while MDA shows a positive correlation ($P < 0.001$) with AIP in SCD with CKD. There was however no correlation between CAT and AIP. Decreased activity levels of antioxidant enzymes and low HDL-cholesterol concentration were confirmed in adult SCD with CKD in Nigerians. The increase oxidative stress and high atherogenic index in CKD may accelerate the process of cardiovascular complications in adult SCD patients. Atherogenic index of plasma was negatively correlated with antioxidant enzymes and positively with MDA.

1. Introduction

Sickle cell disease (SCD) is a haemoglobinopathy which is characterized by red blood cell rigidity, compromised perfusions, and tissue infarction [1]. The kidney of patients with SCD is affected both by haemodynamic changes of chronic and by consequences of vaso-occlusion within the renal medulla [2, 3]. Renal abnormalities in structure and function occur with increasing age of subject with SCD. The pathogenesis of SCD is due to polymerization of sickle red blood cell causing chronic haemolytic anaemia, vaso-occlusive crisis, and intravascular haemolysis. Sickle cell disease patients are susceptible to increased oxidative stress due to constant haemolysis of mutant red blood cells since haemoglobin acts as powerful catalyst for initiation of peroxidative reaction [4, 5]. Proteinuria is common in adult patients with SCD and we earlier reported a 28% prevalence of proteinuria in this group of patients in Nigeria [6]. Proteinuria is a progression factor in chronic kidney disease heralding a further deterioration in renal function [6]. Metabolic abnormalities, inflammation, and ischaemia may increase oxidative stress in sickle cell nephropathy (SCN). Increased oxidative stress in SCN due to increased pro-oxidative activity may lead to diminished antioxidant system [4, 7]. Increased oxidative stress is considered as an important pathogenic mechanism in the development of cardiovascular, cerebrovascular, and peripheral vascular complications [8, 9]. Autoperoxidation of polyunsaturated fatty acids (PUFAs) is initiated by free radicals, and the products which are oxidized in vivo to form malondialdehyde are capable of damaging membrane of biomolecules [9, 10]. Lipid abnormalities and increased oxidative stress in

Table 1: Biochemical and lipid profile of sickle cell disease controls, macroalbuminuria, and chronic kidney disease.

Variables	SCD controls	SCD macroalbuminuria	SCD CKD	Reference range
Number of subjects	65	30	15	
Age (years)	21.1 ± 6.0	24.5 ± 7.0	31.8 ± 2.0*	
Urea (mmol/L)	2.6 ± 0.9	8.3 ± 2.0*	14.2 ± 2.6*	1.7–8.3
Creatinine (μmol/L)	57.3 ± 9.8	260 ± 25*	498 ± 75*	53–116
eGFR (mL/min)	101 ± 2.3	72 ± 5.0*	15.1 ± 2.0*	90–128
Triglyceride (mmol/L)	1.16 ± 0.4	1.20 ± 0.2	1.7 ± 0.25*	<1.7
Total cholesterol (mmol/L)	3.06 ± 0.5	3.45 ± 0.6*	3.9 ± 0.34*	3.1–6.2
HDL-cholesterol (mmol/L)	0.72 ± 0.2	0.70 ± 0.3	0.06 ± 0.08*	0.8–1.9
LDL-cholesterol (mmol/L)	1.90 ± 0.5	1.8 ± 0.32*	1.82 ± 0.06*	<3.99
VLDL-cholesterol (mmol/L)	0.47 ± 0.06	0.52 ± 0.06*	0.74 ± 0.06*	<0.8
AIP	0.22	0.23	0.45	<1.44
TC: HDL	4.25	4.92	6.50	<4.9
LDL: HDL	2.67	2.57	3.03	<2.4

eGFR: estimated glomerular filtration rate; AIP: atherogenic index of plasma; * $P < 0.001$.

SCN may accelerate the process of atherosclerosis in patients with SCN. This study evaluates the association of oxidative stress and atherogenic index of plasma in order to assess the cardiovascular risk in SCN especially when lipoprotein levels are lower in SCD than non-SCD patients.

2. Materials and Methods

The study population was 110 confirmed SCD patients attending sickle cell disease clinic of Aminu Kano Teaching Hospital. They consisted of 65 males in steady state, aged 21.1±6.0 years, 30 males with macroalbuminuria aged 24.5 ± 7.0 years, and 15 with chronic kidney disease (CKD), aged 31.8 ± 2.0 years. Demographic and clinical examination findings were obtained using structured questionnaires. The study protocol used was approved by the institute's ethical committee and the patients gave informed consent before enrolment in the study. Random urine was obtained for analysis using combi-9 commercial dipstick, which was used to test for biochemical urinalysis. Five milliliter of blood was collected aseptically and dispensed into a plain tube after 12-hour fast. The blood was allowed to clot and serum obtained after centrifugation at 3000 rpm for 10minutes. The sera were stored at −20°C and analysis was done within two weeks of collection. Urea was determined using urease colorimetric technique, creatinine was assayed using sodium hydroxide-picric acid technique, and superoxide dismutase (Cu/ZnSOD), and glutathione peroxidase (GPx) were assayed using ELISA kits supplied by Northwest life science specialities, Vancouver, Canada. Catalase was estimated using kit by SIGMA (St. Louis, Missouri, USA) and malondialdehyde was determined using thiobarbituric acid reacting substance kit supplied by Northwest life science specialties. Total cholesterol and triglyceride (TG) were determined using enzyme-catalyzed colorimetric methods by Randox laboratories, UK. HDL cholesterol was assayed using the supernatant after precipitation with magnesium chloride-phosphotungstic acid solution, while LDL cholesterol was calculated using Friedewald formula [11]. Cardiovascular risk ratio was calculated using atherogenic index of plasma (AIP) [12], which was defined as log(TG/HDL-c) with TG and HDL-c expressed in molar concentration. Glomerular filtration rate was estimated using Cockroft-Gault formular [13]. Chronic kidney disease was defined as estimated glomerular filtration rate (eGFR) of <60 mL/min and presence of macroalbuminuria. Macroalbuminuria was defined as presence in urine of albumin concentration of ≥300 mg/L. A two-sample t-test was used to determine the statistical significance of the means between the different groups. A P value of 0.05 or less was considered statistically significant. Pearson correlation coefficient was used to show the levels of association of antioxidant enzyme levels with AIP in SCD patient with CKD.

3. Results

The results are as indicated in Tables 1, 2, and 3. Table 1 shows biochemical and lipoprotein levels of SCD subjects in steady state used as controls, SCD with macroalbuminuria, and CKD. The mean serum urea and creatinine in SCD with macroalbuminuria and CKD were significantly higher ($P < 0.001$) than those of the SCD control subjects while the mean eGFR was significantly lower ($P < 0.001$) in SCD with macroalbuminuria and CKD compared with control subjects. The mean levels of triglyceride, total cholesterol, LDL cholesterol, and VLDL cholesterol were significantly higher ($P < 0.001$) in CKD compared with SCD controls while HDL cholesterol was lower ($P < 0.001$) in CKD compared with control subjects. The atherogenic index significantly increased in SCD patients with CKD compared with SCD with macroalbuminuria and control group.

Table 2 indicates changes in oxidative stress markers in the study group. The mean activity levels of GPx, Cu/ZnSOD, and CAT were significantly lower ($P < 0.001$) in SCD with macroalbuminuria and CKD while MDA was higher

Table 2: Oxidative stress markers of sickle cell disease control, macroalbuminuria, and chronic kidney disease.

Oxidative markers	SCD controls	SCD with macroalbuminuria	SCD with chronic kidney disease	Reference range
Number of subjects	65	30	15	
Glutathione peroxidase (mU/mL)	9.2 ± 0.9	5.54 ± 3.5*	3.01 ± 0.24*	9.2–19.6
Superoxide dismutase (ng/mL)	30.6 ± 5.1	21.3 ± 5.2*	18.5 ± 2.6*	22.5–103
Catalase (μmol/min/L)	154 ± 7.9	150 ± 2.58	147 ± 1.06*	156–182
Malondialdehyde (μmol/L)	2.5 ± 0.4	3.8 ± 1.7*	5.30 ± 0.3*	0.025–0.98

* $P < 0.001$.

Table 3: Correlation between antioxidant enzymes and atherogenic index of plasma in SCD with chronic kidney disease.

Correlation between parameters	R value	P value
GPx and AIP	−0.760	0.001
Cu/ZnSOD and AIP	−0.621	0.02
CAT and AIP	−0.416	NS
MDA and AIP	0.943	0.001

GPx: Glutathione peroxidase; Cu/ZnSOD: Superoxide Dismutase; CAT: Catalase and MDA: Malondialdehyde.

($P < 0.001$) in SCD with macroalbuminuria and CKD compared with controls.

Table 3 shows the levels of association of antioxidant markers with AIP in SCD with CKD. There were negative correlations between GPx ($P < 0.001$), Cu/ZnSOD ($P < 0.02$), and AIP in SCD with CKD, while MDA shows a positive correlation ($P < 0.001$) with AIP in SCD with CKD. The correlation between CAT and AIP was however not significant.

4. Discussion

The data showed that there were increases in oxidative stress and lipoprotein levels in SCD patients with macroalbuminuria and CKD. Atherogenic index of plasma was negatively correlated with antioxidant enzymes and positively with MDA in CKD. Studies have shown decreases in the activity levels of antioxidant enzymes in SCD patients in steady state [4] and in non-SCD patients with acute renal failure [14]. The present study associated the decreases in the antioxidant enzyme activity levels with AIP, used as cardiovascular risk, especially as lipoprotein levels in SCD patients are lower than non-SCD individuals in both normal and renal disease. In this study, we observed increased oxidative stress in SCD patients with CKD. Oxidative damage is due to redox imbalance between production of reactive oxygen species (ROS) and the countering effects of the various antioxidants in the body. The increased production of ROS in SCD can be grossly amplified in response to a variety of pathophysiological conditions including renal disorders, inflammation, immunologic disorders, hypoxia, metabolism of drugs or alcohol, and deficiency in antioxidant enzymes [1, 4, 15]. Reactive oxygen species can cause significant damage to biomolecules since membrane lipids readily react with ROS resulting in lipid peroxidation [16–18]. Increased oxidative stress has been reported to mediate most of the risk factors involved in kidney disease [19]. Free radical mediated injury is the primary event leading to renal injury and progressive renal insufficiency and may lead to increased levels of lipid peroxidation. This occurs because cell membrane contains large quantity of PUFAs. These PUFAs react with ROS to form peroxide derivatives [20, 21]. Oxidative damage may alter both structure and function of the glomerulus due to its effects on mesangial and endothelial cells. Overexpression of ROS by both enzymatic and nonenzymatic pathways (Fenton chemistry) promotes intravascular oxidant stress that can disrupt nitric oxide homeostasis and produce the highly oxidative peroxynitrite [22]. Previous reports have showed that renal disease in SCD has the capacity to diminish arginine bioavailability through the loss of de novo arginine synthesis from citrulline which occurs primarily in the kidneys. Renal insufficiency may impair the major route for endogenous arginine biosynthesis [23] since it plays some roles in the regulation of nitric oxide production [24]. Adults with SCD are arginine deficient even at steady state [25, 26].

The observed increases ($P < 0.001$) in the levels of serum triglyceride, LDL, VLDL, and decrease ($P < 0.001$) of HDL-cholesterol levels in SCD patients with CKD are consistent with other studies elsewhere and in Nigeria [27–30]. The lipid profile of SCD patients is quite lower than that in subjects with normal haemoglobin and SCD patients in Nigeria have even lower levels than SCD patients in America and the Middle East [27–29]. Several reasons have been proposed as to why there are inconsistencies between lipid studies; these include differences in age, diet, weight, smoking, gender, small sample sizes, different ranges of disease severity, and treatment regimen [31–33]. Shores et al. [28] proposed the reasons why lipoprotein levels are lower in SCD than nonSCD even in the same environmental condition to be partly due to a decrease in red blood cell volume leading to a dilution effect on plasma constituents. It may also result from strong downregulation of the synthetic pathways or downregulation of the enzymes in lipid biosynthesis. Others suggested that it is the haemolytic stress in SCD patients that is associated with a significant reduction in plasma lipids in SCD patients [29, 34].

5. Conclusion

Decreased activity levels of antioxidant enzymes and low HDL-cholesterol concentration were confirmed in adult SCD with CKD in Nigerians. The increase oxidative stress

and high atherogenic index in CKD may accelerate the process of cardiovascular complications in adult SCD patients. Atherogenic index of plasma was negatively correlated with antioxidant enzymes and positively with MDA.

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Research Article

Sickle Cell Disease Activates Peripheral Blood Mononuclear Cells to Induce Cathepsins K and V Activity in Endothelial Cells

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Sickle cell disease is a genetic disease that increases systemic inflammation as well as the risk of pediatric strokes, but links between sickle-induced inflammation and arterial remodeling are not clear. Cathepsins are powerful elastases and collagenases secreted by endothelial cells and monocyte-derived macrophages in atherosclerosis, but their involvement in sickle cell disease has not been studied. Here, we investigated how tumor necrosis alpha (TNFα) and circulating mononuclear cell adhesion to human aortic endothelial cells (ECs) increase active cathepsins K and V as a model of inflammation occurring in the arterial wall. ECs were stimulated with TNFα and cultured with peripheral blood mononuclear cells (PBMCs) from persons homozygous for sickle (SS) or normal (AA) hemoglobin. TNFα was necessary to induce cathepsin K activity, but either PBMC binding or TNFα increased cathepsin V activity. SS PBMCs were unique; they induced cathepsin K in ECs without exogenous TNFα ($n = 4$, $P < 0.05$). Inhibition of c-Jun N-terminal kinase (JNK) significantly reduced cathepsins K and V activation by 60% and 51%, respectively. Together, the inflammation and activated circulating mononuclear cells upregulate cathepsin activity through JNK signaling, identifying new pharmaceutical targets to block the accelerated pathology observed in arteries of children with sickle cell disease.

1. Introduction

Sickle cell disease is a genetic disorder that causes *in vivo* polymerization of hemoglobin molecules into rigid fibers within red blood cells, deforming them in the canonically described "sickle" shape. Rigid, sickled red blood cells and the byproducts of their hemolysis cause chronic vascular damage and increase systemic levels of inflammatory cytokines, mobilized mononuclear cells [1], and pathological levels of increased monocyte adhesion to the endothelium [2, 3]. Overall, these pathological inflammatory conditions and mononuclear cell-endothelial cell interactions may contribute to intimal thickening, and lumen narrowing seen in pulmonary hypertension and stroke lesions of children; pulmonary hypertension is responsible for 20–30% of sickle-cell-related deaths in adult patients [4, 5] and 11% of children with sickle cell disease will suffer from a major stroke by the age of 16.

Both of these clinical syndromes are characterized by vascular remodeling [6–8]. Vascular remodeling analogous to stroke lesions in sickle cell disease has been observed in atherosclerosis, the major cardiovascular disease, where mononuclear cell infiltration of the subendothelial space, degradation of the elastic lamina, and subsequent smooth muscle cell proliferation mediate lesion progression and luminal narrowing [2]. These similarities suggest that common mechanisms for arterial remodeling may exist between the well-studied, well-characterized atherosclerosis, and the less understood mechanisms of sickle cell disease.

Arterial remodeling can be defined as changes in the composition of proteins, cell types, and even cell phenotypes that induce chronic effects on the structure, mechanical properties, and total health of the artery [6–8]. This includes degradation of old matrix by newly activated proteases as well as synthesis and deposition of new extracellular matrix proteins. Cysteine cathepsins, one such family of proteases upregulated in arterial remodeling [6, 9], belong to the papain superfamily of proteases and contain the most potent human collagenases and elastases [10]. Increased cathepsin activity has been linked to tissue destruction in

the cardiovascular system with atherosclerotic elastic lamina degradation [11–13], stent restenosis [14, 15], abdominal aortic aneurysm formation [16], and heart valve remodeling under hypertensive conditions [9].

Two cathepsins in particular have gained significant interest in their role in arterial remodeling in cardiovascular disease. Cathepsin K is the most potent human collagenase yet identified [17], as well as an extremely powerful elastase [18, 19]. Additionally, cathepsin K has been shown to be highly expressed in atherosclerotic lesions where it degrades arterial collagen and subendothelial elastic lamina [12, 13]. Cathepsin V is the most powerful mammalian elastase yet identified and is expressed in human monocyte-derived macrophages [10]. Studies have shown that the human cathepsin V homolog, murine cathepsin L [20, 21], significantly contributes to cardiovascular disease in mouse models [9, 22]. Neither of these two enzymes has been linked to sickle-cell-disease induced vascular wall remodeling and pathology.

In this study, we evaluated the potential involvement of cathepsin-mediated arterial remodeling in sickle cell disease by studying the effects of TNFα stimulation and adhesion of mononuclear cells isolated from whole blood of individuals homozygous for the sickle mutation on endothelial cell expression and activation of cathepsins K and V. We employed a novel, multiplex cathepsin zymography technique to simultaneously quantify the active forms of cathepsins K, L, S, and V in response to the different stimulation and coculture conditions [23]. Furthermore, we investigated the phosphorylation of key kinases to identify intracellular signaling cascades linking TNFα stimulation and mononuclear cell binding to increased levels of active cathepsins K and V as a proposed model for the unique and accelerated tissue remodeling observed in arteries of children and adults living with sickle cell disease.

2. Materials and Methods

2.1. Ethics Statement. All protocols were reviewed and approved by the Georgia Institute of Technology Institutional Review Board, and informed consent was received from all participants. In the case of minors, assent was provided by parents/guardians.

2.2. Cell Culture. Human aortic endothelial cells (HAECs) (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% endothelial cell growth serum (ECGS). Cells were maintained with 5% CO_2 at 37°C.

2.3. TNFα ELISA. Whole blood samples were allowed to coagulate for 6 hours, followed by centrifugation at 900 g for 30 minutes to remove platelets and cells. The supernatant was collected, and TNFα levels were quantified using an enzyme-linked immunosorbent assay (ELISA) specific for soluble, human TNFα (R&D Biosystems). Absorbance values were recorded using Synergy 4 (Biotek) at 450 nm with correction readings at 540 nm. Quantification of TNFα protein levels was calculated by generating a four-parameter logistic standard curve using Gen5 software (Biotek).

2.4. Peripheral Blood Mononuclear Cell Isolation. Whole blood samples were obtained from males and females homozygous for sickle (SS) or normal (AA) hemoglobin; patients on hydroxyurea, chronic transfusion, or who had experienced a recent crisis were excluded from this study. Whole blood samples were centrifuged against a Ficoll-Paque density gradient (density: 1.077 g/mL; GE Healthcare) for 30 minutes at 2450 rpm to separate the buffy coat layer. After centrifugation, peripheral blood mononuclear cells (PBMCs) were aspirated, washed in PBS, and pelleted by centrifugation for 10 minutes. The isolated cells were then washed with a red blood cell lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA) for seven minutes to remove any contaminating RBCs. Cell number and viability were determined using a Vi-Cell (Beckman Coulter).

2.5. PBMC Adhesion Assay. HAECs were preconditioned in normal growth media in the presence or absence of 10 ng/mL recombinant human TNFα (Invitrogen) and cultured for 4 hours prior to the addition of 500,000 PBMCs/mL. Isolated PBMCs were allowed to adhere for 45 minutes prior to washing three times with PBS, and then cocultures were maintained for an additional 20 hours. For JNK inhibition studies, endothelial cells were preconditioned with 10 μg/mL of SP600125 (EMD Biosciences) for one hour prior to addition of media containing vehicle, 10 ng/mL TNFα, and/or 10 μg/mL of SP600125.

2.6. Phosphorylated Kinase Screening. Cell lysates were prepared per BioPlex Suspension Array System instructions (BioRad). Lysates were incubated overnight with fluorescently labeled beads specific for the phosphorylated forms of Akt (Ser473), extracellular signal-regulated kinases 1 and 2 (Thr202/Tyr204, Thr185/Tyr187), c-Jun NH_2-terminal kinase (JNK) (Thr 183 /Tyr 185), and c-Jun (Ser63) (BioRad). The samples were then washed and incubated with kinase-specific, biotinylated antibodies for 2 hours, followed by treatment with avidin/streptavidin tagged with phycoerythrin. Phosphorylated kinase levels were measured using a BioPlex 200 System (BioRad).

2.7. Multiplex Cathepsin Zymography. Cathepsin zymography was performed as described previously [24]. Determination of cathepsin V band required incubation in acetate buffer, pH 4 [25]. Gels were imaged using an ImageQuant 4010 system (GE Healthcare). Images were inverted in Adobe Photoshop and densitometry was performed using Scion Image.

2.8. Statistical Analysis. Each experimental condition was repeated with a minimum of three biological replicates, and each data point is presented as the mean value and standard error of the mean. Representative images are shown.

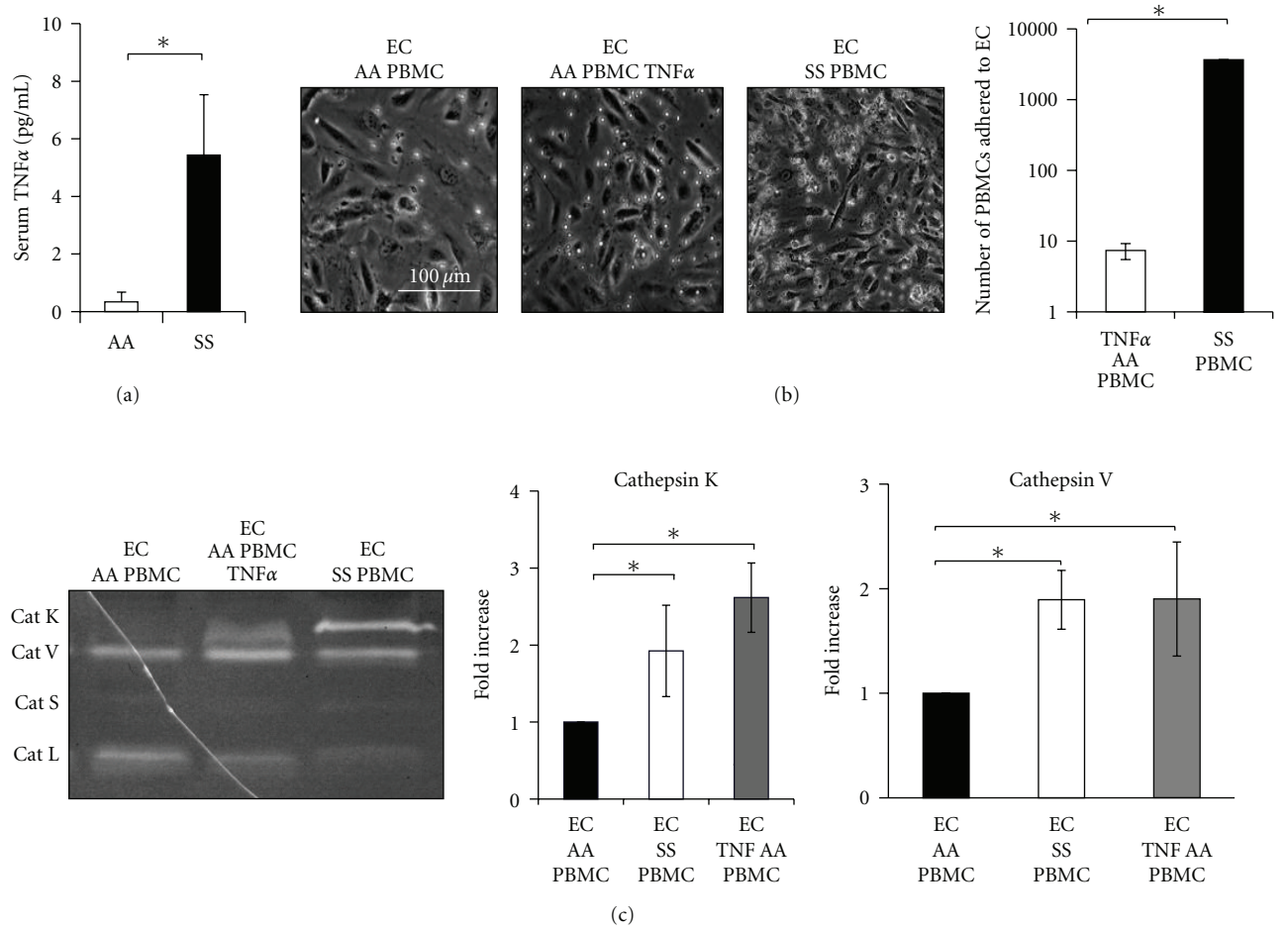

Figure 1: Sickle cell disease preconditions circulating peripheral blood mononuclear cells to induce cathepsin K activity. Whole blood samples were obtained from donors homozygous for the normal β-globin allele (AA) and homozygous for the sickle allele (SS). (a) Baseline serum levels of TNFα were quantified using an ELISA specific for human TNFα ($n = 3$, $^*P < 0.05$, SEM bars shown). (b) PBMCs were isolated via differential centrifugation through a density gradient. For cocultures, confluent EC cultures were preconditioned with 10 ng/mL TNFα for 4 hours, prior to the addition of either AA or SS PBMCs. Nonadherent cells were washed away, and cocultures were maintained for an additional 20 hours. Representative images of cocultures were used for mononuclear cell adhesion counts. (c) Cells were lysed and cathepsin K activity was assessed using multiplex cathepsin zymography and quantified via densitometry ($n = 10$, $^*P < 0.05$).

Unpaired student t-tests were used to determine statistical significance ($^*P < 0.05$) between most experimental groups.

3. Results

3.1. Sickle Cell Disease Preconditions Circulating PBMCs to Induce Cathepsin K Activity. Whole blood samples were obtained from donors homozygous for normal (AA) or sickle (SS) hemoglobin. First, an ELISA was run to quantify blood serum levels of TNFα. SS donors had 5.43 ± 2.3 pg/mL of TNFα compared to 0.3 ± 0.3 pg/mL of TNFα in AA controls ($n = 3$, $P < 0.05$), an almost 20-fold increase (Figure 1(a)). TNFα stimulation of endothelial cells increased the adhesion of AA PBMCs, compared to unstimulated EC cultures (Figure 1(b)); however, the number of adhered SS PBMCs was 100 times higher than TNFα stimulated AA PBMC cocultures (Figure 1(b); $n = 3$, $P < 0.001$). Cells were cultured together for an additional 20 hours for cathepsin induction, prior to lysing, collection, and multiplex cathepsin zymography. SS PBMCs significantly increased levels of active cathepsins K and V when cocultured with endothelial cells, and without exogenous TNFα stimulation (Figure 1(c)), suggesting that the SS PBMCs were preconditioned to induce this activity. AA PBMC cocultures in the absence of TNFα lacked detectable bands of active cathepsin K (Figure 1(c), left lane).

3.2. TNFa Stimulation and PBMC Interactions with Endothelial Cells Activate JNK Signaling. To investigate the intracellular signal cascades increasing the levels of active cathepsins K and V downstream of TNFα and PBMC adhesion cues, we measured phosphorylation of JNK, c-jun, Akt, and ERK1/2 using Bioplex/Luminex technology, a quantitative bead-based immunofluorescent assay that allowed measurement of all four signals in one cell extract after 24 hours of coculture. JNK and its downstream signaling protein substrate, c-Jun, showed the greatest activation in response to TNFα stimulation with or without AA or SS PBMCs (Figures 2(a)

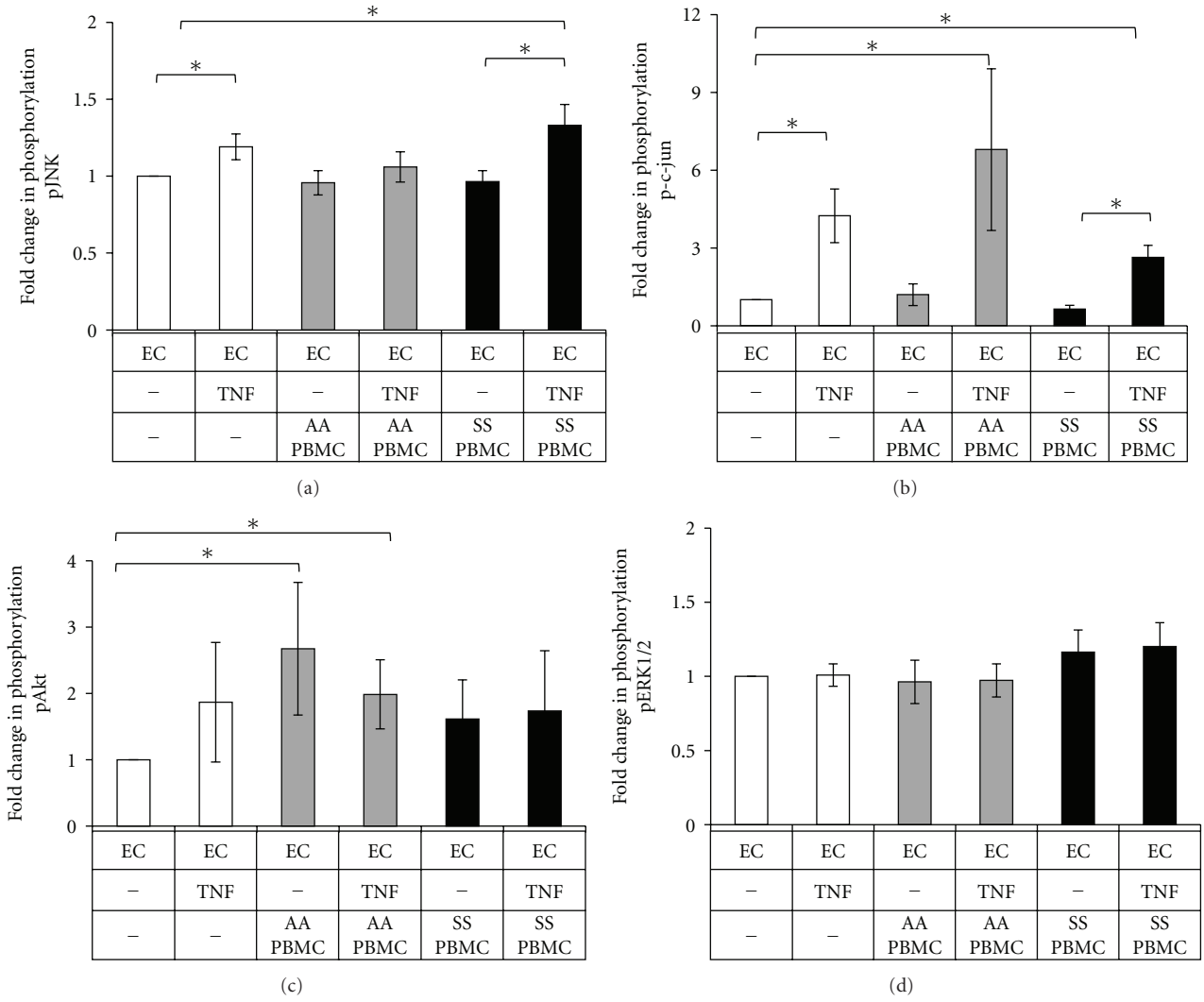

Figure 2: TNFα and PBMC interactions increase JNK and Akt phosphorylation. Confluent HAECs were cocultured with peripheral blood mononuclear cells isolated from AA or SS donors, and lysates were collected for kinase analysis. Levels of phosphorylated (a) JNK, (b) c-Jun, (c) Akt, and (d) ERK1/2 were measured, and phosphorylated kinase signals were normalized to unstimulated HAEC control ($n = 3$, $^*P < 0.05$, SEM bars shown).

and 2(b), $n = 3$, $P < 0.01$) with c-Jun activation as high as 6-fold that of the EC controls. Akt phosphorylation was significantly increased by AA PBMC binding alone even without TNFα stimulation (Figure 2(c), $n = 3$, $P < 0.01$). There were no changes in ERK 1/2 phosphorylation in any condition for all time points measured (Figure 2(d)).

3.3. Cathepsins K and V Activities Induced by Sickle Cell Disease PBMCs Were Significantly Reduced by JNK Inhibition. Since JNK and c-jun phosphorylation were significantly upregulated, we tested if inhibiting this signal cascade would block the increase in levels of active cathepsins K and V by endothelial cells after adhesion and coculture with SS PBMCs. HAECs were cultured with or without SP600125, a JNK inhibitor, for 1 hour prior to addition of 10 ng/mL TNFα or vehicle. AA or SS PBMCs were subsequently added, and nonadhered cells were washed away. Cell lysates were collected after 24 hours, and cathepsin activity was assessed through multiplex cathepsin zymography. SP600125 significantly reduced the upregulated cathepsin K and cathepsin V activities of unstimulated SS PBMCs when cocultured with endothelial cells by 48% and 29%, respectively (Figure 3; $n = 5$, $P < 0.05$).

4. Discussion

Endothelial cell expression of cathepsins and increased cathepsin-mediated elastase activity are upregulated during atherosclerotic development and induced by inflammation and altered hemodynamics [9, 12, 13, 26, 27], which are both present in sickle cell disease [26], leading to our hypothesis that elevated TNFα and increased circulating mononuclear cells would stimulate increased endothelial cell cathepsin activity. This elevated activity may contribute to arterial

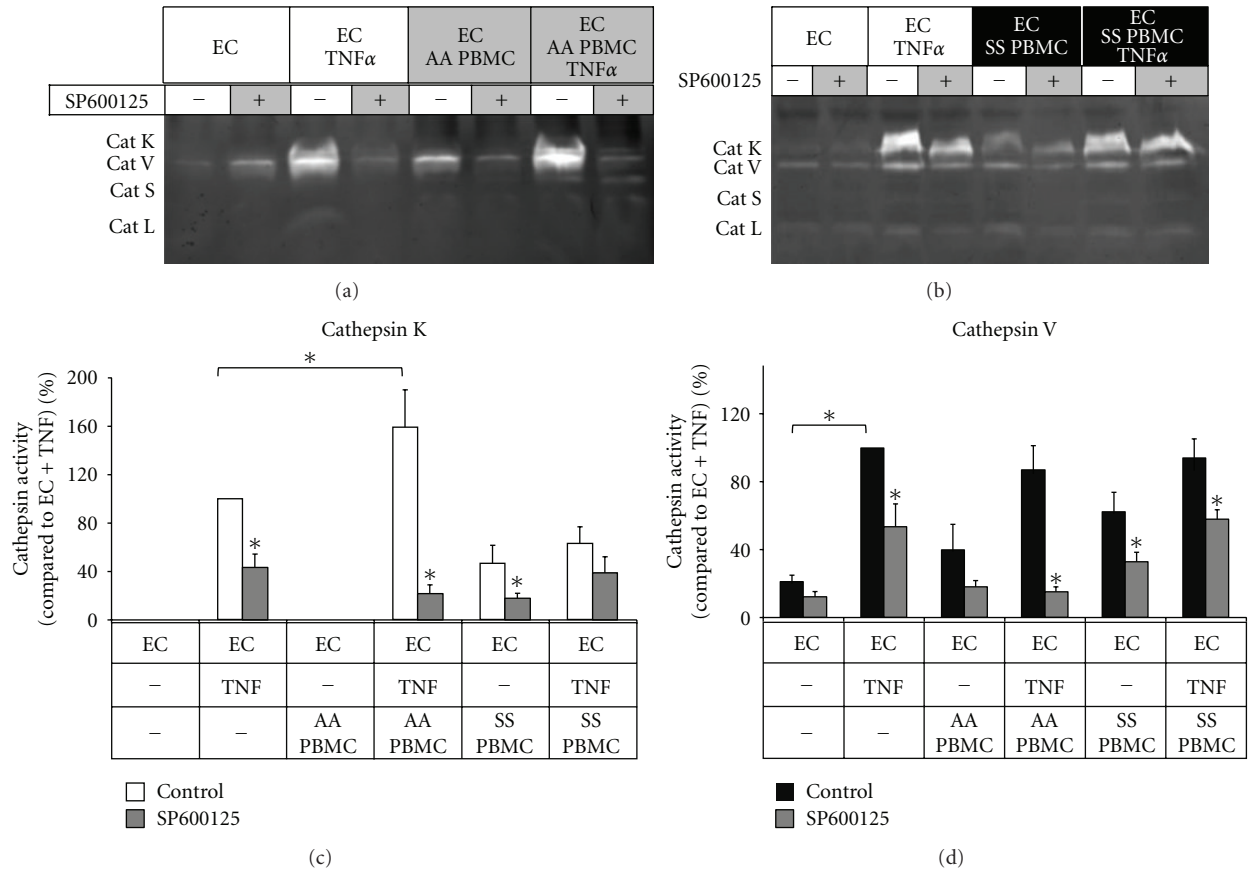

Figure 3: Cathepsins K and V activities induced by sickle cell disease PBMCs are significantly reduced by JNK inhibition with SP600125. HAECs were incubated with or without 10 μM of the JNK inhibitor, SP600125, 1 hour prior to TNFα stimulation, as described previously. Cocultures with AA or SS PBMCs were maintained for an additional 20 hours. Cell lysates were collected and analyzed via multiplex cathepsin zymography. Densitometric analysis quantified active cathepsins K and cathepsin V ($n = 3$, $^*P < 0.05$, SEM bars shown).

remodeling in sickle cell disease. The findings of this study specifically implicate TNFα and mononuclear cell binding to endothelium as key mediators, and that circulating mononuclear cells in sickle cell disease are predisposed to induce cathepsin proteolytic activity.

Here, we have specifically shown that TNFα stimulation increased the expression and activity of the most potent mammalian collagenase and elastase, cathepsins K and V, respectively (Figure 1). Additionally, SS PBMCs significantly increased cathepsin K activity in endothelial cells in the absence of TNFα, suggesting that they were preconditioned in the blood for adhesion to endothelium and cathepsin K induction (Figure 1); AA PBMCs required TNFα stimulation to reach these higher levels of cathepsin K and V (Figure 1). These findings are consistent with reports that circulating sickle erythrocytes increase mononuclear cell activation and adhesion to endothelial cells [28] and support our hypothesis that the blood milieu of people living with sickle cell disease predisposes circulating mononuclear cells to adhere to endothelium and promote arterial remodeling. Previous studies have already established that the circulatory environment in sickle cell disease preconditions peripheral blood mononuclear cells into a pathologically activated state, where these cells produce 139% more TNFα per cell than control mononuclear cells [28, 29]; these mechanisms may be at play here leading to increased active cathepsins K and V.

Inhibition of JNK signaling with SP600125 reduced the inflammation-induced activation of cathepsins K and V in AA and SS PBMC cocultures with endothelium (Figure 3). These findings highlight the role of JNK signaling as an integration control point and as a therapeutic target to inhibit the initiation of gene and protein expression in response to inflammatory stimuli resulting in endothelial cell upregulation of cathepsins K and V protein and activity. More importantly, the predisposition of SS PBMCs to induce these effects suggests that these novel mechanisms may be occurring constantly in the vasculature of individuals with sickle cell disease. It will be important to continue these studies quantifying cathepsin activation of SS donors with and without stroke or with high transcranial Doppler velocities known to be a risk factor for stroke to parse differential activation mechanisms potentially responsible for the increased risk. Such investigations may reveal novel biomarkers relevant to stroke risk prediction in pediatric patients and open new avenues for pharmaceutical therapies to prevent the arterial remodeling and luminal narrowing that cause cardiovascular complications and death.

5. Conclusion

Elevated inflammatory factors and circulating mononuclear cells inherent to sickle cell disease induce pathologically high levels of cathepsins K and V activity when binding to and stimulating endothelial cells, increasing proteolytic activity that may be involved in arterial wall remodeling to increase risk of stroke and pulmonary hypertension. There is a pressing need for novel pharmaceutical targets to inhibit these activities, and from this work, we propose that JNK, cathepsin K, and cathepsin V are three new targets for inhibition to reduce pathological arterial remodeling in sickle cell disease.

Conflict of Interests

The authors have declared that no competing interests exist.

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