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Punam Malik
John Tisdale *Editors*

Gene and Cell Therapies for Beta-Globinopathies

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Editors

Gene and Cell Therapies for Beta-Globinopathies

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Preface

The β-hemoglobinopathies and thalassemias are inherited genetic diseases which result in a pathologic or underproduction of one or more of the globin chains. Genetic modifiers of disease severity have defined new potential treatment modalities. Indeed, transfer of the normal β-globin gene through hematopoietic stem cells (HSCs) by allogeneic bone marrow transplantation has already proven curative in a select group of pediatric patients. However, the requirement of an HLA-matched sibling donor and the toxicity of myeloablative conditioning have historically limited this approach to a fraction of affected individuals. A non-myeloablative conditioning regimen designed intentionally to allow for stable mixed chimerism was recently developed to allow application in the severely affected demonstrating stable mixed chimerism and disease reversion in around 90% of adults with a suitable matched sibling donor. These data support mixed chimerism as a suitable goal for HSC transplantation in sickle cell disease (SCD), and efforts to extend this potentially curative approach to individuals with half-matched donors or autologous correction using gene therapy have now emerged.

Autologous HSC "gene therapy" remains a logical alternative to allogeneic transplantation approaches as the patient serves as his/her own donor, eliminating the risk of graft versus host disease and graft rejection. Significant progress in the field of HSC gene transfer has resulted in proof of concept through clinical successes in a number of diseases. Recently, incorporation of large elements of the human β-globin locus control region (LCR) became feasible with the development of lentiviral vector systems based upon HIV1, and clinical trials are now under way testing this approach in patients with both thalassemia and sickle cell disease. Finally, new molecular tools which allow editing of the genome have introduced the prospect for creating disease-modifying genetic changes or for correcting the underlying genetic defect in induced pluripotent stem cells or primary hematopoietic stem cells.

These exciting new developments are described by leaders in these fields and offer hope for a new era of treatments for these devastating disorders.

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Punam Malik received her M.D. degree from Lady Hardinge Medical College, University of Delhi, India. She completed a pediatric residency in Delhi and trained in hematology-oncology at Children's Hospital Los Angeles (CHLA), University of Southern California, where she served as a fellow, an assistant professor and a tenured associate professor. She joined Cincinnati Children's Hospital Medical Center (CCHMC) and the University of Cincinnati thereafter as the program director of the Gene and Cell Therapy Program, and the director of the Translational Core Laboratories. Subsequently, she became a professor of pediatrics, the director of CCHMC's Comprehensive Sickle Cell Center, and the Marjorie Johnson endowed professor of gene and cell therapy. Her group focuses on the pathobiology of sickle cell disease and thalassemia, identifying mechanisms of organ pathologies in sickle cell disease, and developing curative strategies for hemoglobinopathies and other hematopoietic stem cell disorders through transplantation of genetically modified autologous bone marrow stem cells.

Chapter 1
Clinical Features of β-Thalassemia and Sickle Cell Disease

Patrick T. McGann, Alecia C. Nero, and Russell E. Ware

Abstract Sickle cell disease (SCD) and β-thalassemia are among the most common inherited diseases, affecting millions of persons globally. It is estimated that 5–7% of the world's population is a carrier of a significant hemoglobin variant. Without early diagnosis followed by initiation of preventative and therapeutic care, both SCD and β-thalassemia result in significant morbidity and early mortality. Despite great strides in the understanding of the molecular basis and pathophysiology of these conditions, the burden of disease remains high, particularly in limited resource settings. Current therapy relies heavily upon the availability and safety of erythrocyte transfusions to treat acute and chronic complications of these conditions, but frequent transfusions results in significant iron overload, as well as challenges from acquired infections and alloimmunization. Hydroxyurea is a highly effective treatment for SCD but less so for β-thalassemia, and does not represent curative therapy. As technology and use of cellular and gene therapies expand, SCD and thalassemia should be among the highest disease priorities.

Keywords Sickle cell disease • β-thalassemia • Beta globin • Hemoglobin • Iron overload

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Introduction

Inherited disorders of hemoglobin, primarily sickle cell disease (SCD) and β-thalassemia are among the most common monogenetic diseases in the world [1]. The importance of these hematological disorders cannot be overstated; it is estimated that 5–7% of the world's population are carriers of a significant hemoglobin variant [2, 3].

SCD has often been described as the "first molecular disease" following Linus Pauling's discovery in 1949 that the disorder was due to an abnormal hemoglobin molecule [4]. This landmark report was followed several years later by a crucial elucidation by Vernon Ingram that sickle hemoglobin (HbS) is due to a single amino acid change in the β-globin chain of the hemoglobin molecule [5]. These reports were the first to describe the specific molecular basis for a human disease, and led to a flurry of scientific investigation of normal and abnormal hemoglobin, including several seminal findings describing the genetic basis of the thalassemias [6–9]. The genetic nature of these hemoglobin disorders has been further defined over the past 50 years, such that now the complete DNA sequences of the normal α- and β-globin gene loci and over 1000 hemoglobin variants are known.

Despite this long history that has led to a detailed understanding of the molecular basis of hemoglobin disorders such as SCD and thalassemia, the global burden of these conditions remains enormous and continues to increase [10, 11]. With the imminent arrival and expansion of cellular and gene therapy technologies, there is no question that SCD and β-thalassemia should be among the highest priorities for these novel and exciting therapies. In this chapter, we will provide an introduction to the global epidemiology, pathophysiology and clinical features of SCD and β-thalassemia, establishing a framework for subsequent discussion of the potential gene and cellular therapies that aim to ameliorate and cure these serious and life-threatening hematological disorders.

Hemoglobin: Structure and Function

Hemoglobin is a protein found abundantly in erythrocytes, with the primary function of oxygen transport; hemoglobin primarily transports oxygen from the lungs to the tissues, and subsequently returns oxygen (in the form of carbon dioxide) back to the lungs. The hemoglobin molecule is a tetramer, consisting of two α-like globin chains (141 amino acids) and two β-like globin chains (146 amino acids). Each polypeptide chain is attached non-covalently to a heme group, which binds oxygen and facilitates gas transport and exchange.

Two tandem α-globin genes (*HBA2*, *HBA1*) regulate α-globin production and are located on chromosome 16 along with *HBZ*, which regulates expression of the embryonic α-like ζ (zeta) globin chains. Each β-globin chain consists of 146 amino acids and is regulated by a single β-globin gene (*HBB*) located on chromosome 11. *HBB* is part of a gene cluster on chromosome 11 that regulates expression of four other β-like globin proteins that can pair with α-globin: embryonic ε-globin (*HBE1*) gene, tandem fetal or γ-globin (*HBG2* and *HBG1*) genes, and the δ-globin (*HBD*) gene.

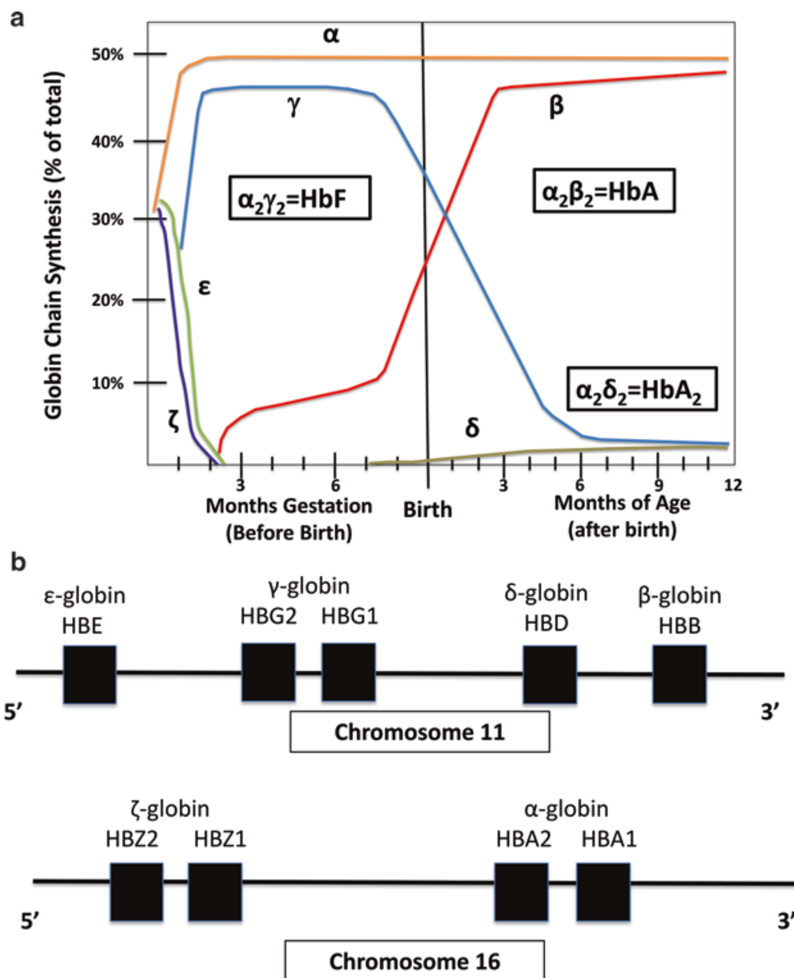

Fig. 1.1 Panel (**a**) illustrates the patterns of globin chain synthesis during normal development. In each setting, two α-like globin chains will normally pair with two β-like globin chains to form a mature hemoglobin heterotetramer (Adapted from Oliveri NF. NEJM 1999, [104]). Panel (**b**) illustrates the α- and β-globin gene clusters on chromosomes 11 and 16, respectively

The production of globin chains is tightly regulated during ontogeny, with important variations in gene expression occurring throughout normal development and in different disease states. Figure 1.1 illustrates the globin gene clusters and the resulting patterns of globin chain synthesis during normal development. In each setting, two α-like globin chains will normally pair with two β-like globin chains to form a mature hemoglobin heterotetramer. In some instances, however, particularly like those found in thalassemia with quantitatively reduced globin chain production, unpaired globin chains produced in excess can form homotetramers that are unstable and lead to serious clinical consequences.

Types of Hemoglobin

In the early stages of human embryonic development (2–3 weeks of gestation), erythropoiesis occurs primarily in the yolk sac and the dominant globin chains include ζ-globin and ε-globin. The resultant earliest embryonic hemoglobin is thus Hb Gower I (ζ_2/ε_2) [12]. By the 5th or 6th week of gestation, the primary site of erythrocyte production is the liver and the synthesis of α-globin and γ-globin chains begins to increase, resulting in Hb Portland (ζ_2/γ_2), Hb Gower II (α_2/ε_2) [13], and ultimately HbF (fetal hemoglobin, $\alpha_2\gamma_2$) [14]. By 12 weeks of gestation and throughout early infancy, HbF is the predominant hemoglobin within circulating erythrocytes.

The unique characteristics of fetal hemoglobin are important to understand, since HbF has key effects on the pathophysiology of patients with SCD and β-thalassemia, and thus serves as a therapeutic target. HbF has an increased oxygen affinity compared to normal adult hemoglobin (HbA, $\alpha_2\beta_2$), so that HbF has a "left-shifted" oxygen dissociation curve; in the fetus, this allows extraction of oxygen from the mother's HbA and still provides sufficient tissue oxygenation despite the relatively low oxygen content in the fetal circulation. After birth, coincident with the post-natal increase in ambient oxygen concentration, this increased oxygen affinity is no longer physiologically necessary and HbF production declines.

Toward the end of gestation and continuing over the first 6–12 months of life, the expression of γ-globin genes is reduced in favor of β-globin gene expression. By 6 months of post-natal age, HbA predominates and by 2 years of age, HbF expression is effectively repressed. This important "hemoglobin switch" has been extensively studied and a number of potent genetic regulators have been identified, most notably BCL11A [15–17]. The importance and potential therapeutic implications of targeting the hemoglobin switch for the treatment of SCD and β-thalassemia, and will be discussed in more detail in subsequent chapters.

HbA_2 ($\alpha_2\delta_2$) is a minor hemoglobin expressed beginning at about 6 months of age, but only represents 2–3.5% of total hemoglobin, since the δ-globin gene is inefficiently transcribed due to a faulty promoter region [18]. Hb A_2 does not have much clinical significance in healthy persons, but is increased in the setting of β-chain imbalance, and thus serves as an important diagnostic feature of β-thalassemia trait.

Classifying the Hemoglobin Disorders

The disorders of hemoglobin can be classified broadly into two distinct categories: quantitative or qualitative disorders. Quantitative disorders include those in which there is an absence or significant decrease in the production in one of the globin chains; these disorders are commonly referred to as the thalassemias. Although not the focus of this chapter, α-thalassemia features quantitative decrease of alpha globin chains, typically the result of gene deletion events resulting in the loss of one or both tandem α-globin genes on chromosome 16. The analogous β-thalassemia most commonly results from point mutations within the *HBB* promoter, exonic, or

intronic sequences; the result is reduced or absent β-globin chain synthesis. In contrast, the sickle cell disorders are qualitative defects of hemoglobin that result from a structural defect in the β-globin chains. The sickle mutation (β^S) is by far the most common qualitative hemoglobin disorder, but hundreds of qualitative hemoglobin mutations causing significant clinical sequelae have been described. For the purposes of this chapter, we will focus on the two most common and most pathological hemoglobin disorders: β-thalassemia and sickle cell disease.

β-Thalassemia

Overview and Historical Perspective

In 1925, at the American Pediatric Society Meeting, Detroit pediatrician Thomas Cooley described five children of Italian origin with severe anemia, splenomegaly, a "peculiar appearance" secondary to "yellowish discoloration of the skin and… thickening of the cranial bones" and a blood smear notable for poikilocytosis, anisocytosis, and target cells [19]. Cooley noted that the clinical condition of these children was similar to the chronic anemia previously named "anemia infantum pseudoleucaemica" a generation earlier [20, 21]. The term "thalassemia," which is derived from the Greek words for sea (thalassa) and blood (haema), was first coined in 1932 by Nobel laureate pathologist George Whipple and his pediatrician colleague William Bradford in their seminal report of autopsy findings in children with Cooley's anemia, most notably the wide deposition of iron [22, 23]. The genetic basis of thalassemia was further described over the next decade by several investigators who noted both the recessive nature of the disease and the distinction between a "minor" and "major" phenotype [24–26].

Epidemiology and Global Burden

β-thalassemia represents one of the world's most common inherited conditions, with estimates that up to 1.5% of the world's population are carriers of a pathological β-thalassemia mutation, and over 40,000 infants are born each year with either β-thalassemia major or HbE/β-thalassemia [1, 2]. The β-thalassemia gene mutations originated primarily in the Mediterranean region and extended eastward in a band across the Middle East, India and Southeast Asia, but subsequent migration has resulted in a widespread global distribution.

The geographic origins of the β-globin mutations responsible for β-thalassemia clearly overlap the geographic distribution of malaria endemicity [27], although protection against severe malaria for β-thalassemia carriers has not been demonstrated as clearly as for carriers of the HbS mutation. The genetic mutations leading to β-thalassemia (including HbE), similar to the HbS mutation, are examples of balanced

genetic polymorphisms in which the heterozygous state offers a survival advantage, while the homozygous state results in significant morbidity and early mortality [28, 29]. The relative survival advantage conferred by the heterozygous state provides genetic pressure that leads to persistence of these deleterious mutations.

Molecular Basis and Pathophysiology

β-thalassemia includes a heterogeneous group of inherited anemias characterized by defective β-globin synthesis. Over 270 different β-globin gene mutations (mostly point mutations) have been described that result in a reduction or absence of β-globin production [30, 31]. Some mutations, notably gene deletions and nonsense point mutations, are so damaging to β-globin gene expression that there is a total absence of β-globin production (β^0-thalassemia), while others result in an incomplete quantitative reduction in β-globin production (β^+-thalassemia). The clinical heterogeneity of β-thalassemia is primarily dependent upon the severity of the genetic mutation and the resultant imbalance between α- and β-globin chains. The molecular basis of β-thalassemia will be discussed in further detail in Chap. 2.

Globin gene synthesis is tightly regulated through the series of α- and β-globin (or β-like) genes on chromosomes 16 and 11, respectively. The pathophysiology of β-thalassemia has been well described, and is due not only to insufficient production of β-globin chains, but also the relative excess of α-globin chains that are deleterious when not bound in a hemoglobin tetramer. During fetal development and early infancy, when ɣ-globin is still expressed, any excess α-globin chains can pair with ɣ-globin to produce HbF. When ɣ-globin expression is repressed, the α-chains are in excess without a sufficient number of available β-like globin chains resulting in α:β chain imbalance and a reduction in hemoglobin formation. Excess α-globin chains precipitate within erythrocytes as hemichromes forming reactive oxygen species that are toxic to both developing erythroblasts and mature erythrocytes. This leads to inadequate erythroid differentiation and increased apoptosis of erythrocyte precursors within the marrow, a process referred to as ineffective erythropoiesis. The anemia is only exacerbated further by hemolysis secondary to erythrocyte membrane damage [32, 33].

The chronic anemia occurring in β-thalassemia leads to an increase in serum erythropoietin, which is not fully compensated in the setting of ineffective erythropoiesis. Clinical sequelae of ineffective erythropoiesis include massive expansion of the bone marrow erythroid compartment with intramedullary destruction, extramedullary hematopoiesis, splenomegaly, and significantly increased gastrointestinal iron absorption. The degree of globin chain imbalance in β-thalassemia is closely linked to the severity of disease. This is most obvious in the setting of co-inherited α-thalassemia, in which the clinical severity is ameliorated by a closer balance of α- and β-globin chains [34]. In addition to co-inheritance of α-thalassemia, there are additional genetic modifiers of β-thalassemia that directly impact the clinical manifestations of disease, which will be addressed in Chap. 2.

Iron and β-Thalassemia

Dysregulation of iron homeostasis is another important component of the pathophysiology of β-thalassemia. Ineffective erythropoiesis and the associated upregulated erythropoietic drive result in increased intestinal absorption of iron, causing a wide variety of deleterious consequences including iron deposition into many internal organs with resulting parenchymal dysfunction. Over the past 15 years, the hepatic peptide hormone hepcidin has been firmly established as a key regulator of iron homeostasis. Hepcidin binds to the membrane-bound iron export protein, ferroportin, causing its degradation and subsequent reduction in the export of cellular iron. This reduction in surface ferroportin expression results in decreased iron influx into plasma from the gastrointestinal tract (dietary iron), macrophages (recycled iron), and hepatocytes (stored iron) [35, 36]. When plasma and stored iron levels are high, hepcidin production is increased and inhibits further iron loading by blocking intestinal iron absorption; conversely when iron is needed, hepcidin levels fall and accelerate uptake of intestinal iron.

Erythropoiesis, due to its dependence upon iron, is perhaps the most potent physiological regulator of hepcidin. In an effort to support increased erythropoietic demand, and potentially mediated by erythroferrone [37], hepcidin production is decreased in β-thalassemia and as a result, there is rapid absorption and release of iron into the plasma [38]. The combination of accelerated and ineffective erythropoiesis allows the increased absorption of iron in the gut to continue unabated. Elevated plasma iron, especially labile (non-transferrin-bound) iron, leads to deposition into viscera (particularly the heart, liver, and endocrine organs) with significant tissue damage and organ dysfunction [38]. The mainstay of treatment of β-thalassemia, chronic erythrocyte transfusions, alleviates anemia and reduces the erythropoietic drive, but compounds the hemosiderosis due to the significant iron load from each transfusion. Iron overload, which results from both increased intestinal absorption and from repeated transfusions, remains the most significant cause of morbidity and mortality for patients with β-thalassemia**.**

Classification and Clinical Manifestations of β-Thalassemia

β-thalassemia is a clinically heterogeneous group of conditions that are typically classified into three groups based on clinical severity: β-thalassemia major, β-thalassemia intermedia, and β-thalassemia minor.

β-Thalassemia Major

The most severe form of β-thalassemia is referred to as β-thalassemia major (TM), typically the result of inheritance of homozygous (β^0/ β^0) or compound heterozygous (β^0/ β^+) mutations that feature minimal (if any) β-globin production and severe α:β chain imbalance, with consequent erythrocyte transfusion dependence. As with other forms of β-thalassemia, however, TM has a variable clinical phenotype dependent

upon the specific β-globin gene mutations, other inheritable genetic modifiers and environmental factors.

TM most commonly presents within the first 1–2 years of life due to severe anemia, failure to thrive, and the need for regular blood transfusions. Infants with TM may not develop clinical symptoms in the first 4–6 months of life due to high levels of HbF. However, as the expression of γ-globin is repressed and the absolute amount of fetal hemoglobin declines, hemolytic anemia becomes clinically apparent making the effects of reduced or absent β-globin chains clearly evident. These children can present with anemia, pallor, poor growth, and hepatosplenomegaly due to extramedullary hematopoiesis.

Hematological Manifestations

The defining hematological feature of TM is severe anemia with an inability to maintain a hemoglobin concentration above 7–8 g/dL. Hemolytic anemia leads to the presenting features of TM with pallor, mild jaundice, and without early diagnosis and initiation of chronic transfusion therapy, TM is a lethal condition.

Early initiation of transfusion therapy is critical for normal growth and development and is also protective of significant early organ damage and the aforementioned physical deformations. Hypertransfusion to maintain a hemoglobin concentration above 10 g/dL affords these infants and children an opportunity for normal growth and development [39]. Chronic erythrocyte transfusion therapy should always be combined with aggressive iron surveillance and iron chelation therapy.

Skeletal Manifestations

Ineffective erythropoiesis in TM results in significant extramedullary hematopoiesis that clinically manifests as skeletal abnormalities, particularly in the skull (e.g., frontal bossing, maxillary hyperplasia). Chronic erythrocyte transfusion therapy (approximately every 3–4 weeks) suppresses ineffective erythropoiesis and prevents these types of abnormalities. In addition to the aesthetic complications of bony abnormalities, these patients demonstrate decreased bone mass starting early in life, which leaves them prone to fractures [40]. Routine surveillance of bone mineral density and general bone health (such as vitamin D monitoring) should be performed in all patients with β-thalassemia major.

Endocrine Manifestations

One of the most critical benefits of initiating early transfusion therapy is improved growth and development, which are significantly delayed in untreated TM. Despite the significant and life-saving benefits of chronic transfusion therapy for persons

with TM, this therapy presents severe endocrine complications if adequate attention to iron burden and aggressive chelation therapy are not maintained. Endocrine complications are often the first clinical manifestations of iron overload due to iron deposition in the anterior pituitary, which can lead to hypogonadism, growth retardation and short stature, hypothyroidism, hypoparathyroidism, and diabetes mellitus [41]. Routine screening and attention to symptoms related endocrine function are essential for all patients with TM.

Hepatic Manifestations

The excess total body iron characteristic of TM results in significant iron deposition within the liver. Without adequate chelation therapy, hepatic iron deposition begins in the macrophages (Kupffer cells) later moving to the parenchyma and sinusoids, which eventually results in hepatic fibrosis and significant liver dysfunction. Cirrhosis and hepatocellular carcinoma are two serious complications of the chronic hepatitis from longstanding severe iron overload.

Over the past decade, there have been significant improvements in the diagnostic methods of measuring liver iron content. Liver biopsy is now replaced by advanced MRI imaging techniques for quantitative measurement of liver iron content [42]. Non-invasive serial measurements of liver iron content are used to assess the dosing and effectiveness of iron chelation therapy. Frequent discussions with patients to optimize compliance with chelation therapy, however, remain essential to prevent the significant morbidity and even mortality associated with hepatic iron overload.

Cardiac Manifestations

The cardiac complications from iron overload are the most common cause of death for persons with TM. Several studies have demonstrated cardiomyopathy as the cause of approximately 70% of deaths in persons with thalassemia [43–45]. The development of effective iron chelating agents has substantially reduced the frequency and severity of fatal cardiomyopathy. Despite these available therapies cardiac dysfunction remains a significant problem when iron chelation therapy is suboptimal. Myocardial iron deposition most commonly results in left ventricular dilatation and systolic dysfunction. It is critical to diagnose and treat myocardial involvement in persons with TM as early as possible, including referral to heart failure specialists as necessary. Similar to the advancements in assessing liver iron content, noninvasive MRI techniques can accurately quantify myocardial iron that and should routinely be performed in all patients with TM.

β-Thalassemia Intermedia

β-thalassemia intermedia (TI) is also a serious blood disorder that requires routine medical care and therapy. The distinction between TI and TM is determined by the clinical severity of the disease. Operationally, patients with TI do not require regular blood transfusions while those with TM do; however, it is possible that a person can initially be diagnosed with TI, but subsequently could become dependent upon transfusions, thereby reclassifying the diagnosis as TM.

The two defining characteristics of TI are that both β-globin gene loci are affected (typically β^+/β^+ or β^0/β^+, but occasionally β^0/β^0 with ameliorating genetic modifiers) and that chronic transfusion therapy is not necessary to maintain an acceptable hemoglobin concentration and reasonable quality of life [46]. A special example of TI is heterozygous HbE with β-thalassemia, found most commonly in persons from Southeast Asia (see Chap. 2). In TI, because patients with TI are often relatively asymptomatic early in life, they often present later in childhood than those with TM.

Despite the transfusion independence, TI is not a benign condition. For the same pathophysiological reasons as described in TM, ineffective erythropoiesis is a key feature of TI and results in chronic hemolytic anemia, hepatosplenomegaly, extramedullary erythropoiesis, and hemosiderosis secondary to increased gastrointestinal iron absorption. Although patients with TI can maintain a hemoglobin concentration of 8–10 g/dL, and do not require chronic transfusions for anemia and its sequelae, regular comprehensive assessments of growth, development, iron burden and organ function are required to ensure that the classification as TI remains appropriate and that they would not benefit from chronic transfusion therapy. Although by definition, patients with TI do not require routine blood transfusions, there are some patients who benefit from intermittent transfusion therapy; indeed, a growing body of evidence suggests hydroxyurea therapy could play a role in improving hematological parameters and reduce the need for transfusions in patients with TI, or even convert some patients from a diagnosis of TM to TI [47–49]. It is important to recognize that TI remains a serious hematological condition, and requires routine clinical surveillance of organ function and liver and cardiac iron content, in order to initiate chelation therapy in a timely manner to prevent the serious long-term health consequences.

β-Thalassemia Minor

The heterozygous β-thalassemia state, also known as β-thalassemia minor or β-thalassemia trait, occurs when one abnormal β-globin mutation leads to ≤50% decrease in β-globin chain production. The typical scenario for β-thalassemia minor is the inheritance of a single β^0- or β^+-thalassemia mutation. There are no significant clinical manifestations of β-thalassemia minor, but classic hematological features can suggest the diagnosis. Since persons with β-thalassemia minor have a mild imbalance between α- and β-globin chains, some excess α-globin chains combine with δ-globin chains and increase the percentage of hemoglobin A_2 above the normal value of 3.5%. Increases in HbA_2 to 5–7% are pathognomonic of β-thalassemia trait.

Complete blood counts of persons with β-thalassemia minor demonstrate mild microcytosis, hypochromia, and mild anemia; typical laboratory values include mean corpuscular volume (MCV) 60–75 fL, mean corpuscular hemoglobin (MCH) 22–28 pg, and hemoglobin concentration 10–12 g/dL, respectively. The degree of anemia is dependent upon the specific β-globin gene mutation, but the anemia of β-thalassemia minor is rarely symptomatic or clinically relevant. When considering the diagnosis of β-thalassemia minor, it is important to also investigate the possibility of concomitant iron deficiency anemia, which is extremely common and demonstrates many of the same hematological manifestations [50]. While clues from the Mentzer index can help distinguish these two diagnoses, the diagnosis of β-thalassemia trait is often made after an unsuccessful trial of iron replacement in the setting of mild microcytic anemia. Hemoglobin electrophoresis usually distinguishes these two conditions, although concomitant iron deficiency anemia as well as α-thalassemia can lower HbA_2 levels [51].

Sickle Cell Disease

Overview and Historical Perspective

Sickle cell disease (SCD) refers to a group of inherited hemolytic anemias characterized by the predominance (>50%) of abnormal sickle hemoglobin (HbS, $\alpha_2\beta^S{}_2$) within the erythrocytes. This operational definition thus distinguishes SCD from sickle cell trait, a typically benign condition where HbS represents approximately 30–40% of the total cellular hemoglobin. SCD is therefore not a single entity, but a constellation of blood diseases that all feature intracellular deoxy-HbS polymerization and subsequent erythrocyte deformation into a sickled shape.

SCD has been described clinically for generations in West Africa with the first documented reports in the 1800s. SCD was first reported in the Western medical literature in 1910 by James Herrick (with a majority of the work done by his intern Ernest Irons), who reported the abnormal “sickle-shaped” red blood cells of a severely anemic Chicago-area dental student born in Grenada [52, 53]. As discussed earlier, the HbS mutation was identified by Linus Pauling in 1949 resulting in SCD being labeled as the first molecular disease. Although these important discoveries are now more than 50–100 years old, the genetic or environmental factors contributing to the phenotypic variability of SCD remains largely unknown.

Over the past 30 years, the management of patients with SCD has greatly improved, due to careful prospective natural history studies in the United States and Jamaica leading to early recognition of clinical complications, widespread use of pneumococcal immunization, and the judicious use of safe blood transfusions. In addition, landmark clinical trials have proven the lifesaving effects of prophylactic penicillin, the efficacy of hydroxyurea therapy for both adults and children, and the importance of transcranial Doppler (TCD) screening and transfusion therapy for the

prevention and management of acute stroke [54–58]. Together with other important pivotal research studies, the morbidity and mortality of SCD has been substantially improved, though much work is still needed, particularly in the global setting.

Epidemiology and Global Burden

SCD is one of the most common monogenic diseases in the world, with an estimated 312,000 annual HbSS births globally, most occurring within sub-Saharan Africa [59]. The sickle mutation (β^S) in *HBB* has its origins in sub-Saharan Africa and the Indian subcontinent. The HbS mutation arose independently in at least four regions within sub-Saharan Africa and one that includes the Arab peninsula and Indian subcontinent (Fig. 1.2) [60, 61]. Although each β-globin haplotype has the same sickle mutation, the flanking genetic polymorphisms result in unique phenotypes for each haplotype [62, 63]. For example, the Senegal (SEN) and Arab-Indian (AI) haplotypes are associated with higher fetal hemoglobin (HbF) levels and an overall less severe phenotype, while the Central African Republic (CAR) haplotype is associated with lower HbF levels and a more severe clinical course.

The multicentric geographical origins and subsequent persistence of the deleterious sickle mutation is due to tremendous genetic selective pressure from *P. falciparum* malaria. Fig. 1.3 demonstrates that the geographic areas with the highest HbS allele frequency are nearly identical to the areas with the highest malaria endemicity [64]. This striking geospatial overlap is the result of the

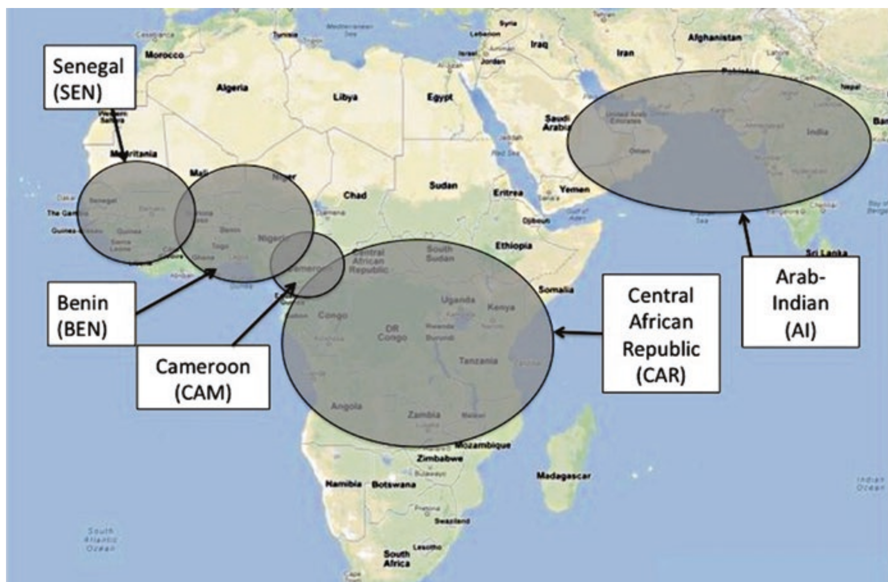

Fig. 1.2 The HbS mutation arose independently in at least four regions within sub-Saharan Africa and one that includes the Arab peninsula and Indian subcontinent

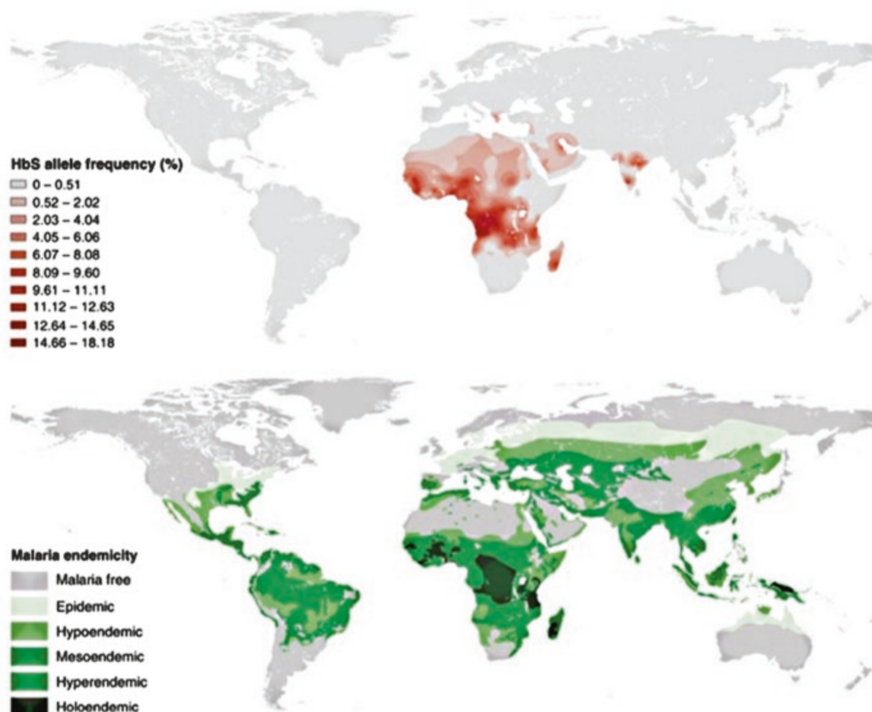

Fig. 1.3 The geographical distribution of the HbS allele is nearly identical to the geographical prevalence of *Plasmodium Falciparum* malaria, due to the protective effect of the HbAS carrier state. From Piel FB et al. *Nature Communications* 2010 [105], reprinted with permission from Nature Publishing Group

significant protection provided by the heterozygous HbS carrier state against severe and lethal malaria infection. Strong epidemiological evidence has demonstrated that children who inherit a single HbS allele (i.e., have sickle trait, HbAS) have lower parasite burdens and are 50–90% less likely to die from severe malaria than children with HbAA [65]. The homozygous HbSS state also confers relative protection against malaria infection, but because of the baseline severe anemia, the clinical severity and mortality of malaria in children with HbSS is high [66]. This balanced genetic polymorphism results in relative selection for the sickle cell trait within areas of malaria endemicity but unfortunate persistence of SCD.

Molecular Basis and Pathophysiology

HbS results from a single point mutation, which causes a substitution at the sixth amino acid in the β-globin protein. The sickle mutation results in a change from a hydrophilic residue (glutamic acid) to a hydrophobic one (valine), causing

modification of the hemoglobin tetramer such that under low oxygen tension, the normally soluble hemoglobin rapidly polymerizes within the erythrocyte and stretches the cell membrane into a curvilinear shape. This "sickling" event is the *sine qua non* of SCD; although there are many other facets to the pathophysiology involving erythrocytes (dehydration, hemolysis, and adhesion), leukocytes (adhesion, leukocytosis), and endothelium (damage, dysfunction, and inflammation), erythrocyte sickling marks the primary phenomenon and is uniquely characteristic of this blood disorder.

The erythrocytes in SCD do not circulate in a permanent deformed state, however, but cycle between their sickled and non-sickle shapes as reversibly sickled cells. After repeated cycles, the cell membrane becomes irreversibly sickled. These cells become fragile, are prone to hemolysis, and have a markedly shortened lifespan that leads to a partially compensated hemolytic anemia that is characteristic in SCD.

Classification of Sickle Cell Disease

SCD encompasses a variety of genotypes, including both homozygous HbSS disease and compound heterozygous sickling conditions such as HbSC and HbSβ-thalassemia. Sickle cell anemia (SCA) is a term that refers to the most severe forms of SCD, such as HbSS or HbSβ^0-thalassemia. Table 1.1 provides representative blood counts and hemoglobin electrophoresis patterns that are commonly observed in persons with various forms of SCD.

Table 1.1 Laboratory findings and clinical phenotype for common sickle genotypes (hematological parameters represent typical median values)

Genotype	Hb (g/dL)	MCV	Reticulocyte (%)	HbS (%)	HbA (%)	HbC (%)	HbF (%)	HbA_2 (%)	Clinical phenotype
HbAS (sickle trait)	Normal	Normal	1–2	≤40	>60	0	<1.0	<3.5	Normal
HbSS	8.3	90	11.8	>90	0	0	<10	<3.5	Severe
HbSβ^0 thalassemia	8.9	72	10	>80	0	0	<20	>3.5	Severe
HbS β$^+$ thalassemia	10.8	68	4.1	>60	10–30	0	<20	>3.5	Mild-Moderate
HbSC	10.6	77	4.2	50	0	45	0	<3.5	Moderate

HbAS (sickle cell trait) is included for comparison but is NOT a form of SCD [67–69]

Clinical Manifestations of Sickle Cell Disease

Although primarily a disorder of hemoglobin and red blood cells, SCD is a systemic disorder that has an effect upon all organ systems because of the importance of hemoglobin in tissue oxygenation. There are certain clinical manifestations that present early in childhood, while there are others that do not typically develop until adolescence or early adulthood. There is also wide clinical heterogeneity among the different SCD genotypes, with SCA (most commonly due to Hb SS and Hb Sβ^0 thalassemia) having a more clinically severe phenotype but with extensive variability among individual patients. Currently, there are no reliable ways to predict which clinical symptoms will happen to which patients at which point in their life. The discussion in this chapter will focus mostly on the clinical manifestations of SCA, but will also briefly summarize the unique clinical complications seen in the other types of SCD.

Hematologic Manifestations

There is significant variability in the hematologic parameters for persons with SCA, but patients tend to maintain their own stable baseline hemoglobin concentration, which typically ranges from 6 to 9 g/dL. In addition to moderate to severe anemia, persons with SCA typically have significant reticulocytosis and elevated white blood cell, absolute neutrophil, and platelet counts due to chronic inflammation. The hematologic abnormalities of HbSC and HbS β^+thalassemia are similar to, though not as severe as, those for patients with SCA and are outlined in Table 1.1 [67–69].

Vaso-Occlusive Manifestations

Vaso-occlusive painful crisis (VOC) or vaso-occlusive event (VOE) is the most common cause for hospital admission in adults with SCD. Dactylitis is an example of a vaso-occlusive painful event and is often the first clinical manifestation of SCA; it occurs in very young patients as the result of the infarction of bone marrow within the small bones, with an intense local inflammatory response in the surrounding tissues. Clinically, infants and toddlers present with acute and painful swelling of the dorsum of their hands and/or feet. In older children and adult patients, infarcts often manifest as acute painful episodes that are localized to a very specific site such as the sternum, ribs, arms or legs. Swelling may or may not be present and imaging is not always diagnostic.

Pain is a very common and even daily complication of SCA and can be difficult to manage. Accurate quantification and evaluation of pain can be challenging, as there may be no accurate measures other than patient report. Unfortunately, it is not uncommon for patients with SCA to be accused of misrepresenting their pain, or reporting pain as part of drug-seeking behavior. This is an important misperception among clinical providers who do not recognize how frequent and severe sickle cell pain can be.

Infectious Manifestations

Due to the splenic dysfunction and several other abnormalities in innate immunity, patients with SCD have a significantly increased risk of serious and life-threatening infections. Invasive bacterial infections from encapsulated organisms (e.g., *Streptococcus pneumoniae, Haemophilus influenza* type b, and *Salmonella species)* are the most common and severe infections, particularly for young children with SCD. Without early diagnosis of SCD allowing for initiation of vaccination and antibiotic prophylaxis, these infections can result in early mortality for young children with SCD. The combination of diagnosis by newborn screening and early interventions such as vaccinations and penicillin prophylaxis has significantly reduced the early mortality of SCD in the US and Europe [70, 71].

Transient aplastic crisis is another common and serious infectious complication of SCA seen most commonly in young children. Children typically present with fever, non-specific signs and symptoms of a viral illness, significant pallor and lethargy. It is most commonly associated with parvovirus B19, but can occur in association with other viral conditions. Parvovirus specifically targets erythroid progenitor cells in the bone marrow and effectively shuts down erythropoiesis for approximately 5–7 days. In healthy children, this temporary decrease in erythrocyte production is not clinically apparent, but for patients with SCA, who have 10–15% turnover in their RBC volume each day, clinical complications are apparent and severe. Dramatic reticulocytopenia is the hallmark laboratory finding that distinguishes this condition from anemia due to other infections or acute splenic sequestration crisis. With significant symptomatic anemia and reticulocytopenia, patients with SCA who develop aplastic crisis commonly require a transfusion of packed red blood cells to maintain an adequate hemoglobin level until reticulocytopenia resolves. Once the viremia is cleared and reticulocytes recover, patients have measurable IgG antibodies to parvovirus and are considered immune against subsequent infection.

Neurological Manifestations

Cerebrovascular disease contributes significantly to the morbidity and mortality of SCA, and can range from poor school performance to overt clinical stroke. The dramatically increased risk of stroke for children with SCA compared to otherwise healthy children has been well documented. In the era before TCD screening, the risk of stroke by age 20 years for children with SCA was 11% and increased to 24% by age 45 years [72]. In the US Cooperative Study of Sickle Cell Disease, ischemic strokes were more common in patients less than 20 years of age and for those greater than 30 years of age, while hemorrhagic events were more common between the ages of 20 and 30 years [72].

Recently, there has been increasing body of literature describing the frequency and clinical significance of silent cerebral infarcts (SCI). SCI is a common neurologic finding in SCA, affected up to one-third of young patients; by definition, neuroimaging (most commonly MRI) reveals parenchymal lesions that are not

associated with focal neurological deficits. The term "silent" is a misnomer since SCI has been associated with cognitive and intellectual deficits [73], and is a recognized risk factor for overt stroke [74, 75]. Recently the randomized controlled Silent Cerebral Infarct Transfusion (SIT) Trial demonstrated that for children with documented SCI, regular blood transfusions can help prevent further cerebrovascular disease including more SCI, transient ischemic attack, and stroke [76]. Last year, a multi-center trial demonstrated the efficacy of hydroxyurea to prevent stroke in children with abnormal TCD velocities [77], thus offering a useful alternative to lifelong blood transfusions for children with high risk for primary stroke.

Cardiac Manifestations

There are many cardiac findings and complications associated with SCD, although a distinct sickle cell cardiomyopathy has not yet been clearly defined. Many of the common findings, including cardiac flow murmurs and cardiomegaly, are often secondary to the chronic anemic state. Common clinical symptoms include fatigue and dyspnea. One study documented over half of the pediatric participants met the New York Heart Association Class I criteria for functional cardiac disease [78]. A more recent study, which evaluated sudden death that occurs in SCA patients, identified the abnormal heart rhythm of pulseless electrical activity arrest [79] while autonomic dysfunction may also be present [80]. Ongoing research is needed to better define the sickle cell cardiomyopathy and to identify effective treatment modalities for this important cause of morbidity and mortality for adults with SCA. Recently a new model of restrictive cardiomyopathy due to fibrosis, which manifests as disastolic dysfunction, has been proposed that may help explain much of the sickle cell cardiac complications [81].

Pulmonary Manifestations

Pulmonary manifestations are among the most frequent and severe complications for both adults and children with SCD and collectively are associated with increased mortality. Both acute and chronic pulmonary complications can occur in patients with SCD, and seem to be exacerbated in the presence of the clinical diagnosis of asthma [82, 83].

Acute chest syndrome (ACS) is the most common cause of hospitalization and death among patients with SCA [84]. ACS is defined by the presence of new onset respiratory symptoms, fever, elevated leukocyte count, and associated radiographic changes representing a severe and often rapidly progressive inflammatory process in the lung. This clinical event can be triggered by many different causes, including infection, infarction, and fat embolism, although a clear etiologic trigger is not always found [84]. Regardless of its origin, ACS is a life-threatening condition for both children and adults with SCD, and a high index of suspicion must be maintained to initiate early interventions to prevent worsening respiratory symptoms

and more serious complications. It is also important to recognize that ACS often develops while a patient is hospitalized for another sickle cell complication, most commonly vaso-occlusive pain. Initiation of incentive spirometry and aggressive pulmonary toilet should be routinely employed for all patients with SCD admitted to the hospital.

Pulmonary hypertension (PH) is a serious and potentially life-threatening complication that can develop in a subset of adults with SCD. Some investigators estimate that 6–11% of patients meet the diagnostic criteria for PH and have a significantly increased risk of early mortality [85]. The American Thoracic Society recently released official clinical practice guidelines regarding the diagnosis, risk stratification and management of PH in SCD [85], although the newer NHLBI evidence-based guidelines recommend evaluation and referral only for symptomatic patients [69]. Right heart catheterization (RHC) is the gold standard for the diagnosis of PH, but there is limited use of RHC in SCD due to the risks of anesthesia for this invasive procedure. In lieu of RHC, tricuspid regurgitant jet velocity from echocardiography is often used to identify elevated right-sided pulmonary pressures, despite having a low positive predictive value for PH due to concomitant cardiac complications [86]. Although a consensus algorithm for screening patients with SCD has not been established, RHC-proven diagnosis of PH appears to be a strong risk factor for early mortality and should be managed aggressively.

Splenic Manifestations

The spleen is one of the earliest organs to sustain damage in the young child with SCD. Its milieu is favorable to sickling, due to the slow circulation and hypoxic conditions within the splenic pulp. Most children with SCA begin a process known as "auto-infarction" of the spleen within the first 2 years of life, due to recurrent damage to the splenic microvasculature by the abnormal sickled red blood cells; this results in functional asplenia with significantly increased susceptibility to infections, particularly by encapsulated bacterial organisms. In patients with SCA, the spleen may be palpable over the first 2–3 years of life due to trapping of sickled erythrocytes, but this does not indicate normal immunological or filtrative function.

Over time, the spleen in SCA is usually no longer palpable once this autoinfarction occurs. In patients with less severe sickle genotypes, however, particularly in HbSC disease, splenomegaly often persists into adulthood. Some adolescents and young adults with HbSC disease develop hematological abnormalities (e.g., worsening anemia, thrombocytopenia, and leukopenia) from hypersplenism and require splenectomy in order to improve their blood counts. Splenic infarction can also occur in patients with persistent splenomegaly.

Acute splenic sequestration crisis (ASSC) is a common and serious complication that occurs most commonly in young children with SCA during the first 2 years of life. Due to the persistence of an enlarged spleen, ASSC can occur later in childhood and even in adulthood for persons with HbSC disease. ASSC typically presents with a clinical picture of severe anemia that should not be mistaken

for transient aplastic crisis, since the management is quite different. Laboratory findings in both settings are notable for significant reduction in hemoglobin concentration, but with ASSC there is an increased reticulocyte count and increased numbers of circulating nucleated red blood cells, since the marrow is unaffected. Physical exam in ASSC is notable for pallor and tender splenomegaly that may extend beyond the umbilicus. If untreated, ASSC can be fatal, so it is important to educate parents about the importance of palpating the spleen regularly and seeking medical attention immediately for splenic enlargement that may portend a life-threatening episode of ASSC. Children with ASSC often require hospitalization and urgent erythrocyte transfusion due to anemia and hypovolemia, but transfusion should be provided carefully in small volumes (e.g., 5 mL/kg) due to the risk of an "overshoot phenomenon" that results from auto-transfusion of sequestered erythrocytes, with potential hyperviscosity and serious neurovascular complications. After recovery, at least half of the children with ASSC will have a recurrence, and many of those will require splenectomy.

Hepatobiliary Manifestations

Due to the chronic hemolysis that occurs in SCD, the development of pigmented gallstones is a very common occurrence, often beginning in childhood. Asymptomatic gallstones do not necessarily require any acute intervention, but symptomatic gallstones and acute cholecystitis benefit from elective cholecystectomy. In most centers, patients with asymptomatic gallstones will also receive surgical intervention, due to the risks of performing emergent surgery in a patient unprepared with preoperative transfusions. Transmission of viral hepatitis from the blood supply is an uncommon occurrence in the United States due to rigorous screening methods, but for older patients or those receiving blood products from regions of the world with potentially unsafe blood supplies, a suspicion for the possibility of viral hepatitis should be raised. In addition to gallstones, there are a number of other rarer hepatobiliary manifestations of SCD, including hepatic crisis, intrahepatic cholestasis, and hepatic sequestration.

Renal Complications

SCD is associated with many renal complications that lead to significant morbidity and mortality within the SCD population. Increased glomerular filtration and the inability to appropriately concentrate the urine are renal manifestations that begin at a very early age [87]. Albuminuria and proteinuria are among the more severe early indications of significant renal organ damage, and may precede progression to more significant end-stage renal disease. Sickle nephropathy can progress to significant renal dysfunction and renal failure. Among adults with SCD, sickle nephropathy is an important contributor to morbidity and mortality and recently losartan was found to reduce urinary albumin excretion [88].

Skeletal Complications

Avascular necrosis (AVN), or osteonecrosis, is a severe complication of SCD, occurring in up to 50% of patients with SCD [89]. AVN most commonly occurs in the femoral or humeral head and results in limited range of motion, as well as chronic debilitating pain. Early and aggressive recognition and management of AVN is essential to prevent a more chronic and refractory painful condition. It is notable that AVN is a common complication for patients with HbSC disease, likely due to a higher hemoglobin concentration and hyperviscosity.

Other Complications

Due to the critical role of hemoglobin in delivering oxygen to all organs, SCD can affect nearly every organ in the body, with a long list of acute and chronic disease manifestations. These clinical complications will not be discussed in detail here but include growth and pubertal delay, priapism, retinopathy, and chronic leg ulcers [90–95].

Sickle Cell Disease Treatment Options

The treatment of SCD has advanced dramatically over the past 30 years, although most therapeutic options are limited to countries with adequate health resources. As discussed earlier, early diagnosis by newborn screening allows timely initiation of lifesaving interventions, including immunizations and prophylactic penicillin therapy. Blood transfusion (either simple packed erythrocytes or exchange transfusion) is an effective treatment for many acute complications of SCD including stroke, acute chest syndrome, splenic sequestration crisis, and aplastic crisis. Chronic transfusion programs, in which patients received blood transfusion on a regular basis, have become the standard of care for both primary and secondary stroke prevention [96, 97].

Over the past two decades, hydroxyurea has been demonstrated to be both safe and effective for the prevention of many complications of SCA. The new NHLBI sickle cell guidelines include strong recommendations for the use of hydroxyurea in adults with SCA, and also recommend that hydroxyurea now be offered to all children with SCA regardless of clinical symptoms [69]. Hydroxyurea is proven to reduce the frequency and severity of painful crises, acute chest syndrome, and the need for transfusion and hospitalization, and a growing body of evidence suggests that hydroxyurea may also prevent or delay chronic organ damage and even improve patient survival [58, 68, 98–102].

Summary

Sickle cell disease and β-thalassemia are common and life-threatening hematological conditions with a large global burden. Over the past century, tremendous scientific discoveries have solved the molecular and genetic basis of these conditions, elucidated the clinical spectrum of disease, and identified potential therapeutic targets. Despite these significant scientific advances, however, effective therapies remain limited and quality of life remains poor for millions of affected persons. Blood transfusion remains the primary treatment by which to manage the acute and chronic complications of SCD and β-thalassemia. Hydroxyurea is now established as the only other effective disease-modifying therapy, especially for SCD, but is not yet prescribed to most patients. Despite the proven merits of blood transfusions and hydroxyurea for the clinical management of these conditions, neither therapeutic option cures the underlying hematological disorder. Hematopoietic stem cell transplantation is the only "curative" treatment option, but transplantation is neither widely available nor affordable, and concerns remain about transplant-related morbidity and mortality [103]. Newer cellular and gene therapies are emerging, and these exciting possibilities are now under investigation in careful clinical trials, leading to real hope for curative therapy for SCD and β-thalassemia in the near future.

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Chapter 2
Genetic Basis and Genetic Modifiers of β-Thalassemia and Sickle Cell Disease

Swee Lay Thein

Abstract β-thalassemia and sickle cell disease (SCD) are prototypical Mendelian single gene disorders, both caused by mutations affecting the adult β-globin gene. Despite the apparent genetic simplicity, both disorders display a remarkable spectrum of phenotypic severity and share two major genetic modifiers—α-globin genotype and innate ability to produce fetal hemoglobin (HbF, $\alpha_2\gamma_2$).

This article provides an overview of the genetic basis for SCD and β-thalassemia, and genetic modifiers identified through phenotype correlation studies. Identification of the genetic variants modifying HbF production in combination with α-globin genotype provide some prediction of disease severity for β-thalassemia and SCD but generation of a personalized genetic risk score to inform prognosis and guide management requires a larger panel of genetic modifiers yet to be discovered.

Nonetheless, genetic studies have been successful in characterizing some of the key variants and pathways involved in HbF regulation, providing new therapeutic targets for HbF reactivation.

Keywords Sickle cell disease • β-thalassemia • Genotype/phenotype correlation • Fetal hemoglobin • Genetic prediction

Introduction

The inherited disorders of hemoglobin (Hb) production are the most common human monogenic disorders, among which those affecting the adult β globin gene (*HBB*)—β-thalassemia and sickle cell disease (SCD)—are the most clinically significant [1, 2]. β-thalassemia is caused by a spectrum of mutations that results in a quantitative reduction of β globin chains that are structurally normal [3], while SCD is caused by a single nucleotide substitution (G**A**G to G**T**G) in the sixth codon

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of *HBB* gene, substituting valine for glutamic acid in adult β globin (βGlu6Val), resulting in an abnormal Hb variant, HbS ($\alpha_2\beta_2^S$) [1, 4]. This change allows HbS to polymerize when deoxygenated, a primary event indispensable in the molecular pathogenesis of SCD.

Both β-thalassemia and SCD occurs widely in a broad belt of tropical and subtropical regions including the Mediterranean, parts of North and sub-Saharan Africa, the Middle East, Indian subcontinent and Southeast Asia, with some variations in frequencies in the two diseases. For example, SCD is predominant in sub-Saharan Africa but it is also prevalent in small pockets in the Mediterranean region, Middle East and the Indian sub-continent. It appears that heterozygotes for both thalassemia [5] and the β^S gene [6] are protected from the severe effects of falciparum malaria, and natural selection has increased and maintained their gene frequencies in these malarious regions. In these prevalent regions, gene frequencies for β-thalassemia range between 2 and 30%; in sub-Saharan Africa, the β^S gene frequency is uneven but rarely reaches 20% [7]. However, owing to population movements in recent years, both β-thalassemia and SCD are no longer confined to these high-incidence regions, but have become an important part of clinical practice posing an important public health problem in many countries, including North America and Europe [1].

The β-Globin Gene (*HBB*) and Normal Expression

Beta-globin is encoded by a structural gene found in a cluster with the other β-like genes on chromosome 11 (11p 15.15) [8]. The cluster contains five functional genes, ε (*HBE*), Gγ (*HBG2*), Aγ (*HBG1*), δ (*HBD*), and β (*HBB*), which are arranged along the chromosome in the order of their developmental expression to produce different Hb tetramers: embryonic (Hb Gower-1 ($\zeta_2\varepsilon_2$), Hb Gower-2 ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\beta_2$)), fetal ($\alpha_2\gamma_2$), and adult (HbA, $\alpha_2\beta_2$ and HbA_2, $\alpha_2\delta_2$) [8]. Expression of the globin genes is dependent on local promoter sequences as well as the upstream β-globin locus control region (β-LCR) which consists of five Dnase 1 hypersensitive (HS) sites (designated HS1 to HS5) distributed between 6 and 20 kb 5′ of *HBE* gene [9–11]. There is one HS site at approximately 20 kb downstream of *HBB* gene. All these regulatory regions bind a number of key erythroid-specific transcription factors, notably GATA-1, GATA-2, NF-E2, KLF1 (also known as EKLF), and SCL as well as various co-factors (e.g., FOG, p300), and factors that are more ubiquitous in their tissue distribution, such as Sp1 [8, 12, 13].

The β-like globin genes are each expressed at distinct stages of development through a process referred to as hemoglobin switching (embryonic → fetal → adult). Transcription of *HBE* in the embryonic yolk sac during the initial period of pregnancy switches during the second month of gestation to transcription of the γ-globin genes, and then around the time of birth, to that of the adult β-globin gene. At 6 months after birth, fetal Hb (HbF, $\alpha_2\gamma_2$) which comprises less than 5% of the total Hb, continues to fall reaching the adult level of <1% at 2 years of age, when

adult Hb becomes the major Hb [8]. It is at this stage that mutations affecting the adult *HBB* gene, i.e. β-thalassemia and SCD become manifested [14–16].

The tissue- and developmental-specific expression of the individual globin genes, i.e. hemoglobin switching, relies on a timely and direct physical interactions between the globin promoters and the β-LCR, the interaction being mediated through binding of erythroid-specific and ubiquitous transcription factors [17]. Tissue-specific expression may be explained by the presence of binding sites for the erythroid-specific transcription factors [12]. The binding of hemopoietic-specific factors activates the LCR, which renders the entire β-globin gene cluster transcriptionally active. Transcription factors which bind to enhancer and local promoter sequences within each gene, work in tandem to regulate the expression of the individual genes in the clusters. Some of the transcription factors are developmental stage specific and may be involved in the (still poorly understood) differential expression of embryonic, fetal and adult globin genes. A dual mechanism has been proposed for the developmental expression: (a) gene competition for the upstream β-LCR, conferring advantage for the gene closest to the LCR [18], and (b) autonomous silencing (transcriptional repression) of the preceding gene [19, 20]. The ability to compete for the β-LCR and autonomous silencing depends on the change in the transcriptional factor environment—in the abundance and repertoire of various transcription factors—favouring promoter-LCR interaction. While the ε and γ-globin genes are autonomously silenced at the appropriate developmental stage, expression of the adult β-globin gene depends on lack of competition from the upstream γ-gene for the LCR sequences. Concordant with this mechanism, when the γ-gene is upregulated by point mutations in their promoter causing a non-deletion hereditary persistence of fetal hemoglobin (HPFH), expression of the downstream *cis* β-gene is downregulated [21]. Further, mutations which affect the β globin promoter, which removes competition for the β-LCR, are associated with higher than expected increases in γ (HbF) and δ (HbA_2, $\alpha_2\delta_2$) expression [22–24]. A thorough understanding of the switch from fetal to adult Hb expression may provide insights on strategies of delaying the switch to allow persistent expression of the fetal globin genes for treating both SCD and β-thalassemia.

So far, the best-defined example of a developmental stage-specific regulatory factor is the erythroid Krüppel-like factor (EKLF, also known as KLF1) without which the β genes cannot be fully activated in the definitive cells [25–27]. Not only is KLF1 expression restricted mainly to erythroid cells [28] but it is also a highly promoter-specific activator, binding with high affinity to the β-globin CACCC box [24]. Its greater affinity for the β-globin than the γ-globin promoter accelerates the silencing of the γ-genes [29]. A genetic network regulating the switch from γ- to β-globin expression has emerged involving the interaction of KLF1, BCL11A and MYB with each other, and other transcription factors (e.g., GATA-1) and co-repressor complexes that involve chromatin modeling and epigenetic modifiers [30, 31]. BCL11A, previously known as an oncogene involved in leukemogenesis, was ‘discovered’ as an important genetic locus regulating HbF through genome-wide association studies (GWAS) [32, 33]. Downstream functional studies in cell lines, primary human erythroid cells and transgenic mice, have shown that BCL11A

is a repressor of γ-globin expression [34–36]. KLF1 is a direct activator of *BCL11A* [37, 38]. KLF1 is key in the switch from γ-globin to β-globin expression; it not only activates the β-globin gene directly, providing a competitive edge, but also silences the γ-globin genes indirectly via activation of *BCL11A*. KLF1 may also play a role in the silencing of the embryonic globin genes. KLF1 has emerged as a major erythroid transcription factor with pleiotropic roles underlying many of the previously uncharacterized anemias (e.g., congenital dyserythropoietic anemia type IV) [39–41]. KLF1 variants have also been associated with variable increases in HbF and HbA_2 levels, as a primary phenotype [39–41]. A recent case report indicated that complete absence of KLF1 causes hydrops fetalis and nonspherocytic hemolytic anemia [42].

Genetics of β-Thalassemia

Almost 300 β-thalassemia alleles have now been described (http://globin.cse.psu.edu) but only about forty account for 90% or more of the β-thalassemias worldwide. This is because in the areas where β-thalassemia is prevalent, only a few mutations are common, possibly reflecting local selection due to malaria. Each of these populations thus has its own spectrum of β-thalassemia alleles.

Downregulation of the β-globin gene can be caused by a whole spectrum of molecular lesions ranging from point changes to small deletions limited to the β-gene or extensive deletions of the whole β globin cluster or βLCR (Fig. 2.1) [3]. Deletions causing β-thalassemia, however, are rare; the vast majority (~250 of the 300) of mutations causing β-thalassemia are non-deletional.

Functionally, the β-thalassemia alleles can be considered as β^0 where no β-globin is produced, or β^+ in which some β-globin is produced, but less than normal. A range of severity is encountered within the β^+ thalassemia group; the less severe forms are sometimes designated β^{++} to reflect the minimal deficit in β chain production. Some β^{++} alleles are so mild that they are 'silent'; carriers do not display any evident hematological phenotypes; their red cell indices and HbA_2 levels are within normal limits, the only abnormality being an imbalanced α:non-α chain synthesis [43]. These β-thalassemia alleles have usually been 'discovered' in individuals with thalassemia intermedia who have inherited a silent β-thalassemia allele in compound heterozygosity with a severe β-thalassemia allele. In this case, one parent has typical β-thalassemia trait, and the other (with the β^{++} thalassemia) is apparently normal.

Non-deletion β-Thalassemia

These non-deletional mutations, i.e. single base substitutions, small insertions or deletions of one to a few bases are located within the gene or its immediate flanking sequences. They downregulate the β-globin gene via almost every known stage of

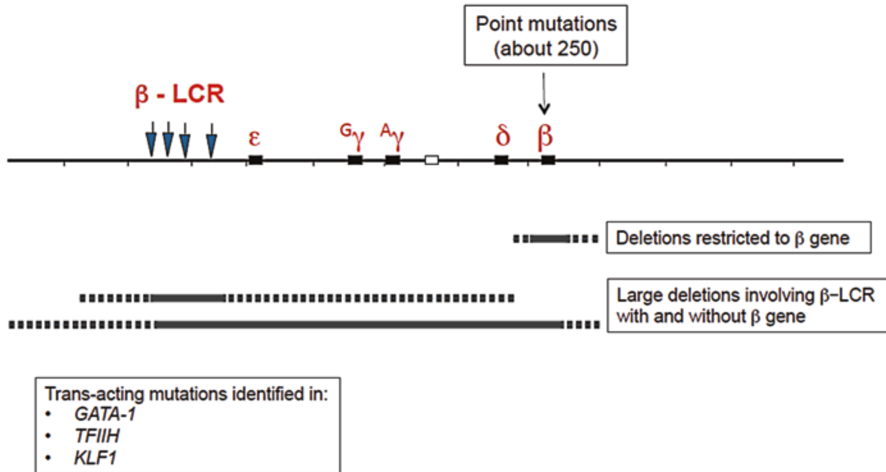

Fig. 2.1 Mutations causing β-thalassemia. A summary of the mechanisms downregulating β-globin gene expression. The upper panel depicts the β-globin gene (*HBB*) cluster with the upstream β-locus control region (βLCR). The vast majority are point mutations affecting the structural *HBB* gene. Deletions downregulating *HBB* are rare and are either restricted to the gene or extensive, involving the βLCR with or without *HBB*. *Dashed lines* represent variation in the amounts of flanking DNA removed by the deletions

gene expression, from transcription to RNA processing and translation of β-globin mRNA. Approximately half of the non-deletional mutations completely inactivate the β-gene with no β globin production resulting in β^0 thalassemia.

Transcriptional Mutations

Transcriptional mutants involve the conserved DNA sequences that form the β-globin promoter (from 100 bp upstream to the site of the initiation of transcription, including the functionally important CACCC, CCAAT and ATAA boxes) and the stretch of 50 nucleotides in the 5′UTR. Generally, these transcriptional mutants result in a mild to minimal reduction of β globin output i.e. β^+ or β^{++} thalassemia alleles, and occasionally they are 'silent'. A silent β-thalassemia allele which has been observed fairly frequently in the Mediterranean region is the −101 C→T mutation where it interacts with a variety of more severe β-thalassemia mutations to produce milder forms of β-thalassemia [44]. Other 'silent' mutations include those in the 5′ UTR; the extremely mild phenotype is exemplified in a homozygote

for the +1 A→C mutation who has the hematologic values of a thalassemia carrier, heterozygotes are 'silent' [45].

Within this group of transcriptional mutants, ethnic variation in phenotype has been observed. Black individuals homozygous for the −29 A→G mutation have an extremely mild disease [46], while a Chinese individual homozygous for the same mutation had severe anemia and was transfusion-dependent [47]. The cause of this striking difference in phenotype is not known but likely to be related to the different chromosomal backgrounds on which the apparently identical mutations have arisen. One difference is the C-T polymorphism at position –158 upstream of the $^{G}\gamma$ globin gene (*Xmn*1-$^{G}\gamma$ site) present in the β chromosome carrying the −29 A→G mutation in Blacks but absent in that of the Chinese. The *Xmn*I-Gγ site, considered to be a quantitative trait locus for HbF, is associated with increased HbF production under conditions of erythropoietic stress (see later on 'Update on the genetic control on HbF).

Mutations Affecting RNA Processing

A wide variety of mutations interfere with processing of the primary mRNA transcript. Those that affect the invariant dinucleotide GT or AG sequences at exon-intron splice junctions prevent normal splicing altogether, and cause β^0 thalassemia. Mutations involving the consensus sequences adjacent to the GT or AG dinucleotides, allow normal splicing to varying degrees and produce a β-thalassemia phenotype that ranges from mild to severe. For example, mutations at position 5 IVS1 G→C, T or A, considerably reduce splicing at the mutated donor site compared with the normal β allele [21]. On the other hand, the substitution of C for T in the adjacent nucleotide, intron 1 position 6, only mildly affects normal RNA splicing. Although the IVS1-6 T-C mutation is generally associated with milder β-thalassemia, studies have shown differential severities for apparently identical mutations; again this is presumably related to the chromosomal background on which the mutations have arisen [48].

Both exons and introns also contain 'cryptic' splice sites which are sequences very similar to the consensus sequence for a splice site but are not normally used. Mutations can occur in these sites creating a sequence that resembles more closely the normal splice site, such that during RNA processing the newly created site is utilized preferentially, leading to aberrant splicing; incorrectly spliced mRNA is not functional because spliced intronic sequences generate a frameshift and a premature termination code. Such mutations have been identified in both introns 1 and 2, and exon 1 of *HBB* gene. The associated phenotype may be either β^+ or β^0 thalassemia, depending on the proportion of normal and abnormal mRNA species generated. One such mutation is the (**G**AG → **A**AG) mutation in codon 26 in exon 1 [49], that results in the HbE variant. The single base substitution leads to a minor use of the alternative pathway; as the major β mRNA that codes for the variant is normally spliced, HbE has a mild β^+ thalassemia phenotype. Clinical phenotypes of

compound HbE/β-thalassemia heterozygotes resemble those of homozygous β-thalassemia ranging from severe anemia and transfusion-dependency to non-transfusion dependent states (i.e. thalassemia intermedia) depending on the non-HbE β-thalassemia allele and other genetic factors [50].

Other RNA processing mutants affect the polyadenylation signal (AATAA) and the 3′ UTR. These are generally mild β^+ thalassemia alleles [3].

Translational Mutations

About half of the β thalassemia alleles completely inactivate the gene mostly by generating premature stop codons (PTCs), either by single base substitution to a nonsense codon, or through a frameshift mutation. As part of the surveillance mechanism that is active in quality control of the processed mRNA, mRNA harboring a PTC are destroyed and not transported to the cytoplasm in a phenomenon called (nonsense mediated RNA decay or NMD) to prevent the accumulation of mutant mRNAs coding for truncated peptides [51]. However, some in-phase PTCs that occur later in the β sequence, in 3′ half of exon 2 and in exon 3, escape NMD and are associated with substantial amounts of mutant β-mRNA leading to a synthesis of β chain variants that are highly unstable and non-functional with a dominant negative effect (see Dominantly inherited β-thalassemia) [52]. Other mutations of RNA translation involve the initiation (ATG) codon. Nine of these have been described; apart from an insertion of 45 bp all are single base substitutions and again they result in β^0 thalassemia [21].

Deletions Causing β-Thalassemia

β-thalassemia is rarely caused by deletions, 15 restricted to the *HBB* gene itself have been described, of which two remove the 3′ end of the gene but leave the 5′ end intact [3]. The 0.6 kb deletion at the 3′ end of the β-gene is relatively common, but restricted to the Sind populations of India and Pakistan where it accounts for about one-third of the β-thalassemia alleles [53]. The other deletion which removes 7.7 kb 3′ from the second intron of *HBB*, was described in compound heterozygosity with β^S gene in a woman with SCD from Cape Verde islands [54]. The other thirteen deletions differ widely in size (from 290 bp to >67 kb) but remove in common a region in the β promoter (from position −125 to +78 relative to the mRNA CAP site) which includes the CACCC, CCAAT, and TATA elements. They are extremely rare, but of particular clinical interest because they are associated with an unusually high levels of HbA_2 and HbF in heterozygotes. These deletions result in β^0 thalassemia, yet the increase in HbF is adequate to compensate for the complete absence of HbA in homozygotes for these deletions [55–57]. It has been proposed that the mechanism underlying the elevated levels of HbA_2 and HbF is related

to deletion of the β promoter removing competition for the upstream β-LCR and limiting transcription factors, resulting in an increased interaction of the LCR with the γ- and δ-genes in *cis*, thus enhancing their expression. This mechanism may also explain the unusually high HbA_2 levels that accompany the point mutations in the β promoter region [23].

Dominantly Inherited β-Thalassemia

In contrast to the common β-thalassemia alleles that are inherited typically as Mendelian recessives, some forms of β-thalassemia are dominantly inherited, in that inheritance of a single copy of the β-thalassemia allele results in clinical disease. Carriers have moderate to severe anemia, splenomegaly and the hallmarks of heterozygous β-thalassemia—elevated HbA_2 and imbalanced globin chain synthesis [58]. More than thirty dominantly inherited β-thalassemia alleles have now been described; they include a spectrum of molecular lesions—single base missense mutations and minor insertions / deletions that result in truncated or elongated β-globin variants with abnormal carboxy-terminal ends [59]. The underlying denominators of these variants are the production of highly unstable and non-functional β chain variants that are not able to form viable tetramers with α globin. These precipitate in the erythroid precursors and together with the redundant α chains, overload the proteolytic mechanism causing premature death of these cells, and accentuating the ineffective erythropoiesis. Unlike the recessive forms of β-thalassemia that are prevalent in the malarious regions, the dominantly inherited β-thalassemia variants are rare, and found in dispersed geographical regions where the gene frequency for β-thalassemia is very low. Furthermore, many of these variants are unique to the families described, and occur as de-novo mutations.

Unusual Causes of β-Thalassemia

The unusual causes of β-thalassemia are extremely rare and are mentioned here, not just for the sake of completeness but also to illustrate the numerous molecular mechanisms of downregulating the β-globin gene. Transposable elements may occasionally disrupt human genes and result in their activation. The insertion of such an element, a retrotransposon of the LI family into intron 2 of the β-globin gene has been reported to cause β^+ thalassemia [60].

Almost all variants downregulating *HBB* are physically linked to the gene and behave as alleles of the β-globin locus (i.e. they are *cis*-acting). Rarely, mutations in other genes distinct from the β-globin complex can downregulate β-globin expression. Such *trans*-acting mutations have been described affecting the XPD protein that is part of the general transcription factor TF11H [61], and the erythroid-specific GATA-1 [62]. Somatic deletion of the β globin gene contributed to thalassemia

intermedia in three unrelated families of French and Italian origins [63, 64]. The affected individuals with thalassemia intermedia were constitutionally heterozygous for β^0 thalassemia but subsequent investigations revealed a somatic deletion of chromosome 11p15, including the β globin gene complex, in *trans* to the mutation in a subpopulation of erythroid cells. This results in a somatic mosaic—10–20% of the cells were heterozygous with one normal copy of the β-globin gene, and the rest hemizygous, i.e. without any normal β-globin gene. The sum total of β-globin product is ~25% less than the asymptomatic β^0 carrier; these observations offer great promise for potential gene therapy as it shows that expression of a single β-gene in a proportion of red blood cells appears to be sufficient to produce a non-transfusion dependent state. Late presentation of β-thalassemia and transfusion dependency has been reported in a Chinese patient [65], and a Portuguese woman at the age of 15 years [66]. In both cases, the phenotype was caused by uniparental isodisomy of the paternal chromosome 11p15.5 that encompassed the β-thalassemia allele.

Genetics of Sickle Cell Disease

SCD describes a clinical syndrome caused by the presence of HbS (HbS $G^{Glu6Val}$) [4]. The main genotypes that contribute to SCD include homozygosity for the β^S allele (HbSS, specifically referred to as sickle cell anemia), followed by compound heterozygous states of HbS with HbC (HbSC disease). The third genotypic group includes compound heterozygotes of HbS with β-thalassemia, HbS β^+ thalassemia or HbS $\beta^{\mathbf{0}}$ thalassemia depending on whether there is some, or no HbA, respectively. In African-descended populations, HbSS typically accounts for 65–70%, and HbSC 30–35% of the cases of SCD, with most of the remainder having HbSβ-thalassemia (Table 2.1(A)). Other genotypes of SCD have been described, including compound heterozygotes of HbS with HbD, HbO-Arab, but these are rare (Table 2.1(B)). In all affected populations, the β^S gene is caused by the same molecular defect (β codon 6 G**A**G to G**T**G), found on a genetic background of four different β^S haplotypes: three distinct African haplotypes—Senegal, Benin and Central African Republic (or Bantu), and a fourth Indo-European β-haplotype (Arab-Indian) that is linked to the β^S gene in Saudi Arabian and Asian Indian patients [67]. The evidence suggests multiple independent origins of the β^S mutation although gene conversion on regionally specific β-haplotypes cannot be excluded.

Genetic Modifiers of β-Hemoglobinopathies

SCD and β-thalassemia are caused by mutations affecting a single gene, and yet, despite the apparent genetic simplicity, both disorders display remarkable diversity in the severity of their disease [4, 68, 69]. The mutations are detectable by DNA analysis, and although the information provides a basis for genetic counselling,

Table 2.1 Common genotypes of SCD

Genotype	Electrophoresis	Comments
(A) Common genotypes of SCD		
HbS/S (βGlu6Val/βGlu6Val)	HbS 80–95%, No HbA, HbF 1–30%, HbA_2 normal	Most severe and most common SCD ~65% in African patients
HbS/C (βGlu6Val/βGlu6Lys)	HbS 45–50%, HbC 45–50% No HbA, HbF 1–6%,	Moderately severe. Frequency ~30% in African patients [152]
HbS/β^0 thalassemia (βGlu6Val/β^0 thalassemia	HbS 80–95%, No HbA, HbA_2 4–6%,	Phenotype almost indistinguishable from HbSS (included as sickle cell anemia). Most prevalent in Mediterranean and Asian Indian patients [116]
HbS/β^+ thalassemia (βGlu6Val/β^+ thalassemia)	HbS 50–80%,HbA 5–30% HbA_2 4–6%,HbF 1–20%	Mild in African but severe in Mediterranean and Asian Indian patients [116]
HbS/E (βGlu6Val/βGlu26Lys)	HbS 55–60%,HbE 30–35% HbA_2 not distinguishable from HbE electrophoretically HbF 1–5%	Moderately severe, an increasingly common genotype [153]
HbS/HPFH	HbS 60–70%, HbF 25–35% No HbA if deletion HPFH Some HbA if non-deletion HPFH HbA_2 1.5–2.5% (decreased)	Very mild due to increased and pancellular HbF distribution [116]
(B) Rarer genotypes of SCD		
HbS/O Arab (βGlu6Val/βGlu121Lys)		Severe SCD, relatively rare. Reported in north Africa, Middle East and the Balkans [116]
HbS/D Punjab (βGlu6Val/βGlu121Gln)		Moderate to severe SCD, relatively rare. Predominant in north India but occurs worldwide [116]
HbS/C Harlem (βGlu6Val/βGlu6Val, βAsp73Asn)		Very rare double mutation of βAsp73Asn on β^S allele; electrophoretically resembles HbS/C but severe SCD [154]

(continued)

Table 2.1 (continued)

Genotype	Electrophoresis	Comments
HbS Antilles/C (βGlu6Val,βVal23Ile/βGlu6Lys)		Second mutation on β^S allele and results in severe disease when inherited with HbC; very rare [155]
HbS/Québec-CHORI (βGlu6Val/Ile, βThr87Ile)		Resembles sickle trait with standard electrophoresis. Two cases described [156]
HbS/Hb Lepore (βGlu6Val/Hb Lepore) (fusion Hb)		Rare, first described in Greece but occurs worldwide. Hb Lepore migrates in same position as HbS on electrophoresis but can be separated by isoelectric focusing. Mild to severe SCD [157]
(C) Unusual and very rare genotypes of SCD		
Dominant form of SCD HbA/Hb Jamaica-Plain (β^A/βGlu6Val,βLeu68Phe)		Unique case of acquired new mutation (βLeu68Phe) on paternal βGlu6Val allele. Double mutant (Hb Jamaica-Plain) has markedly low oxygen affinity [158]
Dominant form of SCD HbA/S Oman β^A/βGlu6Val, βGlu121Lys)		Dominant form of SCD caused by 2nd mutation on β^S allele; very rare [159]
Uniparental disomy. Genetic heterozygote but functional hemizygote		Patient had paternal isodisomy of chromosome 11p that contained β^S allele resulting in subpopulation of erythroid progenitors homozygous for βGlu6Val [160]
Apparent heterozygosity for HbS βGlu6Val/Deletion of β LCR		Two cases reported. In both cases, HPLC showed no HbA and sequence analysis heterozygosity for β6 gene [161]

predicting disease severity remains difficult. Identification of the genetic modifiers could provide more precise estimates of severity of disease, contributing to the ethos of precision medicine. Further, defining the molecular mechanisms linking the genetic factors could reveal new targets for therapeutic intervention.

Historically, the genetic modifiers in both β-thalassemia and SCD have been derived from an understanding of their pathophysiology, and subsequently validated by family and case control studies. Two important modifiers—co-inheritance of α

thalassemia and variants associated with increased synthesis of HbF in adults have emerged from such clinical genetic studies. The modifying effects of HbF and α-thalassemia on SCD and β-thalassemia at the molecular level could not be more different, and yet, elucidation of these genetic modifiers has not been too difficult as these loci have a major clinical effect and the genetic variants are common, and thus would contribute substantially to disease burden. However, these two modifiers do not explain all the clinical heterogeneity. Recent advances in technology and reducing costs have prompted numerous genome-wide association studies (GWAS) in an attempt to derive some of the genetic modifiers in such complex traits [70].

GWASs involve an unbiased scan of the whole human genome and, by design, are more likely to reveal unsuspected interactions [71]. A case in point is the application of GWAS in the highly successful discovery of *BCL11A* (an oncogene that hitherto, was not known to have a role in erythropoiesis) as a quantitative trait locus (QTL) controlling HbF [32].

Update on the Genetic Control of Fetal Hemoglobin (HbF)

The production of HbF is not completely switched off at birth; all adults continue to produce residual amounts of HbF, with over 20-fold variation [72]. Twin studies have shown that the common HbF variation in adults is predominantly genetically controlled; 89% of the quantitative variation is heritable but the genetic etiology is complex with no clear Mendelian inheritance patterns [73]. *Xmn*1-*HBG2* (*rs782144*) within the β-globin gene cluster on chromosome 11p, *HBS1L-MYB* intergenic region, (HMIP) on chromosome 6q23, and *BCL11A* on chromosome 2p16 are considered to be quantitative trait loci (QTLs) for the common HbF variation in adults [74–76].

*Xmn*1-*HBG2* (*rs782144*) was the first known QTL for HbF and long implicated by clinical genetic studies [77]. SCD individuals in whom the β^S gene is on the Senegal or Arab-Indian β^S haplotype have the highest HbF levels and a mild clinical course, while SCD individuals with the β^S gene on a Bantu haplotype have the lowest HbF levels and the most severe clinical course [78, 79]. The differences in clinical severity was ascribed to the difference in HbF levels implicating the *Xmn*1-*HBG2* site which is linked to the Senegal and Arab-Indian β^S haplotype but not to the Bantu haplotype [77]. Recent high resolution genotyping, however, suggests that rs782144 is not likely to be the variant itself, but in tight linkage disequilibrium with causal element(s) that remain to be discovered in the β-globin cluster.

Variants in the *HBB*, *HMIP* and *BCL11A* loci account for 10–50% of the variation in HbF levels in adults, healthy or with sickle cell anemia or β-thalassemia, depending on the population studied [32, 80–86]. The remaining variation ('missing heritability') is likely to be accounted for by many loci with relatively small effects, and/or rare variants with significant quantitative effects on γ-globin gene expression that are typically missed by GWAS population studies. An example of the latter is the *KLF1* gene [25, 40].

KLF1, discovered by Jim Bieker in 1993 [25] re-emerged as a key transcription factor controlling HbF through genetic studies in a Maltese family with β-thalassemia and HPFH that segregated independently of the *HBB* locus [37]. Linkage studies identified a locus on chromosome 19p13 which encompassed *KLF1* and expression profiling of erythroid progenitor cells confirmed *KLF1* as the γ-globin gene modifier in this family. Family members with HPFH were heterozygous for the nonsense K288X mutation in *KLF1* that disrupted the DNA-binding domain of KLF1, a key erythroid gene regulator. Numerous reports of different mutations in *KLF1* associated with increases in HbF soon followed (see review by Borg et al. 2011) [40]. The HbF increases occurred as a primary phenotype or in association with red blood cell disorders such as congenital dyserythropoietic anemia [39, 41], congenital nonspherocytic hemolytic anemia due to pyruvate kinase deficiency [87] and sickle cell anemia [40]. Several GWASs of HbF however, including ones in sickle cell anemia patients of African descent, failed to identify common variants [82, 86]. It would seem that *KLF1* does not play an important part in regulation of HbF in SCD. On the contrary, *KLF1* variants were over represented in Southern China where β-thalassemia is prevalent compared to North China. Further, *KLF1* variants were also over-represented in patients with thalassemia intermedia when compared to thalassemia major [88].

KLF1 is a direct activator of *BCL11A* (see below) and is also essential for the activation of *HBB* expression [38, 89]. Collectively, studies suggest that KLF1 is key in the switch from *HBG* to *HBB* expression; it not only activates *HBB* directly, providing a competitive edge, but also silences the gamma-globin genes indirectly via activation of *BCL11A*, and may play a role in the silencing of embryonic globin gene expression [87]. In the light of these findings, KLF1 has now emerged as a major erythroid transcription factor with pleiotropic roles underlying many of the previously uncharacterized anemias [30].

Functional studies in primary human erythroid progenitor cells and transgenic mice demonstrated that *BCL11A* acts as a repressor of gamma-globin gene expression that is effected by SNPs in intron 2 of this gene [34]. Fine-mapping demonstrated that these HbF-associated variants, in particular rs1427407, localized to an enhancer that is erythroid-specific and not functional in lymphoid cells [90]. BCL11A does not interact with the γ-globin promoter but occupies discrete regions in the *HBB* complex [91]. Functional studies in primary human erythroid progenitors and transgenic mice demonstrated that *BCL11A* represses γ-globin and the silencing effect involves re-configuration of the *HBB* locus through interaction with GATA-1 and SOX6 that binds the proximal γ-globin promoters [92, 93].

High resolution genetic mapping and resequencing refined the 6q QTL to a group of variants in tight linkage disequilibrium (LD) in a 24-kb block between the *HBS1L* and *MYB* gene, referred to as *HMIP-2* [94]. The causal single nucleotide polymorphisms (SNPs) are likely to reside in two clusters within the block, at −84 and −71 kb respectively, upstream of *MYB* [95, 96]. Functional studies in transgenic mice and primary human erythroid cells provide overwhelming evidence that the

SNPs at these two regions disrupt binding of key erythroid enhancers affecting long-range interactions with *MYB* and *MYB* expression, providing a functional explanation for the genetic association of the 6q *HBS1L-MYB* intergenic region with HbF and F cell levels [95, 97, 98].

A three-base pair (3-bp) deletion in HMIP-2 is one functional element in the *MYB* enhancers accounting for increased HbF expression in individuals who have the sentinel SNP rs9399137 that was found to be common in European and Asian populations, although less frequent in African-derived populations [99]. The DNA fragment encompassing the 3-bp deletion had enhancer-like activity that was augmented by the introduction of the 3-bp deletion.

The *MYB* transcription factor is a key regulator of hematopoiesis and erythropoiesis, and modulates HbF expression via two mechanisms: (1) indirectly through alteration of the kinetics of erythroid differentiation: low MYB levels accelerate erythroid differentiation leading to release of early erythroid progenitor cells that are still synthesizing predominantly HbF [8], and (2) directly via activation of *KLF1* and other repressors (e.g., nuclear receptors TR2/TR4) of gamma-globin genes [98, 100, 101].

Modulation of *MYB* expression also provides a functional explanation for the pleiotropic effect of the *HMIP-2* SNPs with other erythroid traits such as red cell count, MCV, MCH, HbA_2 levels, and also with platelet and monocyte counts [102–105]. *MYB* expression is also reduced by GATA-1 [106], and miRNA-15a and -16-1 [107]. Elevated levels of the latter have been proposed as the mechanism for the elevated HbF levels in infants with trisomy 13. A delayed HbF to HbA switch, along with persistently elevated HbF levels, is one of the unique features in infants with trisomy 13 [108]. One study has provided compelling evidence that the elevated HbF levels relate to the increased expression of microRNAs 15a and 16-1 produced from the triplicated chromosome 13. The increased HbF effect is mediated, at least in part, through down-modulation of *MYB* via targeting of its 3′ UTR by microRNAs 15a and 16-1 [107].

The *HBS1L-MYB* intergenic enhancers do not appear to affect expression of *HBS1L*, the other flanking gene [95]. Further, one study also excluded *HBS1L* as having a role in the regulation of HbF and erythropoiesis. In whole-exome sequencing of rare uncharacterized disorders, mutations in the *HBS1L* gene leading to a loss of function in the gene were identified in a female child [109]. The child had normal blood counts and normal HbF levels. Thus, *HMIP-2* is likely to affect HbF and hemopoietic traits via regulation of *MYB*. *MYB* was also causally implicated by fine-mapping which identified rare missense *MYB* variants associated with HbF production [110].

The emerging network of HbF regulation also includes SOX6, chromatin-modeling factor FOP and the NURD complex, the orphan nuclear receptors TR2 / TR4 (part of DRED) and the protein arginine methyltransferase PRMT5, involving DNA methylation and HDACs 1 and 2 epigenetic modifiers. Regulators of the key TFs, such as microRNA-15a and 16-1 in controlling MYB, could also have a potential role in regulating HbF levels [111].

Genetic Modifiers of Sickle Cell Disease

Two case reports [112, 113] and a pilot twin study [114] show that despite identical β and α globin genotypes and similarities in growth, hematological and biochemical parameters, the identical twins have quite different prevalence and severity of painful crises and some of the sickle complications. Although sample size of the twin study is small, nevertheless, it does suggest that environmental factors may be of greater importance in determining clinical expression and complications of sickle disease.

SCD should be considered as both a qualitative and quantitative genetic disorder. While presence of HbS is fundamental to the pathobiology, the likelihood of HbS polymerization and sickling is also highly dependent on the concentration of intra-erythrocytic HbS, as well as the presence of non-S Hb [115]. Thus, individuals with HbSS (SCD-SS) or HbSβ^0 thalassemia, where the intra-cellular Hb is almost HbS tend to have the most severe disease followed by HbSC and HbSβ^+ thalassemia (see Table 2.1).

HbA ($\alpha_2\beta_2$) or HbA$_2$ ($\alpha_2\delta_2$) do not participate in HbS polymerization, and since the β^+ thalassemia alleles in Africans are of the milder type with minimal deficit in β-globin production, HbSβ^+ thalassemia in Africans tends to be very mild. In contrast, individuals with HbSβ^+ thalassemia in the Mediterranean, have SCD almost as severe as that of HbSS [116]. Subjects with sickle cell trait (HbAS) with HbF of 35–40% do not normally suffer from symptoms of SCD. Under exceptional circumstances however, such as intense physical activity and dehydration, the consequent increased intracellular HbS concentration can induce vaso-occlusive pain [117].

Other genetic factors that influence the primary event of HbS polymerization include (HbF, $\alpha_2\gamma_2$) and the co-inheritance of α thalassemia (Table 2.2).

Impact of HbF in SCD

HbF inhibits the propensity for HbS polymerization: the hybrid tetramers ($\alpha_2\gamma\beta s$), not only inhibits HbS polymerization, but intra-erythrocytic HbF presence also dilutes the concentration of HbS [118]. The clinical phenotype of SCD becomes evident within 6 months to 2 years of age as HbF levels decline.

Its impact at the primary level of disease pathology predicts HbF levels to have a global beneficial effect. Indeed, HbF levels are a major predictor of survival in SCD [119], and low levels of HbF have been associated with increased risk of brain infarcts in young children [120]. At the sub-phenotype level, apart from the clear benefit of high HbF levels with acute pain and leg ulceration, there are disparities and less conclusive evidence in its effects on complications such as stroke, renal impairment, retinopathy and priapism [121, 122]. The failure of HbF to uniformly modulate all complications of SCD may be related to the different pathophysiology of the different complications and perhaps also to the small sample sizes in genetic studies and even smaller numbers of end complications, and to ascertainment of phenotypes.

Table 2.2 Genetic modifiers of SCD

Modifier	Mechanism
Primary at level of HbS polymerization and sickling	
1. Sickle genotype HbSS, HbSC, HbSβ-thalassemia	Likelihood of HbS polymerization highly dependent on concentration of intracellular HbS
2. Innate ability to produce HbF ($\alpha_2\gamma_2$) through co-inheritance of HbF QTLs	HbF inhibits HbS polymerization and dilutes intracellular HbS concentration
3. Co-inheritance of α-thalassemia	α thalassemia reduces intracellular HbS concentration, increases Hb; clinical effects uneven, protective for some complications but predisposes to others
	Complication
Secondary at level of sub-phenotypes and complications	
1. *UGT1A1* promoter $(TA)_n$ polymorphism	Serum bilirubin and propensity to gallstones
2. *MYH9—APOL1* locus	Proteinuria and sickle cell nephropathy
3. *ADYC9*, *ANXA2*, *TEK* and *TGFBR3*	Stroke risk, mechanisms unclear
4. *GOLGB1* and *ENPP1*	Stroke risk, mechanisms unclear
5. *TGFβ /SMAD /BMP* pathway	Multiple sub-phenotypes including osteonecrosis, acute chest syndrome, pulmonary hypertension, leg ulceration, renal impairment, infarction, priapism
6. *KLOTHO* (*KL*)	Priapism, mechanisms unclear
7. *NPRL3* on Chr16p	Reduces hemolysis, α-thalassemia effect suggested

Note: Most modifiers that affect SCD at the secondary level of sub-phenotypes and complications have not been replicated

Adapted from Thein, S.L. Genetic association studies in β-hemoglobinopathies. Hematology, ASH Education Program Book 2013; 354-61

HbF levels vary considerably from 1% to as high as 25% in individuals with SCA and behave as a quantitative genetic trait as in healthy individuals. As discussed earlier, part of this variation resides in regions linked to the *HBB* complex and is associated, at least in part, with the β^S haplotype (Senegal, Arab-Indian, Bantu and Benin) on which the β^S gene is found. However, a variance of HbF levels is encountered within each β^S haplotype, evidence for the importance of unlinked HbF QTLs such as HMIP-2 on 6q and *BCL11A* on 2p. Their effect on HbF levels varies with the frequency of the HbF boosting (minor) alleles on the three QTLs (β -globin cluster, HMIP-2 and *BCL11A*) in different population groups; in patients of African American patients with SCD, the three loci account for 16%-20% of the variation in HbF levels with a corresponding reduction in acute pain rate [80].

A recent GWAS in Tanzanian patients with SCD also confirmed association SNPs at *BCL11A* and HMIP-2 with HbF but no other HbF QTLs were 'discovered' [86]. Similarly, in patients from Cameroon, while these SNPs had a significant impact on HbF levels and rates of hospitalization, they explained less of the HbF variance than that observed in African American patients [123]. The *Xmn*1-*HBG* (*rs782144*) is virtually absent in Cameroon patients with SCD [123].

Impact of α-Thalassemia on SCD Phenotype

About one third of SCD patients of African descent have co-existing α-thalassemia due to the common deletional variant ($-\alpha^{3.7}$/) [124]. The majority is heterozygous (αα/α-) with 3–5% homozygous for the deletion (α−/α−) [125]. Co-existing α-thalassemia reduces intracellular Hb concentration, decreases the propensity for HbS polymerization, and decrease hemolysis. While the co-existing α-thalassemias have a protective effect against complications associated with severe hemolysis such as priapism, leg ulceration and albuminuria, the increased hematocrit and blood viscosity may account for the increase in other complications associated with microvascular occlusion such as increased acute pain, acute chest syndrome, osteonecrosis and retinopathy [122].

Co-inheritance of α-thalassemia improved hematological indices and was associated with lower rates of hospitalization in Cameroon patients with SCA [126]. In the same study, Rumaney *et al* also showed that in Cameroon, the incidence of α-thalassemia trait in controls (HbAA) was significantly lower than that in patients with SCA (9.1 vs. 30.4%); the authors proposed that the difference in incidence could be explained by a protective effect of α thalassemia on overall survival in SCA patients. Co-inheritance of α-thalassemia was also associated with lower hospitalization rate in SCA patients.

Coexisting α-thalassemia reduces bilirubin with a quantitative effect that is independent to that of the *UGT1A1* promoter polymorphism [125]. In Jamaicans, the absence of α thalassemia and higher HbF levels predicts a benign disease, although a subsequent study reports that α-thalassemia did not promote survival in elderly Jamaicans with SCD-SS [127]. Coinheritance of α-thalassemia blunts the response to hydroxyurea therapy in SCD, that may be explained by its effect on HbF levels and MCV, two key parameters associated with hydroxyurea response [128]. It is quite likely that α-thalassemia carriers could have a poorer response to channel blockers that aim to reduce sickling through preservation of cell hydration [129].

Secondary Modifiers of Sub-phenotypes and Complications

Association studies of candidate genes implicated in the pathophysiology of vasculopathy, such as those encoding factors modifying inflammation, oxidant injury, nitric oxide biology, vaso-regulation and cell adhesion, have been used extensively to identify variants affecting sickle-related complications, such as stroke, priapism, leg ulcers, avascular necrosis, renal disease, acute chest syndrome, gallstones and susceptibility to infection (see following reviews [121, 122, 130]). The majority of these reported associations have not been replicated or validated, and are likely to be false positives. Of the numerous association studies reported, the most robust is the association between serum bilirubin levels and predisposition to gallstones with the 6/7 or 7/7 (TA) repeats in the *UGT1A1* promoter [125, 131]. The influence of *UGT1A1* polymorphism became more evident in patients while on

hydroxycarbamide therapy; children with 6/6 *UGT1A1* genotype achieved normal bilirubin levels while children with 6/7 or 7/7 *UGT1A1* genotypes did not [132].

Numerous genetic and clinical association studies on cerebrovascular complications in SCD have been carried out [133]. Of the 38 published SNPs associated with stroke, the protective effect of α thalassemia on stroke risk and SNPs in four genes (*ADYC9*, *ANXA2*, *TEK* and *TGFBR3*) could be replicated although only nominally significant association results were obtained [134]. More recently, GWAS in combination with whole exome sequencing have identified mutations in two genes—*GOLGB1* and *ENPP1*—associated with reduced stroke risk in pediatric patients but again, this needs validation in independent studies. In an attempt to overcome the small sample size in end-point complications, a study utilized a compound phenotype that included one or more sickle-related complications [135]. Patients with complications had a higher frequency of the platelet glycoprotein allele *HPA-5B*. In this small study, most of the complications were osteonecrosis and only four individuals had more than one complication [135]. As traditional methods are often inadequate in association studies of complex traits, methods of evaluating multi-locus data are promising alternatives. A GWAS was applied to SCD based on a disease severity score that was derived from a Bayesian network that integrates 25 different clinical and laboratory variables [136]. Several genes not known to be related in the pathogenesis of SCD were identified including *KCN6* (a potassium channel protein) and *TNK5* (gene encoding tankyrase-1, a possible telomere length regulator). However it is important to remember that results from such analytical techniques are dependent on the structure of a model which assumes causality and prior probabilities assigned to the different variables. Using a hemolytic score, GWAS identified a SNP (*rs7203560*) in *NPRL3* that was independently associated with hemolysis [137]. *rs7203560* is in perfect LD with SNPs within the α-globin gene regulatory elements (HS-48, HS-40 and HS-33), and also in LD with *ITFG3* that is associated with MCV and MCH in several GWASs of different population groups. It is proposed that *NPRL3* reduces hemolysis through an independent thalassemic effect on the *HBA1*/*HBA2* genes.

Hydroxyurea remains a major treatment option for SCD [138, 139]; its main effect is mediated primarily through induction of HbF. Clinical and laboratory response to HU therapy however, is variable, a main determinant of response appears to be the baseline HbF levels. Numerous association studies on HbF response to hydroxycarbamide have been reported, of which the association with baseline HbF levels and *Xmn*1-*HBG2* seems to be the most robust [140]. Table 2.1 summarizes the genetic modifiers of SCD.

Genetic Modifiers of β-Thalassemia

The central mechanism underlying the pathophysiology of the β-thalassemias relates to the deleterious effects of the excess α-globin chain on erythroid survival and ineffective erythropoiesis [68]. Clinical studies have shown that disease severity correlates well with the degree of imbalance between α and β globin chains and the

size of the free α chain pool. Genetic modifiers can impact the phenotypic severity at the primary level by affecting the degree of globin chain imbalance, and at the secondary level by moderating complications of the disease related to the anemia or therapy e.g. iron chelation.

While the severity of β-thalassemia is primarily determined by the degree of β chain deficiency, for any given β-thalassemia allele the severity of the disease can be alleviated by co-inheritance of α-thalassemia or by co-inheritance of factors that increase γ-globin chain production and HbF levels. In the latter case, γ-globin chains combine with the excess α-globin to form HbF; cells that contain a relatively higher percentage of HbF are protected against the deleterious effect of alpha-globin chain precipitation and premature death, and have selective survival. Thus, all individuals with β-thalassemia have variable increases in HbF due to survival of these F cells.

Certain β-thalassemia mutations, notably those that involve small deletions or mutations of the promoter sequence of the *HBB* gene, are associated with much higher levels of HbF production than mutations affecting other regions of *HBB* (see deletions causing β-thalassemia) [3]. This might reflect the competition between the *HBG* and *HBB* promoters for interaction with the upstream β-LCR and limited transcription factors. Heterozygotes for such types of β-thalassemia mutations have unusually high HbA_2, and although the increases in HbF levels are variable, the increase in HbF production in homozygotes is adequate to compensate for the complete absence of HbA. HbF levels are normal or slightly elevated in β-thalassemia heterozygotes. Higher HbF (and HbA_2) levels are found with mutations that involve promoter of the *HBB* gene, but variations in HbF levels also reflect the genetic background of the individual (e.g. −29 promoter mutation in Blacks and Chinese). In homozygous β-thalassemia, the proportion of HbF ranges from 10%in those with the milder alleles to almost 100% in homozygotes or compound heterozygotes with β^0 thalassemia. In the most severe cases, the absolute amount of HbF is approximately 3–5 g/dL, produced as a result of extreme erythroid hyperplasia, selective survival of F cells, and some increase in *HBG* transcription. Non-transfusion dependent β^0 thalassemia intermedia with hemoglobin levels of 8–11 g/dL and 100% HbF has been observed. In some cases, the increase in HbF production reflects the type of β-thalassemia allele, but in others co-inheritance of QTLs associated with increased *HBG* expression might explain their more benign clinical features [114].

Effect of the Primary Modifiers: HbF Quantitative Trait Loci and α-Globin Genotype

While selection of F cells provides an explanation for the increases in HbF in β-thalassemia, the mechanism does not explain the wide variation in the amount produced. Much of this variability is genetically determined, in part from the co-inheritance of one or more of HbF-boosting alleles of the *Xmn*1-*HBG2*, *HMIP-2* and *BCL11A* HbF quantitative trait loci (QTLs) [33, 81, 84, 142, 143]. The *Xmn1-HBG2* QTL is a common sequence variation in all population groups, present at a frequency of approximately 0.35. Although increases in HbF and F cells associated

with *Xmn1-HBG2* are minimal or undetectable in healthy adults, clinical studies have shown that under conditions of stress erythropoiesis, as in homozygous β-thalassemia, the presence of *Xmn*1-*HBG2* leads to a much higher HbF response [141]. This could explain why the same mutation on different beta chromosomal backgrounds, some with and others without the *Xmn1-HBG2* variant, are associated with different clinical severity. High resolution genotyping studies suggest that *Xmn*1-*HBG2* may not be the causal element but in tight linkage disequilibrium to another, as yet undiscovered, variant(s) on chromosome 11p.

Together *Xmn*1-*HBG2*, *BCL11A*, and HMIP-*2,* and perhaps other loci, linked and unlinked to the *HBB* complex, constitute the loosely-defined entity of heterocellular HPFH. These HbF QTLs play an important role in fine-tuning γ-globin production in healthy adults and in response to the stress erythropoiesis of sickle cell anemia and β-thalassemia. The three QTLs are associated with HbF and severity of thalassemia in diverse population groups including Sardinian, French, Chinese, and Thai [33, 84, 143, 144]. More than 95% of Sardinian β-thalassemia patients are homozygous for the same codon 39 β^0 thalassemia mutation but have extremely variable clinical severity. Co-inheritance of variants in *BCL11A* and HMIP-*2*, and α-thalassemia accounts for 75% of the differences in disease severity [81]. In France, a combination of the beta thalassemia genotype, Xmn1-*HBG2* and SNPs in *BCL11A* and HMIP-2, can predict up to 80% of disease severity [84]. In a cohort of 316 β^0 thalassemia patients, delayed or absent transfusion requirements correlated with status of the three HbF QTLs and the α-globin genotype [145]. Using a combination of the HbF QTLs, the type of β-thalassemia mutations, and the α-globin genotype, a predictive score of severity has been proposed [146].

In many populations where β-thalassemia is prevalent, α-thalassemia also occurs at a high frequency and hence it is not uncommon to co-inherit both conditions [15]. Homozygotes or compound heterozygotes for β-thalassemia who co-inherit α-thalassemia will have less redundant α globin and tend to have a less severe anemia. The degree of amelioration depends on the severity of the β-thalassemia alleles and the number of functional α-globin genes. At one extreme, patients with homozygous β-thalassemia who have also co-inherited HbH (equivalent of only one functioning α-globin gene) have thalassemia intermedia [68].

In individuals with one β-thalassemia allele (heterozygotes), co-inheritance of α thalassemia normalizes the hypochromia and microcytosis but the elevated HbA_2 remains unchanged. Increased α globin production through co-inheritance of extra α-globin genes (triplicated—ααα/αα or ααα/ααα, quadruplicated—αααα/αα, or duplication of the whole α globin gene cluster—αα/αα/αα) with heterozygous β-thalassemia tips the globin chain imbalance further, converting a typically clinically asymptomatic state to thalassemia intermedia [14, 68, 147]. Again, the severity of anemia depends on the number of extra α globin genes and the severity of the β-thalassemia alleles.

At the primary level of chain imbalance, the proteolytic capacity of the erythroid precursors in catabolising the excess α globin has often been suggested, but this effect has been difficult to define. Alpha hemoglobin stabilizing protein, a molecular chaperone of α globin has also been suggested as another genetic modifier but its impact on disease severity has been inconclusive [148].

Secondary Modifiers of Complications of β-Thalassemia

These modifiers do not affect globin imbalance directly but might moderate the different complications of β-thalassemia that are directly related to the anemia, or to therapy such as iron overload [69, 149, 150]. They include genetic variants which affect bilirubin metabolism, iron metabolism, bone disease and cardiac complications. Jaundice and a predisposition to gallstones, a common complication of β-thalassemia, are associated with a polymorphic variant in the promoter of the *UGT1A1* gene. Individuals who are homozygous for 7 [TA]s, also referred to as Gilbert's syndrome, have higher levels of bilirubin and increased predisposition to gallstones, an observation that has been validated at all levels of β-thalassemia. Several genes involved in iron homeostasis have now been characterized, including those encoding HFE, (*HFE*), transferrin receptor 2 (*TFR2)*, ferroportin (*FPN*), hepcidin (*HAMP*) and hemojuvelin (*HJV*) [151]. The H63D variant, a common polymorphism in the *HFE* gene, appears to have a modulating effect on iron absorption. β-thalassemia carriers who are homozygous for *HFE* H63D variant, have higher serum ferritin levels than carriers without the variant (see Table 2.3 for modifiers of β-thalassemia).

Table 2.3 Genetic modifiers of β-thalassemia

Modifier	Mechanism
(A) Primary at level of α : non-α globin chain imbalance	
1. β globin genotype (one or two, and severity of β-thalassemia alleles)	Directly affects output of β globin and chain imbalance
2. α globin genotype	
α thalassemia	Reduces chain imbalance and α globin excess
co-inheritance of extra α globin genes (ααα/, αααα/, or *HBA* cluster duplication	Increases α globin excess and chain imbalance
3. Innate ability to increase HbF (co-inheritance of HbF QTLs, eg, SNPs in *BCL11A*, *HMIP*, *Xmn*1-*HBG2*	Increased γ chains combine with excess α reducing chain imbalance
4. α hemoglobin stabilizing protein (*AHSP*)	Chaperones excess α globin (studies inconclusive)
(B) Secondary at level of complications related to disease and therapy	
Complication	**Modifier**
1. Serum bilirubin and propensity to gallstones	UGT1A1 promoter $(TA)_n$ polymorphisms
2. Iron loading	*HFE* - H63D variants increase GI absorption of iron
3. Osteopenia and osteoporosis	Variants in *VDR*, *COL1A1*, *COL1A2*, TGFB*1*, modify bone mass
4. Cardiac disease	Apolipoprotein (*APOE*) ε4—risk factor for left ventricular heart failure
5. Cardiac iron loading	Glutathione-S-transferase M1—increased risk of cardiac iron in thalassemia major

Note: Adapted from Thein, S.L. Genetic association studies in β hemoglobinopathies. Hematology, ASH Education Program Book 2013; 354-61

The degree of iron loading, bilirubin levels and bone mass are quantitative traits with a genetic component; variants affect the genes that are involved in the regulation of these traits that contribute to the complications.

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Chapter 3
Current Standards of Care and Long Term Outcomes for Thalassemia and Sickle Cell Disease

Satheesh Chonat and Charles T. Quinn

Abstract Thalassemia and sickle cell disease (SCD) are disorders of hemoglobin that affect millions of people worldwide. The carrier states for these diseases arose as common, balanced polymorphisms during human history because they afforded protection against severe forms of malaria. These complex, multisystem diseases are reviewed here with a focus on current standards of clinical management and recent research findings. The importance of a comprehensive, multidisciplinary and lifelong system of care is also emphasized.

Keywords Sickle cell disease • Thalassemia • Thalassaemia • Clinical care • Standard care • Outcome

Introduction

Thalassemia and sickle cell disease (SCD) are disorders of hemoglobin that affect millions of people worldwide. The carrier states for these diseases arose as common, balanced polymorphisms during human history because they afforded protection against severe forms of malaria. These complex, multisystem diseases are reviewed here with a focus on current standards of clinical management and recent research findings. The importance of a comprehensive, multidisciplinary and lifelong system of care is also emphasized.

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Thalassemia

Thalassemia is the name for a group of disorders of hemoglobin characterized by diminished or absent production of alpha or beta globin chains, the two protein subunits of the hemoglobin molecule. This inherited disorder derives its name from the Greek “Thalassa” (sea) and “Haema” (blood), referring to its discovery among people living around the Mediterranean Sea. It also occurs in high frequencies in parts of Africa, the Asian sub-continent, southeast Asia and the Middle East. Thalassemia is mainly divided into alpha thalassemia (defective or absent alpha chain synthesis) and beta thalassemia (defective or absent beta chain synthesis). This section on thalassemia will largely focus on the clinical management of these patients with a brief introduction to the molecular biology underlying the different forms of thalassemia.

Molecular Basis and Classification

The alpha globin gene cluster is on chromosome 16 and the beta globin gene cluster is on chromosome 11. These gene clusters include regulatory regions as well as distinct genes for embryonic, fetal (for β-like globins), and adults versions of the α-like and β-like globins. Thalassemic mutations can affect any of these genes, but the most common forms of thalassemia occur due to defects in the α and β globin genes and their related regulatory sequences. The main subtypes of alpha and beta thalassemia are presented in Tables 3.1 and 3.2.

Table 3.1 Common genotypes and basic classification of alpha thalassemia

Genotype	α-globin gene number[a]	Name	Phenotype
αα / αα	4	Normal state	None
αα / α–	3	Silent carrier	None (values for Hb and MCV may be near the lower limits of normal)
– – / αα or α– / α–	2	Thalassemia trait	Thalassemia minor: asymptomatic, mild microcytic anemia
– – / α–	1	Hb H disease	Thalassemia intermedia: mild to moderate microcytic anemia
– – / $\alpha^{CS}\alpha$	1	Hb H-Constant Spring	Thalassemia intermedia: moderate to severe microcytic anemia
– – / – –	0	Alpha thalassemia major	Thalassemia major: hydrops fetalis

[a]Number of normal alpha globin genes

Abbreviations: *αα* a chromosome with a normal complement (2) of α-globin genes, *α–* a chromosome with 1 of the 2 α-globin genes deleted, – – a chromosome with both α-globin genes deleted, *αα* a chromosome with the thalassemic Hb Constant Spring mutation affecting 1 of the 2 α-globin genes

Table 3.2 Common genotypes and basic classification of beta thalassemia

Common genotypes	Name	Phenotype
β/β	Normal	None
β/β^0 β/β^+	Beta thalassemia trait	Thalassemia minor: asymptomatic, mild microcytic hypochromic anemia
β^+/β^+ β^+/β^0 β^E/β^+ β^E/β^0	Beta thalassemia intermedia	Variable severity Mild to moderate anemia Possible extramedullary hematopoiesis Iron overload
β^0/β^0	Beta thalassemia major (Cooley's Anemia)	Severe anemia Transfusion dependence Extramedullary hematopoiesis Iron overload

Abbreviations: β^0 a β-thalassemia mutation that eliminates globin transcription or translation, β^+ a β-thalassemia mutation that decreases globin transcription or translation, β^E the β-globin mutation that results in Hb E

Alpha thalassemia is usually caused by deletions of one or more of the 4 alpha globin genes (2 on each chromosome). Small deletions removing one alpha globin gene (α^+) are more commonly seen in people of African ancestry. Larger deletions of DNA removing both alpha globin genes (α^0) on each chromosome are more commonly seen in Southeast Asia. Alpha globin point mutations and structural variants are less common but well-known forms of alpha thalassemia.

Beta thalassemia results mostly from over 200 point mutations, but thalassemic deletions also occur less commonly. These point mutations affect gene regulation and expression to varying degrees [1]. Beta globin is normally synthesized from two beta globin genes (one on each copy of chromosome 11). If a mutation abolishes the expression of beta-globin, it is classified as a beta-zero (β^0) thalassemia allele, while reduced or partial expression is classified as a beta-plus (β^+) thalassemia allele. As shown in Table 3.2, clinically important thalassemia variants can also occur due to interactions of structural beta globin variants (especially Hb E, β^E) with β^0 or β^+ thalassemia mutations.

The Globin Gene Server (http://globin.cse.psu.edu) database of hemoglobin variants continues to be updated on a regular basis with newly identified mutations [2].

Clinical Presentation

The clinical phenotype or severity varies considerably, mainly depending on the type and number of genetic defects of the alpha or beta globin genes and regulatory regions.

In alpha thalassemia, the clinical severity is primarily based on the number of alleles affected (Table 3.1). Many forms of alpha thalassemia are asymptomatic or mild. Individuals who have the alpha thalassemia silent carrier state (α-/αα) or trait

(--/αα or α-/α-) are asymptomatic. Thalassemia trait produces microcytosis, hypochromia and mild anemia. These individuals do not encounter any problems, but awareness of their condition is important to prevent injudicious use of iron therapy to treat their anemia and facilitate genetic and reproductive counseling. Most patients with classical hemoglobin (Hb) H disease (--/α-) are generally transfusion-independent except occasional need during periods of illnesses or surgery. A form of Hb H disease that is more severe is Hb H-Constant Spring. In this variant, a non-deletional alpha globin gene that disrupts the stop codon is co-inherited with a two-gene deletion alpha thalassemia (--/$\alpha^{CS}\alpha$) (Table 3.1). This is an important cause of Hb H disease, especially in Southeast Asia [3]. These patients are more likely to be transfusion-dependent and have other clinical complications (reviewed below). Homozygous alpha thalassemia major (--/--) produces a hydrops fetalis phenotype.

In beta thalassemia, the clinical severity is also a function of the number and type (β^0 or β^+) of genetic defects. Beta chain deficiency results in an excess of unbound alpha globin chains (due to lack of beta chains to pair with to make Hb A; $\alpha_2\beta_2$). These excess alpha globin chains precipitate within and damage erythroid precursors and red blood cells causing ineffective erythropoiesis and hemolysis, respectively [4, 5]. Patients with beta thalassemia trait or minor (β/β^+ or β/β^0) are asymptomatic and have a mild, hypochromic, microcytic anemia. Patients with beta thalassemia intermedia have a quite variable phenotype that ranges between the asymptomatic (minor) phenotype and the transfusion-dependent (major) phenotype. Much of this variability depends on the particular combination of mutations (e.g., β^+/β^+, β^0/β^+, β^E/β^+, β^E/β^0) and alpha globin gene number. Thalassemia intermedia patients typically present around 4–6 years of age with mild to moderate anemia and require intermittent transfusions, but less often than patients with thalassemia major. Children with beta thalassemia major present in infancy, corresponding to the decline in fetal hemoglobin production in infancy. The typical presentation is failure to thrive, progressive anemia that becomes transfusion-dependent, and hepatosplenomegaly as a manifestation of extramedullary hematopoiesis.

It is important to note that thalassemia mutations can be co-inherited with other mutations that cause thalassemia or other types of hemoglobinopathies. As such, it is necessary to know the alpha globin and beta globin genotypes in all patients with thalassemia, because the phenotype depends on the joint interaction of the alpha and beta globin genotypes. For example, the phenotype of patients with a single beta thalassemia mutation is made more severe by the coinheritance of alpha thalassemia triplications. The sickle-β-thalassemia syndromes are forms of SCD discussed elsewhere in this chapter.

Diagnosis

Newborn screening is an important way to identify thalassemia, especially in high-risk populations, before symptoms appear. Common methods of diagnosis in the newborn and later life are the Hb separation techniques, such as gel-based

electrophoresis (especially isoelectric focusing), high-performance liquid chromatography, and capillary electrophoresis. A diagnosis of alpha thalassemia is often inferred by Hb separation techniques by decreased levels of Hb A_2 or decreased ratios of a variant beta-globin to normal Hb A (e.g., decreased Hb S: Hb A ratio). The presence of Hb Barts in the newborn period, Hb H, or Hb Constant-Spring specifically indicate the presence of alpha thalassemia. Beta thalassemia is often inferred by raised levels of Hb A_2 of Hb F. The absence of Hb A indicates homozygous or compound heterozygous beta thalassemia major.

Given the genetic complexity of thalassemia and the limitations of Hb separation techniques, DNA-based testing is key to identify the specific mutations underlying alpha and beta thalassemia. Newer techniques to identify fetal DNA in maternal circulation can permit prenatal diagnosis. Such screening can be beneficial to prevent hydrops fetalis and related maternal complications [6].

Preventive Care and Screening Measures (Table 3.3)

Growth and Development

Children with thalassemia intermedia and major can have growth abnormalities from chronic anemia, a hypermetabolic state, endocrine abnormalities, poor nutrition, and iron overload, among others. Short stature can be seen in over 30% of children with thalassemia major [7, 8]. Assessment of growth with height velocity, weight, along with pubertal assessment during clinic visits is essential. This should be at a minimum of every 6 months. Involvement of nutritionist will be beneficial.

Endocrine Studies

Abnormalities involving gonadal function, glucose intolerance, hypoparathyroidism and hypothyroidism have been noted with increasing frequency. Many of these endocrine abnormalities are the consequence of iron overload. This necessitates monitoring of pituitary hormones, along with testing for thyroid function, glucose tolerance and micronutrients (calcium, folate, Vitamin D, phosphate). Annual monitoring of these is recommended and, if abnormal, it warrants involvement of an endocrinologist for expert care.

Iron Monitoring

Iron loading rate and iron burden are monitored based on volume of pRBC in mL/kg/year transfused, serum ferritin and liver Iron concentration. Serum ferritin is an inexpensive and easily monitored marker of body iron stores, but it underestimates the body iron and liver iron concentration [9]. It is also increased in states of

Table 3.3 Standard care and screening guidelines for thalassemia intermedia and major

Assessment	Ages	Frequency	Comments
Bone mineral density	≥10	Yearly	DEXA scan or quantitative CT
Tanner stage	10–20	Yearly	Perform yearly starting at age 10 and continuing until breast or gonadal Tanner Stage 5 or age 20.
Liver iron content	All	Yearly	MRI method (R2, T2*) or liver biopsy
Cardiac T2*	≥ 10	Yearly (see comment)	To be performed when available for patients with biochemical evidence of iron overload **or** age 10 years or older
Cardiac studies	≥ 10	Yearly	Echocardiography and/or cardiac function by MRI; indicate the need to assess for pulmonary hypertension when ordering an echocardiogram
CBC plus differential	All	Yearly (minimum)	If transfused, preceding each transfusion
Blood chemistries	All	Yearly	BUN, creatinine, calcium, magnesium, phosphorus, and zinc
LFTs	All	Yearly	ALT, AST, total bilirubin, albumin
Ferritin	All	Yearly (minimum)	If transfused, preceding each transfusion
HIV	All	Yearly	Only for transfused participants starting at the first transfusion
Hepatitis testing	All	Yearly	Only for transfused participants starting at the first transfusion. HBV serology. HCV serology and PCR
Plasma ascorbate	All	Yearly	12 h fast
Serum glucose	≥10	Yearly	12 h fast
Endocrine panel I	≥6	Yearly	TSH, free T4, parathyroid hormone, 25-hydroxy vitamin D, and 1,25 dihydroxy vitamin D levels
Endocrine panel II	≥10	Yearly	Testosterone (males only), FSH and LH (males and females), and estradiol (females only)
Ophthalmology and Audiology testing	Children and Adults	Yearly	All patients undergoing chelation. Auditory testing necessary for those on desferroxamine

inflammation and suppressed by deficiency of vitamin C. Serum ferritin should be tested every 3 months to monitor the trend among chronically transfused patients and less frequently in patients who receive fewer transfusions. Liver Iron concentration (LIC) of ≥5 mg/g dry weight is associated with increased morbidity and mortality in patients with thalassemia [10, 11]. MRI methods to measure LIC have largely supplanted serial liver biopsies. Regular monitoring of LIC (at least yearly for chronically transfused patients) will inform the initiation and intensity of iron chelation therapy. Serum ferritin and/or LIC do not reliably predict the cardiac iron overload. Non-transferrin bound iron and labile plasma iron are other markers being studied to correlate with the toxicity of circulating iron [12].

Cardiac Studies

Cardiac pathology, especially due to cardiac iron overload, has become the most common cause of death in thalassemia. Cardiac iron burden and risk of heart failure can be estimated by MRI-based techniques (e.g., T2*) [13]. Cardiac T2* measurements are recommended at least yearly in chronically transfused patients, and less frequently in non-transfusion dependent patients. Cardiac MRI screening should be commenced around 8–10 years of age, or sooner if there is concern for severe iron overload, especially if compliance with chelation is poor. Monitoring of ventricular function is also important and can be done by functional cardiac MRI, echocardiography or both. Pulmonary hypertension has also been reported in patients with thalassemia, which is thought to arise from iron overload and factors such as splenectomy, thrombocytosis and activation of coagulation factors. Monitoring for pulmonary hypertension can be done by echocardiography, and regular transfusion therapy, iron chelation, and hydroxyurea have shown to be protective against pulmonary hypertension [14].

Infection

While screening and closer monitoring have helped in preventing morbidity and mortality due to iron overload, infection-related deaths in developing and developed countries still occur. Some of the important risk factors include chronic transfusion therapy, use of central venous lines, iron overload, splenectomy and immune abnormalities [8, 15]. Hepatitis C still remains one of the important transfusion related infection in thalassemia patients in North America, as the incidence of HIV and Hepatitis B have reduced [16]. Yearly screening for HIV and viral hepatitis infections is necessary for regularly transfused patients.

Bone Mineral Density

Reduced bone mineral density (Z-score $\leq$ 2.0) occurs in over 30% of individuals with thalassemia syndromes despite adequate transfusion therapy. Greater age, males with Collagen type 1A polymorphism, endocrine abnormalities, lower weight, poor nutrition, reduced physical activity and increased bone turnover are some of the risk factors [17, 18]. Annual DEXA scans will help diagnose and institute early management.

Ophthalmologic and Auditory Screening

Children and adults on iron chelation therapy are recommended to get yearly eye assessments whilst on iron chelation therapy though it is unclear if lens opacities are due to underlying thalassemia or iron chelation [19]. Yearly audiograms are also recommended for patients receiving chelation therapy, especially with deferoxamine.

Medical and Surgical Management of Patients with Thalassemia

Current, most effective, and evidence-based management strategies will be highlighted here, including chronic transfusion therapy, iron chelation, induction of fetal Hb, and splenectomy.

Transfusion Therapy

An early and appropriate transfusion program is a pillar of care for children with beta thalassemia major and in some children with beta thalassemia intermedia. Transfusion is beneficial because it alleviates symptomatic anemia, suppresses ineffective erythropoiesis, improves growth and development, reduces iron loading from increased gastrointestinal absorption, and prolongs life [16, 20, 21].

The decision to begin chronic transfusion therapy is often based on clinical characteristics due to complex and incompletely understood genotype-phenotypes correlations in thalassemia [22]. Chronic transfusion is considered in thalassemia patients when the hemoglobin concentration is consistently below 6–7 g/dL, the anemia is symptomatic, quality of life is poor, growth and development is faltering, or there is troublesome extramedullary hematopoiesis. Early initiation of transfusions may also minimize the risk of alloimmunization [20, 23].

The usual transfusion goal is to maintain a pre-transfusion hemoglobin level between 9 and 10 g/dL [20, 21, 24]. Once this goal is reached, transfusions can usually be given every 3–4 weeks, typically using 8–15 mL/kg of packed red blood cells (PRBC). Leukodepleted PRBCs should be used to minimize febrile nonhemolytic transfusion reactions and lessen the risk of cytomegalovirus transmission and alloimmunization. Irradiation of blood products is not necessary unless hematopoietic stem cell transplant is planned in the near future. A central venous catheter can facilitate transfusions.

Detailed documentation of pre-transfusion hemoglobin levels, transfusion reactions, alloimmunization, and volume of all PRBCs received is necessary for high quality care. While chronic (and intermittent) transfusions have remarkably improved the survival and quality of life, of individuals with thalassemia, transfusions do come at a cost. In developing countries, the transfusion-transmitted infections (primarily hepatitis B and C) remain an important cause of morbidity and mortality, and transfusional hemosiderosis affects all transfused individuals.

Fetal Hemoglobin (Hb F) Inducing Agents

In beta thalassemia, increased gamma globin chain synthesis (that is, increased Hb F production) decreases the degree of alpha chain to beta-like chain imbalance. This is associated with increased red cell life span, decreased ineffective erythropoiesis

and hemolysis, and thereby decreases the severity of beta thalassemia [25, 26]. Several genetic polymorphisms are associated with high Hb F levels. Pharmacologic agents can also augment Hb F production, such as demethylating agents histone deacetylase (HDAC) inhibitors, and hydroxyurea.

Demethylating Agents

5-azacytidine and decitabine are cytidine nucleoside analogs that become incorporated into DNA where they sequester DNA methyltransferases (DNMTs) and result in DNA hypomethylation, thereby leading to the expression of previously silenced genes, such as the gamma globin genes. Since Ley and DeSimone first reported the use of 5-azacytidine, a cytosine analogue in a patient with severe beta thalassemia, multiple smaller cohort studies have since been reported [27]. Initial studies identified the potential for toxicity, including mutagenicity, myelosuppression, and others. More recent studies using decitabine (5-aza-2′-deoxycytidine), an analogue of azacytidine, have shown clinical benefits with better safety profile. A pilot study in 2011 by Oliveri et al. for Thalassemia Clinical Research Network treated 5 patients with beta thalassemia intermedia with subcutaneous decitabine given at 0.2 mg/kg two times per week for 12 weeks. They noticed an increase in total hemoglobin from 7.88 to 9.04 g/dL and absolute fetal hemoglobin from 3.64 to 4.29 g/dL. They also reported favorable changes in red blood cell indices of hemolysis. Major side effects noted was an elevation in platelet count [28]. This is an additional concern for thalassemia patients post-splenectomy, who have thrombocytosis and a higher risk for venous and arterial thromboembolism.

Histone Deacetylase (HDAC) Inhibitors

Butyrates and short chain fatty acid derivatives regulate gene expression by inhibition of HDAC, which increases core histone acetylation, modifies chromatin structure, and affects the rate of transcription. Perrine and colleagues demonstrated that butyrates could activate gamma globin gene expression. This discovery was predicated on the observation that infants born to mothers with diabetes had high levels of butyrates and a delayed fetal to adult hemoglobin switch. A subsequent study of butyrates in patients with thalassemia and SCD patients demonstrated increases in fetal Hb production [25]. Though multiple smaller studies have shown some benefit, it is thought that butyrate, in addition to inducing gamma globin expression, also increases alpha globin expression in thalassemia, thus minimizing the beneficial effects in beta thalassemia. This is contrary to the effect seen in SCD where in addition to augmentation of gamma globin mRNA levels, the alpha globin mRNA levels decreased in response to butyrate [29]. Among other newer and ongoing pilot studies, an oral butyrate derivative (HQK-1001) that does not have HDAC2 inhibitory activity has been shown to stimulate gamma globin expression [30].

Erythropoietin

Erythropoietin preparations have shown to increase total hemoglobin levels, thereby decreasing transfusion requirements. While its use may be beneficial in beta thalassemia intermedia [31], inability of erythropoietin or darbepoetin to increase fetal hemoglobin might limit its use in beta thalassemia major patients [32]. There is potential for its use along with hydroxyurea in patients with low erythropoietin levels [21].

Hydroxyurea

Hydroxyurea is a ribonucleotide reductase inhibitor that can induce Hb F production [33]. Hydroxyurea has been shown to increase the total hemoglobin level in beta thalassemia intermedia. A long term study in Iran of its use in patients with beta thalassemia showed dramatic response in 149 of 163 patients with 83 of 106 transfusion-dependent patients becoming transfusion-free [34]. The retrospective OPTIMAL CARE study in spite of its limitations, noted some decrease in complication rates if hydroxyurea was used along with transfusion and iron chelation in thalassemia intermedia patients [35]. Unlike its benefits in SCD where improved red blood cell deformability, reduced oxidative stress and reduced hemolysis were noted, its beneficial effects in thalassemia is limited [10].

Hydroxyurea is often started at a dose of 8–10 mg/kg/day and escalated as tolerated to 20 mg/kg/day. Predictors of good response to hydroxyurea are younger age, higher pretreatment hemoglobin level, associated alpha thalassemia, Hb E/beta thalassemia, and history of splenectomy [36].

Iron Chelation Therapy

Iron overload occurs from chronic transfusion therapy and from increased gastrointestinal absorption in thalassemia patients with ineffective erythropoiesis. Poorly transfused individuals can absorb around 3–5 mg/day or more of iron through their gut. Hemolysis and chronic hypoxia can further increase this gut absorption by decreasing hepcidin. Each 100–200 mL/kg body weight of PRBC delivers about 116–232 mg of iron/kg body weight/year. Therefore, it is transfusion therapy that most rapidly increases the iron overload.

Generally, chelation is initiated for transfusion-dependent thalassemia patients between 2 and 4 years of age, after 200 mL/kg of PRBC have been transfused, with a serum ferritin level > 1000–1500 ng/mL and an LIC >3–7 mg Fe/g dry weight as measured by liver biopsy or by noninvasive hepatic R2 or T2* MRI methods [12, 21]. The regimen of any chelator should be adjusted individually to achieve an iron excretion rate that is commensurate with the rate of ongoing iron loading.

Table 3.4 Iron chelators

Agent	Route half-life	Classification[a]	Dose and regimen	Common or typical adverse effects
Deferoxamine	IV or SQ 8–10 min	Hexadentate	30–40 mg/kg/day over 8–12 h 5–7 day/week	Anaphylaxis Local irritation Retinopathy Hearing loss Renal dysfunction Hepatic dysfunction
Deferasirox	Oral 12–18 h	Tridentate	20–40 mg/kg/day Once daily	Diarrhea Rash Proteinuria Renal dysfunction Hepatic dysfunction
Deferiprone	Oral 1.5–4 h	Bidentate	75–100 mg/kg/day 3 divided doses daily	Arthralgia Arthropathy Agranulocytosis Renal dysfunction Hepatic dysfunction

[a]Indicates the number of coordination sites of the Fe atom that each molecule of the chelator can bind with; Fe has 6 coordination sites

There are three main iron chelators in clinical practice: subcutaneous (or intravenous) deferoxamine, oral deferiprone and oral deferasirox (Table 3.4). Current recommendations in children are to begin with an oral chelator such as deferasirox due to its advantages of high oral bioavailability, longer half-life of 12–18 h enabling once daily dosing, and ability to better chelate cardiac iron. Adverse effects like skin rash, nausea, and diarrhea can be mitigated by starting at 20 mg/kg per day and slowly escalating up to 30–40 mg/kg/day. Serum creatinine, proteinuria and liver function should be monitored monthly during deferasirox treatment.

Transfusion history and serum ferritin are not reliable indicators for iron overload in children with non-transfusion dependent thalassemia intermedia [37]. Non-transfusion dependent thalassemia patients have increased enteral absorption of iron that is not accounted for when considering only transfusions as a source of iron loading. Also, serum ferritin is known to underestimate iron burden in thalassemia intermedia. The THALASSA study investigated the use of deferasirox at 5–10 mg/kg/day in 166 patients with non-transfusion-dependent thalassemia and iron overload (LIC ≥5 mg Fe/g dry weight and serum ferritin levels >300 ng/mL). Compared to placebo, deferasirox significantly reduced LIC, mean serum ferritin levels with a manageable toxicity profile [12, 38].

Combination therapy with deferoxamine and deferasirox or deferiprone has been reported as an option for patients requiring rapid reduction in their cardiac and/or systemic iron burden [12].

Splenectomy

Splenomegaly with hypersplenism is a common problem in thalassemia intermedia and major. The main cause of splenomegaly is extramedullary hematopoiesis. Due to hypersplenism, chronic hemolysis is exacerbated, thereby worsening the anemia and increasing transfusion requirements. Splenectomy is indicated in a small group of patients with annual transfusion requirements exceeding 200–250 mL/kg, with hypersplenism with or without the presence of splenomegaly-related complications such as cytopenias, pain or risk of rupture [21, 23]. Splenectomy should only be strongly considered if it is likely to improve anemia and minimize extramedullary hematopoiesis and growth failure. In addition to risk of post-splenectomy sepsis, multiple studies have highlighted an increased risk of other post-splenectomy complications in thalassemia, including pulmonary hypertension, heart failure, thrombosis, cholelithiasis, leg ulcers, osteoporosis, brain infarcts, and others [23, 35, 39]. Therefore, the benefits of splenectomy should we weighed against the increased risk of post-splenectomy complications. Overwhelming post-splenectomy infection is a clearly documented risk, and splenectomy should be avoided in children less than 5 years of age, when possible, because pneumococcal bacteremia is more common in young children. Immunizations prior to surgery are necessary against pneumococcus, *Haemophilus influenzae* type b, and meningococcus. Post-splenectomy antibiotic prophylaxis should be prescribed for at least several years after splenectomy (if not life-long).

Cure

Hematopoietic stem cell transplantation and gene therapy are discussed in other chapters in this book.

Long Term Outcomes

The life expectancy of patients with thalassemia has improved dramatically over the past 50 years (Fig. 3.1). Improved survival has mainly been achieved by early diagnosis of the disease, intensified transfusion regimens (“hyper-transfusion”), early introduction (especially in the first decade of life) and intensification of chelation therapy, and reduction or elimination of HCV and HIV infection. In developed nations, complication-free survival rates higher than 90% have been documented in children and young adults [40]. Early mortality has not been eliminated, and cardiac disease (heart failure, dysrhythmia, and myocardial infarction) is the most common cause of death. Other common morbidities, and less common causes of mortality, include infection, cirrhosis, thrombosis, malignancy, diabetes, and other

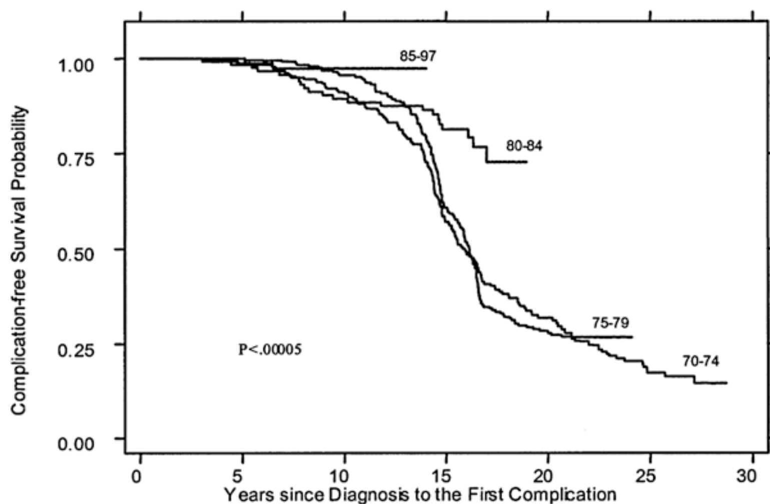

Fig. 3.1 Improving survival in thalassemia major. Complication-free survival for patients with thalassemia major cared for in Italy is shown by birth cohort (1970-74, 1975-79, 1980-84, 1985-97). From: Borgna-Pignatti C, et al. Ann N Y Acad Sci. 2005;1054(1):40-7. Reprinted with permission from John Wiley and Sons

endocrinopathies. Many of these conditions, especially cardiac disease, are directly related to iron overload, highlighting the need for adequate iron chelation therapy. Even despite good, contemporary therapy, health-related quality of life is decreased in patients with thalassemia, indicating that ongoing improvements in therapy and support are needed [40–42].

In conclusion, while significant disparities remain between developed and developing nations, improvements in outcomes over time along with continued research into better therapies with potential for cure–gene therapy and HSCT--will certainly change the face of this common genetic disease.

Sickle Cell Disease

Sickle cell disease (SCD) is the name for a group of disorders caused by a structural abnormality of hemoglobin (Hb) called sickle Hb (Hb S). Since the discovery of sickle-shaped cells in 1910 by Herrick [43], many advances have been made in understanding the genetics and pathophysiology of SCD which is now beginning to guide the development of specific treatments for this condition. SCD is widespread amongst people of African descent as well as in the Middle East, Indian subcontinent, and other Mediterranean regions. This section on SCD will largely focus on the diagnosis and standard clinical management of patients and the associated complications.

Molecular Basis, Pathophysiology and Diagnosis

Hb S is the result of a single amino acid substitution (glutamic acid to valine) at the sixth position of the mature beta globin protein, the gene for which is on chromosome 11. This abnormal hemoglobin is inherited in an autosomal recessive fashion, and SCD can occur due to homozygosity for the Hb S mutation (β^S/β^S) or from compound heterozygous states with certain other β-globin variants (β^S/β^C, β^S/β^0 or β^S/β^+) (Table 3.5).

Hb S is normally functional and soluble when oxygenated, but it forms insoluble polymers upon deoxygenation ("sickling"). This process deforms the red blood cell (RBC), leading to progressive cytoskeletal and other cellular damage that dramatically shortens the lifespan of the RBC. The result is a chronic, partially compensated, hemolytic anemia. This process of RBC "sickling" is initially reversible, but recurrent cycles of oxygenation and deoxygenation lead to cumulative and permanent membrane damage that gives rise to the hallmark irreversibly sickled cell. The RBCs in SCD also participate in abnormal adhesive interactions with platelets, leukocytes [44] and endothelial cells, which leads to stasis in post-capillary venules [45]. Stasis exacerbates the deoxygenation-polymerization process, leading to vaso-occlusion. The consequence is ongoing ischemia-reperfusion injury in nearly all organs, punctuated by acute vaso-occlusive events, leading to chronic organ damage and shortened life-expectancy of affected individuals.

SCD in developed countries is primarily diagnosed at birth by newborn screening for hemoglobinopathies. Common methods of diagnosis in the newborn period and later life are the Hb separation techniques, such as gel-based electrophoresis (especially isoelectric focusing), high-performance liquid chromatography, and capillary electrophoresis. Table 3.5 shows the relative abundance of the main Hbs in different forms of SCD. Like the thalassemias, genetic testing is being increasingly used to provide a definitive diagnosis, and also to identify clinically important phenotypic modifiers, such as alpha globin gene number and genetic determinants of Hb F production.

Table 3.5 Common forms of SCD in order of decreasing relative frequency

Genotype		Name	Main Hbs	Hb (g/dL)[a]	MCV (fL)[a,b]	Retic (%)[a]	Severity[c]
$\beta^S\beta^S$	SS	Sickle cell anemia	S > F	6–9	nl	10–25	4+
$\beta^S\beta^C$	SC	Sickle-Hb C disease	S ≈ C	9–12	nl or ↓	5–10	2+
$\beta^S\beta^+$	$S\beta^+$	Sickle-β^+-thalassemia	S > A	10–13	↓	5–10	1–2+
$\beta^S\beta^0$	$S\beta^0$	Sickle-β^0-thalassemia	S > F	6–9	↓	10–25	4+

[a]Common range of laboratory values in un-treated state (e.g., no hydroxyurea)
[b]Does not consider effect of co-inherited alpha thalassemia or therapeutic effect of hydroxyurea
[c]Population-based generalization
Abbreviations: *nl* normal, ↓ decreased, β^+ a β-thalassemia mutation that eliminates globin transcription or translation, β^+ a β-thalassemia mutation that decreases globin transcription or translation, β^E the β-globin mutation that results in Hb S, β^C the β-globin mutation that results in Hb C

Prenatal diagnosis using chorionic villi sampling (10–12 weeks of pregnancy), amniotic fluid sampling (15–20 weeks) or fetal blood (17 weeks onwards) is also possible. This invasive testing comes with a small but significant risk to the fetus and mother. Newer non-invasive techniques using cell-free fetal DNA in maternal blood allows earlier (6 weeks) and less risky testing [46].

Screening Tests and Prevention of Complications

Bacterial Infection

Infection still poses significant risk for children with SCD, especially in less developed countries. Invasive pneumococcal disease, especially, results from functional asplenia due to progressive ischemic involution of the spleen. Immunizations are essential for children with SCD, as they have over 100-fold risk for invasive pneumococcal infection [47]. All children should complete the routine pneumococcal protein-conjugate vaccine (PCV13) series during infancy and also receive the 23-valent pneumococcal polysaccharide vaccine (PPSV-23) starting at 2 years of age and a booster dose at 5 years of age (some recommend a booster dose every 5 years thereafter). Other recommended vaccines include meningococcal protein-conjugate vaccine (MCV-4) starting at 2 years of age (and consider booster doses every 5 years). Vaccination against *H. influenzae* type b is also critical due to hyposplenism. Hepatitis B virus and yearly influenza vaccines are also prudent [48, 49].

Antibiotic prophylaxis should be commenced at 1–2 months of age as risk for infection from functional asplenia coincides with falling fetal hemoglobin. Children with Hb SS and Hb Sβ^0 should receive oral penicillin: 125 mg twice daily under 3 years of age and 250 mg twice daily over 3 years of age. Prophylactic penicillin is usually stopped at 5 years of age [50], but there are recent reports of invasive pneumococcal disease from non-vaccine serotypes in older children with SCD [51, 52], so ongoing vigilance will be needed. Penicillin prophylaxis may not be needed for young children with Hb SC disease due to the delayed occurrence of hyposplenism [53].

In addition to above, any child or adult with SCD should be evaluated urgently for fever with prompt use of empiric intravenous broad-spectrum antibiotics. The management should also include a complete blood count and a blood culture. Presence of a central venous catheter in these individuals poses an additional risk. Very young age, toxic clinical appearance, laboratory values significantly different from baseline, and respiratory or cardiovascular instability require hospitalization. Most children with fever, however, can be safely managed as outpatients following empiric antibiotic therapy.

Ongoing education and reminders for patients, families and providers of the risk of sepsis is essential in preventing infection related morbidity and mortality. Combined with immunization, antibiotic prophylaxis, and empiric antibiotic treatment for fever episodes have largely eliminated death from infection in developed countries.

Acute Splenic Sequestration

This remains a potentially life-threatening complication in children with SCD. It occurs in younger patients, usually less than 5 years of age, before spleen involution is complete. Potentially reversible sequestration of red blood cells can occur suddenly with acute anemia, hypovolemia, splenomegaly, thrombocytopenia and reticulocytosis. Prompt detection of an enlarged spleen by parents who are taught splenic palpation along with urgent medical evaluation for this finding can usually prevent fatal episodes. A complete blood count and the presence of splenomegaly can help in differentiating this from transient aplastic crisis, which is characterized by reticulocytopenia and a normal or increased platelet count. For moderate to severe episodes, supportive measures with oxygen and fluid resuscitation (if necessary) and judicious PRBC transfusions can be lifesaving.

While acute splenic sequestration usually occurs in younger Hb SS or Hb Sβ^0 patients (<5 years of age) before splenic involution is complete, splenic sequestration can occur in older Hb SC and Hb Sβ^+ patients, in whom splenic involution is delayed. These adolescents and young adults often present with left upper quadrant pain from accompanying splenic infarction. The increasing use of hydroxyurea and chronic transfusion therapy can delay or partially reverse splenic involution, so the occurrence of splenic sequestration may also be delayed in such treated patients.

Primary Prevention of Stroke

In children with Hb SS and Hb Sβ^0, screening for increased risk of overt stroke is performed using transcranial Doppler (TCD) ultrasonography at least yearly starting at 2 years of age until at least 16 years of age. This detects abnormally increased cerebral arterial blood flow velocities that indicate an increased risk of overt stroke. The randomized STOP Trial (Stroke Prevention Trial in Sickle Cell Anemia) demonstrated that chronic transfusions reduced the rate of first overt stroke by 92% in patients with abnormal TCDs. Recently, early, unpublished results from the TWiTCH study (Transcranial Doppler with Transfusions Changing to Hydroxyurea; clinicaltrials.gov: NCT01425307) suggest that hydroxyurea appears to be non-inferior to chronic transfusion therapy (for TCD velocities, not MRI findings or stroke) for patients without severe vasculopathy by MRA after at least 12 months of chronic transfusions. So, chronic transfusions may not have to be indefinite therapy for primary stroke prevention.

Health Maintenance and Screening (Table 3.6)

Growth and Development

Children with SCD can have poor growth in comparison to normal African American children. Pubertal and skeletal maturation delay has been noted in these patients [54, 55]. Chronic anemia, poor nutrition, increased metabolic rate and

renal loss of nutrients have been posited to lead to growth failure in children with SCD. Contemporary management of SCD with early hydroxyurea, monitoring and supplementation of nutrients (such as zinc and vitamin D) and appropriate use of age-, sex-, and ethnicity-matched growth charts can help maintain normal or near normal growth in most patients, thereby potentially improving quality of life [56, 57].

Blood Counts and Serum Chemistries

Monitoring of hematological and biochemical parameters is recommended during clinic visits. The following laboratory studies are recommended at least once or twice yearly: complete blood count, reticulocyte count, differential leukocyte count (if taking hydroxyurea therapy), renal and liver function tests, urinalysis, urine microalbumin and protein quantitation, and ferritin (for a history of transfusions). Routine monitoring will help determine baseline (steady-state) blood count values and detect early evidence renal dysfunction, iron overload, and other complications associated with SCD.

Electrocardiogram and Echocardiography

Routine screening with electrocardiography in asymptomatic children with SCD is currently not recommended. While there is evidence that adults with SCD who have a tricuspid regurgitant velocity (TRV) equal to or greater than 2.5 m/s are at risk of early mortality [58], similar evidence in children is lacking. Given the potential for cardiac or pulmonary disease associated with elevated TRV, we think it is still prudent to screen adolescents by echocardiography before transition to adult medical care. Children with signs or symptoms of cardiopulmonary disease should be tested accordingly.

TCD Ultrasonography

Annual screening with TCD ultrasonography can detect high risk of overt stroke and direct the initiation of chronic transfusion programs for primary prevention of stroke. Please refer to the section on primary prevention of stroke earlier in this chapter.

MRI of Brain and Silent Cerebral Infarction (SCI)

Routine magnetic resonance imaging (MRI) of brain in children with Hb SS is currently not recommended by the NHLBI guidelines [59]. However, silent cerebral infarcts (SCI), which can only be detected by a screening MRI (or incidentally on an MRI for another reason) are associated with neurocognitive deficits, poor school

Table 3.6 Health maintenance and screening for individuals with SCD[a]

Health topic	Recommendations
Prevention of infection	Administer oral penicillin prophylaxis (125 mg for age <3 years and 250 mg for age ≥3 years) twice daily until age 5 in all children with HbSS or and HbSβ^0-thalassemia Discontinue prophylactic penicillin in children with HbSS or and HbSβ^0-thalassemia at age 5 unless they have had a splenectomy or invasive pneumococcal infection. When discontinuing penicillin prophylaxis at age 5, it is important to assure that the child has completed the recommended pneumococcal vaccination series, and if not, complete the series immediately Penicillin prophylaxis may not be necessary for children with HbSC disease and HbSβ$^+$-thalassemia unless they have had a splenectomy Assure that people of all ages with SCD have been vaccinated against *Streptococcus pneumoniae*, *Haemophilus* influenzae type b, and the meningococcus Remind people with SCD, their families, and caregivers to seek immediate medical attention whenever fever (temperature greater than 101.3°F or 38.5°C) occurs, due to the risk for severe bacterial infections
Renal disease	Screen all individuals with SCD, beginning by age 10, for proteinuria. If the result is negative, repeat screening annually. If the result is positive, perform a first morning void urine albumin-creatinine ratio and if abnormal, consult with or refer to a nephrologist
Heart disease	Consider annual echocardiography for adults with SCD Routine echocardiography screening is not recommended for children with SCD Routine ECG screening is not recommended in children and adults with SCD
Hypertension	In adults with SCD, screen for hypertension and treat to lower systolic blood pressure ≤140 and diastolic blood pressure ≤90 according to national guidelines In children with SCD, measure blood pressure, and evaluate and treat hypertension following recommendations from national guidelines
Retinopathy	Refer individuals with SCD to an ophthalmologist for a dilated eye examination to evaluate for retinopathy beginning at age 10 For people with a normal dilated retinal examination, re-screen at 1–2 year intervals Refer people with suspected retinopathy to a retinal specialist
Stroke	In children with HbSS and HbSβ^0-thalassemia, screen annually with TCD according to methods employed in the STOP studies, beginning at age 2 and continuing until at least age 16 In children with abnormal (non-imaging TAMMV >200 cm/s in dICA, bifurcation or MCA) TCD results, begin chronic transfusion therapy aimed at preventing stroke In children with genotypes other than SCA (e.g., HbSβ$^+$-thalassemia or HbSC), do not perform screening with TCD Consider screening MRI for detection of silent cerebral infarction (SCI) in children and adults with HbSS and HbSβ^0-thalassemia For children with SCI, offer chronic transfusion therapy for prevention of recurrent infarction

(continued)

Table 3.6 (continued)

Health topic	Recommendations
Lung disease	In children and adults with SCD, assess for signs and symptoms of respiratory problems (such as asthma, COPD, restrictive lung disease, or obstructive sleep apnea) by history and physical examination In children and adults with SCD found to have signs or symptoms of respiratory problems by history and/or physical examination, further assessment, which includes pulmonary function tests, is recommended to determine the cause and develop a plan to address the problem Do not screen asymptomatic children and adults with pulmonary function tests
Contraception	Progestin-only contraceptives (pills, injections, and implants), levonorgestrel IUDs, and barrier methods have no restrictions or concerns for use in women with SCD

[a]Adapted (modified and extended) from Evidence-Based Management of Sickle Cell Disease, Expert Panel Report 2014, National Heart, Lung and Blood Institute

performance, and increased risk for stroke [60, 61]. Students with SCD who have SCI and decreased cognitive function can be eligible for specific educational resources for which they would not otherwise be eligible. Similar resources are available for young adults who need vocational support or assistance in college. Therefore, the benefits of an MRI of the brain performed without anesthesia or sedation to identify SCI very likely outweigh the few risks of the procedure.

The recently completed Silent Cerebral Infarct Transfusion Trial (SIT Trial) [61] showed that chronic transfusions for children with Hb SS and SCI who are not at high risk of stroke by TCD criteria: (1) decrease the incidence of recurrent or progressive cerebral infarction; (2) decrease the frequency of pain, chest syndrome, AVN and priapism; and (3) improve quality of life. This comes at the cost of an increase in the likelihood of iron overload (ferritin >1500 ng/mL). As such, we believe it would be judicious to perform at least one screening MRI for SCI in young school-aged children and consider it for older individuals with SCD as well.

Immunizations

Immunizations are a vital component of standard medical care for individuals with SCD. Please refer to the section on bacterial infection earlier in this chapter.

Educational Attainment

As a group, children with SCD face educational difficulties from a combination of factors, such as chronic anemia, cerebral infarction, school absenteeism from recurrent illnesses, and hospital visits, limited parenteral education, and low

socio-economic status has been associated with poor academic performance [62, 63]. This highlights the importance of psychosocial, school and occupational intervention in SCD patients as a key component of standard care.

Management of Specific Complications

Acute Painful Episodes

The acute painful episode of SCD, often called vaso-occlusive crisis (VOC), is thought to be the consequence of ischemia and infarction of bone, bone marrow, and other tissues. This can manifest in infants (until about 3 years of age) as dactylitis, which is vaso-occlusive ischemia and infarction of phalanges and metacarpals that produces painful swelling. In toddlers and older children, acute SCD pain can involve extremities, back or chest wall. Pain can be local to one part of the limb, regional (involving multiple contiguous sites), or abdominal. Physical signs (edema, induration, erythema) are only present in about 15% of episodes. Triggers of pain are often not obvious, but known triggers include infection, inflammation, cooling of the skin, and psychological stress.

The management of painful episodes includes analgesia with a combination NSAIDs and opiates that is individualized to the patient and degree of pain. Non-pharmacological adjuncts can also be used (warm compresses, relaxation, massage). It is important to avoid over- and under-treatment of pain. The goal of fluid therapy is to correct dehydration, maintain normal hydration, and avoid over-hydration. Incentive spirometry is needed to prevent acute chest syndrome (ACS), and clinicians need to remain vigilant for the development of fever and ACS. Supportive care also includes laxatives for opiate-related constipation and anti-pruritics for opiate-related pruritus. Acute transfusion is not indicated for uncomplicated pain.

There is ongoing research into drugs that alter cell adhesion, blood viscosity, inflammation, and platelet activity that may prove useful in the treatment or prevention of acute painful episodes.

Acute Chest Syndrome (ACS)

ACS is a non-specific term for an acute pulmonary illness in an individual with SCD. It is defined clinically by a new, radiographic pulmonary infiltrate in conjunction with fever and respiratory signs and symptoms, such as cough, tachypnea, dyspnea, increased work of breathing, hypoxia/desaturation, and chest pain. ACS is not apparent in 30–60% of patients at the time of hospitalization for pain or fever. Incentive spirometry, therefore, is an important adjunct for patients with thoracic or abdominal pain that has been shown to prevent the development of ACS [64].

Common or known antecedents or triggers of ACS include infection (viruses, Mycoplasma, Chlamydia, S. pneumoniae), pulmonary vascular occlusion, hypoventilation/atelectasis, pulmonary edema, bronchospasm, and surgery or general anesthesia. Pre-operative simple transfusion has been shown to prevent post-operative ACS. Prior ACS, younger age, and asthma are known risk factors for the development of ACS in general [48, 65].

Management of ACS is primarily supportive, including empiric antibiotic therapy with a cephalosporin (e.g., ceftriaxone) and a macrolide (e.g., azithromycin). Transfusion is often not needed for young children with mild disease. Simple transfusion can be given for hypoxemia or acute exacerbation of chronic anemia. Exchange transfusion is indicated for severe disease, such as rapid clinical deterioration, widespread pulmonary infiltrates, and hypoxemia despite supplemental oxygen. Other principles of supportive care include correction of hypoxemia, maintenance of normal hydration, adequate analgesia, bronchodilators for wheezing, and support of ventilation for severe disease.

Overt Stroke

TCD screening programs and chronic transfusion therapy for primary prevention of stroke is discussed earlier in this chapter. Without primary prevention, unselected children with Hb SS have a 10% risk of overt stroke by 18 years of age. Although overt stroke is now much less common in developed countries, it can still occur despite the availability of screening programs. Acute stroke should be suspected in the event of focal neurologic deficits, weakness, asymmetric face, changes in speech, or altered mental state. Principles of management for acute stroke include rapid triage, supplemental oxygen, and avoidance of delays to transfusion. Erythrocytapheresis appears to be associated with better long-term outcomes than simple transfusion [66]. Simple transfusion can be given if the Hb concentration is <9 g/dL as a temporizing measure for any delay in erythrocytapheresis. If the diagnosis of stroke is strongly suspected by history and examination, transfusion therapy should not be delayed by imaging. The goal of exchange transfusion should be an end-procedure hematocrit of 28–30%, and Hb S percentage <30%, and a fraction of cells remaining (FCR) of <0.3.

The risk of recurrent stroke is high (50–90%), and chronic transfusions are the mainstay of secondary stroke prevention, reducing the rate to 20% [67]. Because chronic transfusions are burdensome, need to be given indefinitely, and carry the added risk of iron overload, investigation of alternative therapies is ongoing. Stem cell transplantation is indicated in this setting (discussed elsewhere in this book). Hydroxyurea has also been studied for secondary stroke prevention in the SWiTCH Trial. This showed that chronic transfusions and chelation is better than hydroxyurea and phlebotomy for the prevention of recurrent stroke and resolution of iron overload.

Acute Anemic Events

Patients with SCD have a chronic, partially compensated hemolytic anemia. Patients may also experience acute exacerbations of this chronic anemia, referred to as acute anemic events (AAEs). AAEs commonly occur as a consequence of parvovirus B19 infection, acute splenic sequestration, or a "hyperhemolytic" event accompanying other complications of the disease. It is now clear that the degree of chronic anemia and the frequency of AAEs are important risk factors, in particular, for silent cerebral ischemia and infarction [60, 68–70]. Given the association of AAEs with cerebral ischemia, transfusion to correct at least the acute component of anemia might be neuroprotective during AAEs, even in the absence of "symptomatic anemia" based on cardiopulmonary signs and symptoms.

Priapism

Priapism occurs in up to 75% of males with SCD by 20 years of age. It is defined as painful, purposeless and persistent erection of penis [71]. Patients can have brief, intermittent ("stuttering") episodes of priapism or prolonged, severe episodes (usually defined as lasting >4 h). Priapism is recurrent in about 90% of patients, especially in patients with the Hb SS genotype [72]. Home management includes advice to patients to initiate oral hydration, warm compresses or showers, exercise, voiding, oral analgesics and pseudoephedrine. Patients should be instructed to seek urgent care for any priapism lasting >4 h. Principles of care for prolonged priapism include IV analgesia, IV hydration, oral pseudoephedrine (if not recently given), and Urologic consultation for aspiration and irrigation. Acute transfusion not shown to be helpful, and there are old reports that exchange transfusion may be harmful [73]. Surgical management with shunts has shown to have less favorable outcome with erectile dysfunction and should be avoided if possible [71]. Erectile dysfunction and subsequent sexual, reproductive issues are troublesome adverse effects of prolonged priapism [74]. Prevention of recurrent priapism can be attempted by optimizing disease-modifying therapies (hydroxyurea, chronic transfusions). There are also a few reports of successful prevention with leuprolide or bicalutamide.

Disease-modifying Therapies

Hydroxyurea

Hydroxyurea is a ribonucleotide reductase inhibitor that induces Hb F synthesis. Higher concentrations of Hb F in RBCs are beneficial because further polymerization of Hb S fibers is prevented when Hb F is incorporated into a growing polymer. This is the primary beneficial mechanism of hydroxyurea in SCD. Secondary mechanisms of action include improved blood rheology, reduced adhesive interactions or

RBCs with other cells, increased total hemoglobin concentration, and decreases in platelet, reticulocyte and neutrophil counts. This is an FDA approved medication for adults with SCD. There are abundant data supporting its benefit in SCD in reducing the frequency of painful episodes, hospitalizations, ACS, and transfusions, all of which likely prevents some chronic organ damage and decreases mortality [75–77].

Current trends in practice include the early and pre-symptomatic prescription of hydroxyurea to young children, based on the results of BABY HUG trial and other investigations. The current NHLBI recommendation is to offer hydroxyurea to children with Hb SS beginning at 9 months of age and older irrespective of clinical status [59, 76]. The side effect profile of hydroxyurea is quite favorable, mainly including mild, dose-related neutropenia and thrombocytopenia [76, 78]. There seems to be no increase in malignancy in children or adults taking hydroxyurea, even for extended periods of therapy. Likewise, there is no evidence of impaired growth or development in children treated with hydroxyurea. Long term monitoring studies are on-going [56, 76]. Table 3.7 reviews dosing and monitoring guidelines for hydroxyurea.

Chronic Transfusions

Regularly scheduled transfusions of Hb S-negative PRBCs to maintain the percentage of Hb S in the blood <30% can effectively prevent most complications of SCD and minimize the chronic anemia. Common indications for transfusions are listed in Table 3.8. "Top-up" or simple transfusions are usually given in acute anemic states when the hemoglobin is 2–3 g/dL or more below baseline hemoglobin. In patients with SCD who require general anesthesia for surgery, a pre-operative simple transfusion to raise the total hemoglobin concentration to 10 g/dL can decrease the frequency of post-operative complications such as ACS and VOC [79]. Routine transfusion for minor elective procedures (e.g., myringotomy, circumcision) is not required, especially in those whose HbS level is below 30% with chronic transfusion therapy [80].

Chronic indefinite transfusion therapy is currently the treatment of choice for prevention of secondary stroke [81] and in the prevention of recurrent infarction in children with silent cerebral infarction [61]. Once initiated, it should be performed every 3–4 weeks to maintain pre-transfusion (maximum) Hb S of <30%, which usually achieves a nadir hemoglobin concentration of 9–10 g/dL. Complications of transfusions include febrile and allergic transfusion reactions, hemolytic transfusion reactions, transfusion-transmitted infections, alloimmunization, and iron overload. Detailed documentation of pre-transfusion hemoglobin levels, transfusion reactions, alloimmunization, and volume of all PRBCs received is necessary for high quality care. Iron chelation therapy is reviewed in the section above on thalassemia and in Table 3.4. Transfusion using phenotype-matched (ABO, C, c, D, E, e, and K-matched) PRBCs has the potential to prevent over 50% of alloantibodies in SCD patients [82].

Table 3.7 Hydroxyurea dosing and monitoring

Phase of therapy	Dosing regimen	Monitoring
Initiation	20–25 mg/kg/day in one daily dose	Baseline: CBC, differential, reticulocyte count, Hb F, creatinine, LFTs Monthly: CBC, differential, reticulocyte count Monitor for toxicity[a]
Dose escalation	Increase dose by 3–5 mg/kg/day every 4–6 weeks to maximum tolerated dose (MTD) MTD is achieved when the absolute neutrophil count is between 1.5 and 3.0×10^9/L	Monthly: CBC, differential, reticulocyte count Monitor for toxicity[a]
Maintenance	Continue MTD dose	Every 3 months: CBC, differential, reticulocyte count, Hb F Every 6 months: creatinine, LFTs Monitor for toxicity[a]

[a]Common criteria for toxicity include: ANC <1.0×10^9/L; platelet count <80×10^9/L; absolute reticulocyte count <80×10^9/L if hemoglobin <9.0 g/dL; Hemoglobin <5 g/dL or >20% below baseline; serum creatinine >1.0 mg/dL; ALT that is double the patient's baseline or three times the ULN for ALT. If toxicity occurs, consider stopping hydroxyurea for 4–7 days or until toxicity resolves. Hydroxyurea can then be resumed at the same dose or a dose decreased by 2.5–5 mg/kg/day

Table 3.8 Common indications for transfusion in SCD

Episodic transfusions for acute management	Chronic transfusions for long-term prevention
Aplastic crisis	Stroke (primary and secondary prevention)
Acute splenic sequestration	Frequent painful episodes
Acute chest syndrome	Frequent or severe ACS
Acute stroke (exchange transfusion)	Other severe or chronic complications
Pre-operative preparation (prevention of ACS)	

Abbreviation: *ACS* acute chest syndrome

Cure

Hematopoietic stem cell transplantation and gene therapy are discussed in other chapters in this book.

Long-Term Outcomes

With contemporary management in developed nations, almost all children (>95%) with SCD will survive to adulthood (Fig. 3.2) [83, 84]. This excellent overall survival is the result of a many incremental advancements, including newborn screening, prophylactic penicillin, protein-conjugate vaccines, advances in supportive

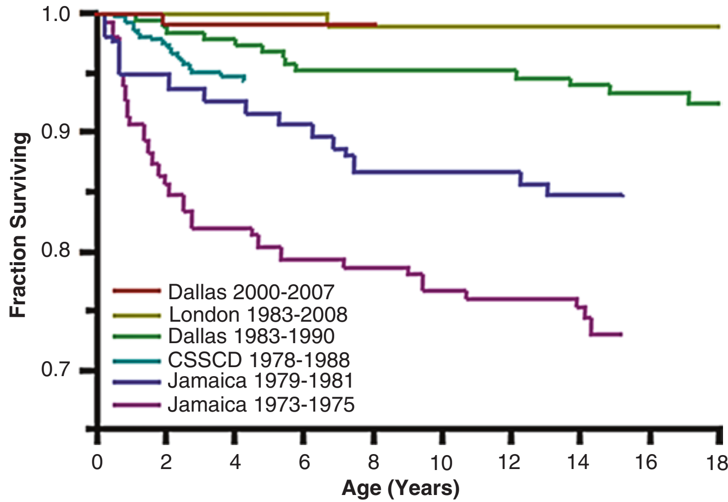

Fig. 3.2 Improving survival in sickle cell disease (SCD). Overall survival curves for patients with sickle cell anemia (Hb SS) and sickle-β^0-thalassemia spanning by years of birth are shown for large SCD cohorts in the US, UK and Jamaica. Figure adapted from: Quinn et al. Blood. 2010;115:3447-3452

care, and the increased use of disease-modifying treatments (hydroxyurea, chronic transfusions, and stem cell transplantation). The burden of mortality in SCD has now shifted to adults, and the transition to adult medical care is a high-risk period for death. There are a number of possible explanations for this vulnerability, including a flawed interface between pediatric and adult medical care and the gradual accumulation of SCD-related chronic organ injury during childhood that becomes manifest in young adulthood. Long-term survival estimates (beyond childhood) are less accurately known, but median survival is estimated to be over 50 years for individuals with Hb SS and Hb Sβ^0. The survival of individuals with Hb SC and Hb Sβ^+ approach that of the general population. Especially for those with Hb SS and Hb Sβ^0, advances in gene therapy and hematopoietic transplantation hold the potential for even greater improvements in survival and quality of life.

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Chapter 4
Allogeneic/Matched Related Transplantation for β-Thalassemia and Sickle Cell Anemia

Françoise Bernaudin, Corinne Pondarré, Claire Galambrun, and Isabelle Thuret

Abstract Allogeneic hematopoietic stem cell transplantation (HSCT) can cure single gene disorders such as thalassemia and sickle cell anemia (SCA). These non-malignant diseases have in common severe hemolytic anemia and high proliferative bone marrow, requiring frequent transfusions. The risk of rejection is high and graft-vs-host disease is not desirable. Important progress has been made in the management of these diseases, including leukocyte depletion of blood products, and chelation therapy, for both diseases, and erythrocytapheresis and hydroxycarbamide for SCA. However, morbidity and quality of life are still of concern. Results have also significantly improved for HSCT, with the reduction of rejection by using anti-thymocyte globulin (ATG), which also decreases the risk of chronic graft-vs-host disease. Current data show a more than 90% chance of cure with myeloablative conditioning in children with hemoglobinopathy and a geno-identical donor. Results are similar whether the cell source is cord blood or bone marrow. Because of the risk of conditioning-related infertility, ovarian and/or testis cryopreservation should be discussed. Non-myeloablative conditioning regimens have also been successfully developed in adults with SCA and organ dysfunction, making cure possible. These encouraging results should incite to perform HLA typing early in families with hemoglobinopathies, and to systematically propose sibling cord blood cryopreservation for those without geno-identical donor.

Keywords Thalassemia • Sickle cell anemia • Allogeneic geno-identical hematopoietic stem cell transplantation • Chimerism • Fertility • Cerebral vasculopathy • Strokes • Pre-implantation diagnosis

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Introduction

Thalassemia and sickle cell anemia (SCA) are the two most widespread hereditary hemoglobinopathies in the world. Worldwide, about 60,000 children with major thalassemia and 250,000 with SCA are born annually, giving a rate of more than 2.4 affected children per 1000 births [1]. The origins of thalassemia were traced to the Mediterranean, Middle Eastern, and Asian countries, while SCA originated throughout African countries; soon, both became globally spread because of imposed and spontaneous migration and represent a growing health problem in many countries, particularly SCA.

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative treatment for β-thalassemia [2–17] and SCA [18–28]. However, its use has been limited in developed countries by the risks of transplant-related mortality (TRM), graft-vs-host disease (GVHD), infertility and the low percentage of patients with geno-identical donor, whereas in countries with low gross domestic product, the principal limitations have been the cost and the lack of transplantation centers.

Both these diseases have the particularity of having proliferative bone marrow (especially β-thalassemia), a history of frequent transfusions with the risk of alloimmunization, and a lack of requirement for a GVHD effect. All these factors have been shown to increase the risk of rejection; nevertheless, progress has been observed with time. Geno-identical HSCT with myeloablative conditioning regimen (CR) now offers an at least 90% chance of cure. Results obtained following cord blood and bone marrow transplants are similar [25, 29–31]. Sibling cord blood cryopreservation [25, 29–32] and preimplantation diagnosis with double selection (HLA-disease) can now be offered [33, 34] to increase the chance of having a geno-identical donor. Fertility preservation is also possible using ovarian [36, 37] and testis [37, 38] cryopreservation. In order to reduce TRM and propose HSCT to adults with organ dysfunctioning, non-myeloablative CR have been successfully developed [39–50] and cure is now also possible in adults. Whereas there is a consensus to transplant all thalassemia patients with a geno-identical donor, the unpredictability of the SCA course has limited this treatment to patients with severe disease. However, based on current data (95% chance of cure), we believe it is time to extend the indications in SCA to improve the patients' quality of life and allow better socio-professional integration. Nevertheless, as conventional supportive therapies for hemoglobinopathies are constantly improving, the development of well-designed clinical studies comparing outcomes between HSCT and conventional treatments will also be necessary.

Thalassemia

Beta-thalassemia major (beta-TM) is a genetic disorder resulting in the absence of β-globin chain synthesis with accumulation of alpha-chains in the erythroid precursors, causing severe apoptosis, ineffective erythropoiesis with massive erythroid hyperplasia in the bone marrow and extramedullar sites, and hemolytic anemia

requiring chronic transfusions. Without transfusions, thalassemic patients experience tremendous skeleton deformities, as well as hepatomegaly and splenomegaly due to the expansion of the hematopoietic system with extramedullar hematopoiesis [51]. Both ineffective erythropoiesis and chronic transfusion therapy inevitably lead to iron overload, resulting in progressive multiple organ damage that causes endocrine deficiencies, liver disease, and cardiac disease, leading to poor quality of life and increased mortality [52]. Regular blood transfusion and iron chelation have significantly improved both survival and quality of life in patients with thalassemia and have changed this previously fatal disease with early death to a chronic disease, which although progressive is nevertheless compatible with prolonged survival [53–57]. Despite prolonged life expectancy, a recent study from the U.K. Thalassemia Registry showed that a steady decline in survival occurs from the second decade, with fewer than 50% of patients still alive beyond 35 years, largely because of poor compliance with chelation therapy [58]. In the developing world where thalassemia is more common, most children barely reach adulthood and die before the age of 20 years because of the lack of safe blood products and/or expensive iron chelating drugs. In recent years, two oral iron-chelators (deferiprone and deferasirox) were developed that could allow better compliance with chelation, and therefore could have a favorable impact on thalassemia patients' survival, although their long-term efficacy compared to desferrioxamine (desferal) has not yet been determined. However, even an ideal iron chelator and rigorous adherence can only substantially reduce, but not eliminate iron overload in patients on lifelong transfusions. Despite the recent promising results of gene therapy for thalassemia [59, 60], in clinical practice, HSCT remains the sole curative option.

The rationale for HSCT in thalassemia resides in substituting the thalassemic HSC bearing ineffective erythropoiesis with allogeneic HSC capable of effective erythropoiesis. This cellular replacement therapy is not limited to the diseased erythropoietic compartment, but leads to the replacement of the entire hematopoietic system. Nevertheless, it is an efficient way to obtain a long-lasting, probably permanent, clinically effective correction of the hemolytic anemia, thereby avoiding transfusion requirements and iron overload [61].

History of HSCT in Thalassemia

Children

Since the first reports on transplantation [2, 3] and after 30 years of experience, HSCT for thalassemia is now accepted as standard clinical practice. In the 1980s and early 1990s more than 1000 beta-TM patients in Pesaro, Italy, were transplanted with an HLA-identical sibling donor, and the 20-year thalassemia-free survival rate was 73% [6]. Analysis of the influence of pre-transplant characteristics on the outcome of transplantation was conducted in 161 patients, aged less than 17 years, who were all treated with the exact same regimen [62]. In multivariate analysis, hepatomegaly more than 2 cm, portal fibrosis and irregular chelation history were associated with a

significantly reduced probability of survival. The quality of chelation was characterized as regular when deferoxamine therapy was initiated no later than 18 months after the first transfusion and administered subcutaneously for 8–10 h continuously for at least 5 days per week. Any deviation from this regimen was defined as irregular chelation. On the basis of these risk factors, patients were categorized into three risk classes. Class 1 patients had none of these adverse risk factors, class 3 patients had all three and class 2 patients had one or two adverse risk factors. However, this stratification is not readily applicable in settings where liver biopsy analysis is not performed routinely. Moreover, this scheme was developed largely in patients with regular access to RBC transfusions, and might not apply to chronically under-transfused children in whom hepatomegaly may not necessarily reflect severe iron overload and can potentially be partially corrected with an appropriate transfusion program. More recently, alternative-risk group assignments that were independent of liver biopsy have been proposed. One such risk stratification scheme, based primarily on age and liver size, reported cure rates exceeding 70% for matched sibling donor (MSD) HSCT in children aged <7 years, and with liver palpation at least 5 cm below the costal margin, regardless of chelation history or liver fibrosis [63].

Current results (Table 4.1) of transplantation with matched related donors in patients aged less than 17 years show 80 –87% probability of cure according to risk classes [52, 64, 65]. However, recent reports using 10–12.5 mg/kg rabbit antithymocyte globulin (ATG) [9, 17] demonstrated 92–94% chance of cure, even in patients transplanted with related phenoidentical or one mismatched donor [17] following a preparation with Protocol 26 [66] (detailed in the footnote of Table 4.1). Therefore, these results should encourage considering HSCT for young thalassemic patients with an available HLA-identical sibling as soon as possible, before development of iron overload and iron-related tissue damage.

Adults

Adult thalassemia patients have more advanced disease, mainly due to prolonged exposure to iron overload. From 1988 through 1996, the probabilities of TRM and thalassemia-free survival in the 107 adult patients transplanted with a sibling matched donor were 34% and 62%. After 1997, 15 adults were transplanted following Protocol 26 (see Table 4.1) and reduced dose of cyclophosphamide (CY; 90 mg/kg), and the probabilities of TRM were still 35% (7% rejection mortality and 28% non-rejection mortality) with a thalassemia-free survival of 65% [52]. Thus, new protocols are needed to improve TRM and survival in adult thalassemia patients.

Conditioning Regimen

Thalassemia patients are not subjected to chemotherapy and their immunologic system is not impaired. Moreover, they have a hypercellular and expanded marrow compartment, and sensitization to red blood cells antigens and possibly anti-HLA

Table 4.1 Matched/related HSCT for thalassemia

Reference			Patients					
BMT/CBT	Period	Conditioning	Category	n	OS	TRM	Rejection	DFS
(a) HSCT teams results								
Lucarelli Pesaro/ Roma (2008) [3, 11, 51, 52, 62, 64–66]	1985–2007	BU 14, CY 200	Class 1 and 2	515 <17 year	88%	12%	4%	85%
In Pesaro	Since 2003 <4 years	With Thiotepa						
		BU 14, CY 200	Class 3		61%	39%	7%	53%
		BU 14, CY 120/160	class 3 <17 years		79%	21%	30%	49%
	1988–1996		Adult 17–35 years	107	66%	37%	4%	62%
		BU 14, CY 200	Class 2	18				
		BU 14/16, CY 120/160	Class 3	89				
[66]	1997–2007	Protocol 26	Class 3 < 17 years	73	87%	12%	7%	82%
	1997–	Protocol 26 but CY 90	Adults	15	65%	28%	7%	65%
In Roma	2004–2007	BU 14, CY 200 ± thiotepa	Class 1–2 <17 years	37	97%	3%	8%	89%
		Protocol 26	Class 3	35	87%	10%	12%	80%
Ghavamzadeh Iran (1998) [13]	1991–1997	BU 14, CY 200	<16 years	70	83%	17%	11%	73%
Di Bartolomeo Pescara (2008) [14]	1983–2006	BU-CY	1–28 years	115	89%	9%	7%	86%
Chandy, India (2001) [15]	1986–1999	BU 16, CY 200	Class 1	7				66%
			Class 2	33				90%
			Class 3	72				55%

(continued)

Table 4.1 (continued)

Reference			Patients					
BMT/CBT	Period	Conditioning	Category	n	OS	TRM	Rejection	DFS
Lawson, UK (2003) [5]	1991–2001			55	94%	5%	13%	82%
Galambrun, France (2013) [10]	1985–2007	BU 16 CY 200 ± rATG		108	87%	13%	23%	69%
		No ATG		49	90%	10%	35%	55%
		With rabbit ATG (Genzyme)		57	93%	7%	10%	83%
Goussetis, Greek (2012) [9]	1995–2009	BU 14–16 mg/kg, CY 150–200 mg/kg with rabbit ATG 12.5 mg/kg	<18 years Class 1–3	75	96%	4%	4%	92%
Gaziev, Roma (2013) [17]	2005–2012	BU, CY 200, thiotepa with rabbit ATG (10–12.5 mg/kg)	Pheno = or 1 mismatch related	16	94%	6%	0%	94%
Registries								
(b) HSCT registries results								
Sabloff, CIBMTR (2011) [8]	1995–2001	BU 16, CY 200 ± ATG	<20 years	179				
			Class 2	75	91%	9%	3%	88%
			Class 3	64	64%	36%	2%	62%
			<7 years and no hepatomegaly	49	98%	2%	4%	94%
			>7 years and no hepatomegaly	29	86%	14%	3%	83%
			<7 years and hepatomegaly	37	78%	22%	2%	76%

			>7 years and hepatomegaly	46	55%	45%	2%	53%
Baronciani, EBMT (2011) [16]	2000–	Not indicated	0.3–45 years	1493	91%	9%	8%	83%
			<2 years	96				90%
			2–14 years	1108				82%
			>14 years	287		25%		72%

Protocol 26 consisted in hypertransfusion to maintain Hb at >14 g/dL, continuous Deferoxamine (40 mg/kg/day), intensified preparation with 3 mg/kg/day azathioprine and 30 mg/kg/day hydroxyurea from day-45 from the transplant, fludarabine (20 mg/m^2) from day-17 through day-13, followed by administration of BU (14 mg/kg) and CY (160 mg/kg total dose) [49]

antibodies may have occurred, at least when undergoing HSCT as adolescent or adult. Under these circumstances, the ideal conditioning regimen should be able of eradicating the diseased marrow and be sufficiently immunosuppressive to allow donor hematopoietic cells to survive and permit a sustained engraftment.

From the early 1980s and for many years, the preparatory regimen for thalassemia patients in Pesaro included oral busulfan (BU) at 14 mg/kg and CY at 120–200 mg/kg. Since 1997, Protocol 26 applied in Pesaro and Roma for high risk patients (class 3), using a preparation with azathioprine, hydroxyurea and fludarabine allowed to significantly improve disease-free survival (DFS) (Table 4.1) [66]. New preparative regimens have been introduced by several teams, such as using intravenous BU or treosulfan associated with thiotepa and fludarabine, with improved results [67, 68]. Others have added rabbit ATG, which allowed to significantly decrease the rejection risk and to improve DFS [9, 10], especially in those patients transplanted with an unrelated donor or an HLA-partially matched relative [17].

As low levels of donor cells are sufficient to cure thalassemia patients with stable mixed chimerism, there was a rationale for using non-myeloablative conditioning regimens. However, the first transplantations were unsuccessful [43] as all patients experienced graft loss. Thereafter, several teams tested the use of reduced intensity conditioning (RIC) regimens in very limited clinical trials [70–72]. In practice, high dose BU was necessary for successful stable engraftment [71], but CY was successfully replaced by Fludarabin and ATG. To date, no series has reported successful and stable engraftment with non-myeloablative CR in thalassemia.

GVHD Prophylaxis

The first transplantations used long-term methotrexate (MTX) (until day 100) and methylprednisolone. Cyclosporine (CSA) was introduced in 1986 and the incidence of acute graft-vs-host (AGVH) >2 was significantly lower with the association CSA + short course MTX (4 doses i.v. on day +1, +3, +6 and +11 post-transplantation) (17%) than with CSA alone (32%) [73]. However, as the use of MTX for GVHD prophylaxis after cord blood transplant (CBT) was associated with a greater risk of rejection, CSA alone is recommended for CBT [29–31].

Stem Cell Source

Bone marrow has almost always been used for transplantation of thalassemia patients, but several groups started using peripheral blood stem cells (PBSC) to reduce the risk of rejection [74–76], especially in high-risk patients. However, all these studies showed an increased risk of chronic GVHD, not suitable in thalassemia. The feasibility of using HLA-identical sibling cord blood for HSCT in hemoglobinopathies was reported [29, 30], and was associated with a decreased risk of both acute and chronic GVHD and of TRM. A recent report [30] compared the

outcome following CBT and BMT in hemoglobinopathies performed between 1994 and 2005. The 6 year-DFS was 86% and 80% following BMT ($n = 259$) and CBT ($n = 66$), respectively, and was not statistically different. However, the cumulative incidence of AGVH > 2 was significantly lower after CBT (10 vs. 21%, $p = 0.04$) whereas the cumulative incidence of chronic GVHD was lower (5 vs. 12%), but not significantly. For unrelated or related CBT, a high dose of nucleated cells (NCs) (i.e., $>3.5 \times 10^7$/kg) was recommended [77]; however, a recent report demonstrated that the cell dose was not a significant risk factor for graft failure in sibling matched CBT, with most patients receiving a high number of NCs (median 3.9×10^7/kg) [30]. Nevertheless, considering the high risk of rejection, a NCs dose $> 3.5 \times 10^7$/kg is recommended for matched related CBT in thalassemia.

Spleen and HSCT

Many children with thalassemia who do not receive optimal care and regular transfusions will have hepatosplenomegaly. Enlarged spleen per se is not associated with higher rejection rates but may increase transfusion requirements and delay engraftment [78], but whether this is an independent risk factor is not clear [63]. In contrast, the French experience [10] reports a better DFS in splenectomised patients. A possible explanation for this discrepancy may be that splenectomy reflects better medical care for the disease before HSCT rather than advanced stage of the disease. Although the benefit of pre-transplantation splenectomy remains unclear, the increased risk of infection associated with this procedure argues against its use [10].

Chimerism

Graft rejection probabilities have been shown to be higher in thalassemia patients with mixed chimerism [79]. Among the patients with early mixed chimerism within the first 2 months after HSCT, 34.7% rejected the graft [80]. However, early mixed chimerism is frequent following CBT, and does not predict rejection [81]. About 10% of patients transplanted for thalassemia develop long-term, stable mixed chimerism after transplantation, consistent with a state of reciprocal tolerance between donor and recipient cells [80, 82–84]. In patients with persistent mixed chimerism, type 1 regulatory cell clones of both donor and host origin were able to inhibit the function of effector T cells of either donor or host origin in vitro [85, 86]. Mixed chimera patients, despite limited engraftment (even no higher than 20%), achieve a functioning graft status characterized by normal hemoglobin level, no red blood cell transfusion requirement, no iron increment, and a limited degree of erythroid hyperplasia that is not of clinical relevance [80, 82–84]. These results suggest that in patients with persistent mixed chimerism after HSCT, a selective advantage of the donor erythroid precursor maturation might successfully contrast the problems related to the recipient ineffective erythropoiesis [84].

Donor Lymphocyte Infusions

In patients with early mixed chimerism, several groups have tried to obtain full donor chimerism using donor lymphocyte infusions (DLI) to prevent graft rejection [87–89]. Risks associated with DLI are cytopenia and GVHD occurrence. In the first published series [88], 7 patients with level 2 ($n = 1$) or level 3 mixed chimerism ($n = 6$) were treated with escalating doses of DLI (1×10^7 and 5×10^7 CD3+/kg at least at 5 weeks interval) at a median time of 167 days (range 90–267 days). Five of the 7 patients experienced transitory cytopenia, but no GVHD, and only 2 Pesaro class II patients still receiving tacrolimus reversed to full donor chimerism. The second series [89] included 19 patients receiving escalating DLI (1.5×10^7–1×10^8 CD3+/kg): 3 patients (16%) evolved to full donor chimerism, 9 (47%) had mixed chimerism with transfusion independency, and 7 (37%) rejected the graft. In this series, contrary to the first one, 3 patients experienced GVHD, which was responsible for one death. The authors suggest that DLI be considered for patients still transfusion-dependent and with less than 75% donor cells at day 30 and in those with 20% decrease in the percentage of donor cells and concomitant decrease in hemoglobin levels between 2 control tests. Nevertheless, more large series are required to give proper recommendations and define the best strategies for tolerance induction and maintenance.

ABO Incompatibility

Blood group incompatibility does not appear to affect the overall outcome in patients undergoing HSCT following myeloablative conditioning with total donor chimerism. However, with non-myeloablative conditioning, and in case of mixed RBC chimerism, ABO mismatch can be responsible for graft rejection, pure red blood cell aplasia and immune hemolysis [90–92]. In a series of transplants for malignant and non-malignant diseases, among the 44 patients with ABO mismatch, 6 (13%) developed pure red cell aplasia [92], which responded to anti-CD20 monoclonal antibody [93]. Other RBC antigen systems, in most cases those of the Rh system, have less frequently been implicated in the development of post-transplant autoimmune hemolytic anemia [94], nevertheless justifying to assess the complete donor and recipient erythroid phenotype before transplant.

Growth and Endocrinal Function

Growth and pubertal anomalies are the most common endocrinopathies in homozygous thalassemia, accounting for significant morbidity in 70–80% children and adolescents globally [95]. In the 1920s–1940s, the cause of early growth failure was the result of severe anemia and ineffective erythropoiesis, and children had multiple endocrinopathies affecting thyroid, parathyroid, and pancreas in early childhood. In the 1960s, adequate transfusion corrected the hypoxia, improving growth during the

first 10 years of life, but children did not attain growth velocity and secondary growth failure was due to iron overload. Adrenal failure, diabetes and compensated primary hypothyroidism were also documented. Effective iron chelation with IM deferoxamine became available in the 1970s, but aggressive chelation was shown to be equally deleterious for growth if started very early at 2–3 years of age, inducing toxicity on body proportion, composition and complex bony lesions with retarded growth. This results in platyspondylisis with flattening of vertebral bodies and spinal height and truncal shortening. In the late 1980s and 1990s, growth and pubertal failure became more frequent with improved prognosis and better survival. Despite significant advances in chelation and transfusion therapy, growth retardation with pubertal aberrations continues to be a significant challenge in thalassemia patients, due mainly to hypogonadotropic hypogonadism (HH) with its consequences on growth and puberty. Hypogonadism is frequent and can be related to iron overload as even a modest amount of iron deposition within the anterior pituitary can interfere with its function, but also to liver disorder, diabetes mellitus and zinc deficiency [96]. Despite a somewhat reduced sensitivity to growth hormone (GH), thalassemia patients may benefit from GH treatment.

Following transplant, most thalassemia patients experience improved height, but 68% still have gonadal dysfunction [96]. Among 25 patients transplanted for thalassemia at an age where the pubertal process should have started, all 12 males patients had normal pubertal development with normal testosterone and, except for one, normal gonadotrophin level, whereas 100% of the post-menarcheal females exhibited amenorrhea and elevated gonadotropin levels [97]. To preserve fertility, ovarian cryopreservation is recommended before transplant as successful pregnancies and births have been obtained after ovarian cortical tissue reimplantation in patients transplanted for stage IV Hodgkin's lymphoma [35], sickle cell anemia [98] and thalassemia [99].

Costs and Cost-effectiveness

The care of thalassemia, both by transplantation and medical therapy, has dramatically improved during recent decades. However, this improvement has been limited to patients living in industrialized countries, while the large majority of patients are born and live outside these countries [100]. Thalassemia care is a complex, multidisciplinary and expensive endeavor, requiring dedicated and experienced centers of excellence. From a global health perspective, thalassemia represents an enormous burden of care in some countries. With the combined cost of blood transfusions, chelation and management of complications the requirements for optimal thalassemia care clearly exceed available health resources in most non-industrialized countries. Although transplantation is also a complex, multidisciplinary approach requiring expert centers, it appears that transplantation, particularly if performed in the early years of life, is more cost-effective, with a total cost approximately comparable to only 4–5 years of transfusion and medical therapy. In countries where thalassemia is most prevalent (>100,000 children born each year in the Middle East

and Southeast Asia), lack of supportive care standards together with mostly insufficient access to dedicated health care facilities results in the majority of affected children not reaching adulthood, further supporting the need for expanded access to HSCT for these patients. As the cost of HSCT is equivalent to that of a few years of supportive but non-curative care, HSCT is justified in low-risk young children with a compatible sibling, not only medically and ethically but also financially. International cooperation can play a major role in increasing access to safe and affordable HSCT in countries where there is a considerable shortage of transplantation centers [101]. For example, the Cure2Children Foundation (C2C), an Italian Non-Governmental Organization, has supported a HSCT network in Pakistan, which was extended to India in 2012 [102]. A total of 100 matched-related HSCT have been performed to date by partner institutions within this C2C-supported network; in the 50 low-risk cases with thalassemia, over 90% DFS was obtained with procedure expenses within 10,000 USD per HSCT, i.e., an outcome comparable to that obtained in affluent countries, but for a fraction of the cost. International cooperation strategies based on shared principles and a common vision may substantially facilitate access to HSCT for thalassemia patients.

Sickle Cell Anemia

Sickle cell anemia (SCA) is a severe recessive genetic disorder resulting from a single nucleotide substitution in codon 6 of the beta-globin gene, which, in the homozygous state, produces an abnormal hemoglobin (HbS) that is prone to polymer formation under deoxygenated conditions. The polymerized HbS leads to decreased red blood cell deformability and sickling within end arterioles, resulting in vaso-occlusion and pain. Despite being a monogenic disease, its clinical expressivity is multigenic. SCA is associated with high-risk complications, including strokes, acute chest syndrome and recurrent severe painful vaso-occlusive episodes with increased risk of early mortality and significant morbidity [103–107].

Since the first report of successful HSCT in a SCA-patient [108], about 1000 children and young adults worldwide have undergone HSCT [109], but only about 250 have been reported so far (Table 4.2) [19–28], a rather limited number of patients considering that hundred of thousands of individuals are affected with SCA worldwide.

Despite progress made in SCA management, such as neonatal screening [110–112], prevention of pneumococcal infections [113–116], introduction of hydroxyurea therapy [117, 118], and early cerebral vasculopathy detection with transcranial Doppler [119, 120], SCA remains a disease with high-risk of morbidity and early death. The probability of death before the age of 18 years was reported to be 6.4% in the Dallas (US) cohort [105] and 2.5% in the Créteil (France) newborn cohorts [107]. While SCA mortality rate has significantly decreased during the last 30 years [121], mortality during adulthood has surprisingly increased [122]. The Cooperative Study of Sickle Cell Disease (CSSCD), which enrolled 2542 SCA-patients (1313

Table 4.2 Myeloablative matched/related HSCT for SCA-patients

	Belgium	International, USA	France		Jackson, USA	Atlanta, USA	Italia
	Vermylen et al.	Walters et al.	Bernaudin et al.		Majumdar et al.	McPherson et al.	Lucarelli
	BMT (1998) [22]	NEJM (1996) [23], Blood (2000) [24]	Blood (2007) [25]	Unpublished	BMT (2010) [26]	BMT (2011) [27]	BMT (2012) [28]
Period	04/86–01/97	09/91–03/99	11/88–12/2004	2005–2010	1997–2005	1993–2007	
Patients (n)	50	50	87	102	10	27	11
Median age	7.5 years	9.4 years	8.8 years	8.6 years	10.1 years	8.6 years	12 years
Age (range)	0.9–23 years	3.3–14 years	2.2–22 years	3.2–27.5 years	2.8–16.3 years	3.3–17.4 years	2–16 years
Strokes n (%)	4 (8%)	14/26 (54%)	36 (41%)	11 (11%)	6 (60%)	13 (52%)	6 (54%)
Ferritin							
Mean (range) ng/ml	?	1542 (58–6795)	911 (13–3820)	986 (8–3834)	ND	1202 (68–3901)	ND
Conditioning regimen	BU-CY-ATG	BU-CY-ATG	BU-CY-ATG	BU-CY-ATG	BU-CY-ATG	BU-CY-ATG	
ATG source	Fresenius	Horse ATGAM	Rabbit ATG	Horse ATGAM	Horse ATGAM	?	
Dose	15–90 mg/kg	90 mg/kg	20 mg/kg	90 mg/kg	90 mg/kg	?	
Stem cell source							?
BM	48	50?	74	88	6	27?	
CB	2		11	12	1		
BM+CB	0		1	2	1		
PBC	0		1	0	2		
Follow-up							
Median (range)	5 (0.9–15 years)	3.2 (0.5–7.9 years)	6 (2–17.9 years)	4.3 (1.6–7.4 years)	5.5 (2.9–11 years)	4.9 (1–10 years)	

(continued)

Table 4.2 (continued)

	Belgium	International, USA	France		Jackson, USA	Atlanta, USA	Italia
Rejections	10%	10%	7%	1%	10%	0%	
TRM	7%	6%	6.9%	2%	10%	4%	10%
DFS	82%	84%	86.1%	97.1%	77%	96%	90%
aGVHD $\geq$ II	20%	15%	20%	25%	40%	12%	64%
cGVHD	20%	12%	13.5%	11%	40%		36%

females and 1229 males) between 1978 and 1988, reported that the median age at death was 42 years for males and 48 years for females [103]; in contrast, a more recent study (1979–2005) reported a mean age at death of 33 years for males and 37 years for females, with an increase in adult mortality rate of 1% each year ($p < 0.001$) during the time period studied whereas the pediatric rate decreased by 3% each year ($p < 0.001$) during the same period [122]. Increased risk of mortality during the transition period from pediatric to adult medical care was reported by several studies [123, 124]. This may reflect a lack of access to high-quality care for adults with SCA or lack of adult hematologists knowledgable about the disease, but more likely in our opinion to the greater number of severe SCA-patients reaching adulthood because of the progress made in the management of the disease during childhood.

Hydroxyurea (HU) therapy, used in SCA children since 1992, has allowed a significant reduction in the rate of vaso-occlusive crises, acute chest syndrome and transfusion requirements in many patients [117, 118, 125–127]. Several studies suggest that long-term use of hydroxyurea is safe and may decrease mortality [128–130], and that HU can be used safely in very young children [131]; nevertheless, its effectiveness on cerebral-vasculopathy has been debated. HU decreases cerebral arterial velocities [132], but does not always prevent the occurrence of abnormal velocities [107, 127]; and is less efficient than transfusion programs for secondary stroke prevention [133]. Moreover, HU does not prevent pulmonary hypertension, which is a major risk factor for early death in adults [134–136]. Unfortunately, this treatment is not curative and only ameliorates the consequences of SCA, leaving an increasing number of adults with accumulating end-organ damage.

Cerebral vasculopathy is the most important complication affecting SCA children, with a risk of stroke occurrence of 11% before the age of 18 years [105, 137]. Overt strokes are due to macrovasculopathy involving the large arteries of the Circle of Willis that can be assessed by MR angiography (MRA). However, the use of transcranial Doppler ultrasonography (TCD), which measures blood flow velocity in the large arteries of the Circle of Willis, has come to be more valuable in young children because it can identify patients at risk of stroke before MRA can detect the occurrence of stenosis [119, 120]. Children with mean maximum cerebral blood flow velocities of 200 cm/s or greater have a 40% risk of stroke within 36 months, which can be reduced by 90% via a transfusion program, as shown by the Stroke Prevention Trial in Sickle Cell Anaemia (STOP 1) (1995–2000) [138]. Moreover, the STOP 2 study showed that discontinuation of transfusion resulted in a high rate of strokes and reversion to abnormal velocities, even in patients with velocities that normalized after a 30-month transfusion program and with normal MRA [139]. However, it was shown in the Créteil cohort that a switch to HU could be successfully implemented in half of these patients with normalized velocities and no stenoses [107, 140]. A US randomized Trial (TWITCH) is ongoing comparing HU to transfusion programs in these patients. In the Créteil newborn cohort, the cumulative incidence of abnormal velocities was 30%, reaching a plateau at 8 years of age while transfusion program in patients with abnormal velocities allowed significant reduction of the stroke risk from 11 to 1.9% by age 18 [107].

In addition to overt strokes, microvasculopathy can result in silent infarcts, which are defined as the presence of ischemic lesions on MRI in the absence of clinical deficits [141–144]. Silent infarcts are associated with cognitive deficiency [141, 142] and with a higher risk of overt stroke or new silent infarcts [143]. In the Créteil newborn cohort early screened by TCD, the cumulative incidence of silent strokes was 37% by age 14 without a plateau effect. Taking into account both macro- and micro-vasculopathy, the probability of exhibiting cerebrovascular disease (abnormal MRA and/or MRI) was around 50% by the age of 18 [107]. Thus, despite progress in its management, SCA remains a very severe disease, justifying the use of more intensive therapies to preserve cognitive functioning and quality of life [25, 145].

Pulmonary hypertension (PH) is a relatively recently described SCA-related complication that is associated with mortality risk [134]. Characterized by the proliferation of medial smooth-muscle cells and endothelial cells in the small pulmonary arteries, it is defined as a mean pulmonary artery pressure (mPAP) >25 mmHg by right heart catheterization (RHC) [146]. Several groups reported a PH prevalence of 6–11% in adults [147, 148]. The use of RHC distinguishes between precapillary (pulmonary arterial hypertension) and postcapillary pulmonary hypertension, which is associated with left-sided heart disease. Patients with SCA and pre-capillary PH have a distinct hemodynamic profile, characterized by a less significant increase in mean pulmonary arterial pressure, a higher cardiac output and lower pulmonary vascular resistance than patients with idiopathic PH [149, 150], and a different response to specific PH therapies. A randomized, double-blinded, placebo-controlled study designed to evaluate the safety and efficacy of the phosphodiesterase-5 inhibitor, sildenafil, was prematurely halted after an interim analysis showed that sildenafil-treated patients were more likely to have acute sickle cell pain crises (35%) compared with placebo-treated patients (14%), and that there was no evidence of treatment-related improvement at the time of study termination [151]. However, a recent report [148, 152] shows that mortality in catheterized adults with SCA is proportional to PH severity assessed by gold-standard hemodynamic measurements of precapillary PH, implying that precapillary PH promotes early mortality in this patient population. Echocardiographic estimation of pulmonary artery pressure by tricuspid valve regurgitant jet velocity (TRJV) has been validated as a useful screening method for PH in adult patients with SCA [134]. A TRJV ≥ 2.5 m/s has been used as a surrogate marker of PH with a prevalence in SCA-adults of about 30% [147]. However, the positive predictive value of this cut-off (TRJV ≥ 2.5 m/s) for the detection of PH was only 25% in the French cohort [147]. With a higher cut-off of 2.9 m/s, the predictive value was better (64%), but had too high negative predictive value (42%). When associating TRJV > 2.5 m/s with other parameters such as NT-proBNP level > 164.5 pg/mL or a 6-min walk distance < 333 m with TRJV > 2.5 m/s, the positive predictive value was 62%, and the false negative rate 7% [147].

The medical costs of this disease are enormous, with estimates of $40,000 per patient per year for chronic transfusion therapy and chelation alone, and the impact on quality of life for those with the disease is significant [153].

HSCT in SCA: Procedure and Results

Myeloablative

Allogeneic HSCT is the only curative treatment for SCA; nevertheless, its use has been limited so far by reports of TRM risks. Worldwide experience with pre-transplant myeloablative conditioning showed similar results with an overall TRM risk for the US (n = 50), Belgium (n = 50) and French groups (n = 87) of 7% and DFS of 82–86% (Table 2) [22, 24, 25]. The reported French experience [25] showed that the outcome of the procedure improved significantly with time as DFS in patients transplanted between 2000 and 2004 with a conditioning regimen consisting of intravenous BU, CY (total dose: 200 mg/kg) and rabbit ATG (total dose: 20 mg/kg) was 95.3%. This conditioning regimen was well tolerated, with no case of vaso-occlusive disease and only one death during aplasia. The significant improvement correlated with the use of ATG, which significantly reduced the graft rejection rate from 22.6 to 2.9%, with the use of cord blood, which reduced the rate of GVHD, and with earlier implementation of HSCT before severe organ damage had occurred [25]. When the total nucleated cell number in the cord blood was $<3 \times 10^7$/kg of recipient, bone marrow from the same donor needed to be associated with the cord blood. As already shown for thalassemia, PBCs should be avoided because of higher risk of GVHD. Between 2005 and 2010, 102 new consecutive patients have been transplanted with the same conditioning and the results (DFS 97.1% at 5 years) confirm that it is possible to offer at least 95% chance of cure with myeloablative conditioning and a geno-identical donor to SCA patients [154]. Survival without SCA (DFS) is similar following CBT or BMT [30], with 100% survival post-CBT but with a higher risk of rejection. The dose of ATG in the conditioning regimen influences also the incidence of chronic GVHD, which is the most feared complication of HSCT in this non-malignant disease. High doses of ATG have allowed decreasing its incidence from 20% with no ATG to 15.6% with 5–15 mg/kg, and only 6.4% with 20 mg of ATG/kg [154]. ATG addition to the conditioning regimen has also significantly reduced the rejection rate despite inducing mixed but stable chimerism. Even with only 20–50% donor chimerism, no SCA-related symptoms (no pain episode or acute chest syndrome) were observed in several patients [20, 21, 155]. These clinical data together with biological studies using a specific marker of erythroblasts [156, 157], suggest that transplanted donor erythroblasts have a competitive advantage over SCA erythropoiesis [158]. However, mixed chimerism may sometimes be responsible for some degree of hemolysis in donor/recipient pairs with ABO incompatibility [159 and personal communication]. In the French series, this myeloablative conditioning has also been successfully applied in young adults (16–29 years of age) without severe organ dysfunction resulting in the same long-term DFS than in children [160].

The principal long-term deleterious effect of transplantation is the risk of infertility, making cryopreservation of ovaries and testis highly recommended. All boys developed normal spontaneous puberty after transplantation. Most of the girls

required oestrogen and progesterone treatment to induce puberty, although girls undergoing HSCT at a younger age were more likely to develop spontaneous puberty [161]. However, pregnancies occurred without the need for treatment in 3 women transplanted more than 10 years before, demonstrating some reversibility of ovarian dysfunction. On the other hand, 2 normal births [81] and one pubertal induction [162] occurred following ovarian fragment reimplantation.

Despite preventive measures such as anticonvulsant prophylaxis, strict control of hypertension, immediate magnesium replacement, an increase in red blood cell (9 g/dL) and platelet transfusion (50,000/mm^3) thresholds [163], seizures and posterior leukoencephalopathy, albeit reversible, remain a particularly frequent adverse effect of cyclosporine and steroid therapy [25]. In 2002, replacing cyclosporine by mycophenolate mofetil in case of GVHD requiring steroid therapy resulted in significant reduction of the rate of these complications [25].

In contrast to the early neurological toxicity observed after HSCT, the outcome of pre-existing cerebrovascular disorders was highly favorable after transplantation [21, 24, 25]. In particular, no new ischemic lesions were observed by MRI after transplantation in the successfully engrafted patients, even in those with cerebral arteriopathy, showing that the most important risk factor for stroke is SCA itself and not the associated macrovascular disease. Moreover, rapid normalization of arterial velocities was observed in two patients whose TCD values had remained abnormal despite a 3-year transfusion program, suggesting that HSCT is more effective than transfusion to prevent cerebrovascular disorders. These results have led SCD centers in France to propose HSCT early in patients with a history of abnormal TCD, even in those with normalized velocities. An ongoing trial compares the outcome of cerebral vasculopathy on transfusion program vs. following HSCT (Clinical Trials.gov number: NCT 01340404).

As HSCT is not as urgent an indication in SCA as in malignant diseases, the chances of finding an identical donor for a sick SCA child will increase with time if the providers discuss HSCT early, and propose prenatal or pre-implantation diagnosis and sibling cord blood cryopreservation when parents are expecting another child. For example, in the Créteil newborn SCA-cohort, in which the cumulative incidence of severity criteria occurrence was 50% by age 5, and 78% by age 14 (50% for cerebral vasculopathy and 28% for recurrent VOC/ACS), the cumulative chance of being transplanted by age 14 was 28% [164]. These percentages are higher than those reported by other groups [155, 165] because the Créteil SCD Center has been systematically followed patients with TCD as soon as 1.5 years of age since 1992, and with MRI/MRA every 2 years since1995, thereby allowing early detection of severity criteria.

Non-myeloablative Geno-identical HSCT (Table 4.3)

Several reports had shown that stable mixed chimerism, even with a low level of donor cells, could have a significant beneficial effect when chimerism remained stable. This was encouraging, and permitted to evaluate non-myeloablative

conditioning regimens in order to reduce TRM and to preserve fertility, but also to recommend HSCT to older adult patients with organ dysfunction. However, contrary to HSCT for hematologic malignancies, the risks of mixed chimerism, rejection and non-engraftment are high in SCA because of the patients' normal immunocompetence, highly proliferative bone marrow, and immunization by multiple transfusions. Unfortunately, the first attempt with non-myeloablative conditioning (200 cGy of total body irradiation (TBI) and fludarabine) and immunosuppression with cyclosporine showed only temporary donor engraftment, with progressive decreasing donor chimerism when cyclosporine was tapered, leading to rejection [43].

After this first step, several teams attempted using "reduced-intensity" regimens, but currently published series (Table 4.3) include only too small of a number of patients to obtain significant results [39–45].

Nevertheless, very encouraging results were obtained in adults by the NIH team [46, 47] who decided to replace cyclosporine, known to block T-cell activation, by sirolimus (rapamycin), which, unlike calcineurin inhibitors (e.g., cyclosporine and tacrolimus), does not block T-cell activation, but renders T cells anergic, promoting T-cell tolerance [166–169]. The conditioning regimen consisted of 300 cGy TBI and alemtuzumab (Campath), and the stem cell source was GCSF-stimulated peripheral blood progenitors from HLA-matched sibling donors. The first 10 adult-patients have been reported [46], with a more recent update as an abstract [47], showing no death and no acute or chronic GVHD occurrence, and 3 rejections between the 2nd and 3rd months with SCA recurrence in the 23 SCA-adult patients (17–64 years of age) transplanted. Conforming to the protocol, sirolimus was stopped in those with > 50% donor chimerism (5/17 evaluable at 1 year), which remained stable. Thus, to date, 87% of DFS seems possible in SCA patients with a genoidentical donor and non-myeloablative conditioning regimen [47]. Among the patients successfully engrafted all had mixed chimerism. However, donor/recipient pairs with ABO incompatibility were excluded from this trial. Very encouraging results were also recently obtained in 8 patients with a reduced-intensity conditioning [170].

Indications for Geno-identical HSCT (Table 4.4)

As the worldwide experience with transplantation for SCA has expanded, the use of HSCT has transitioned from an experimental intervention reserved for the most severely affected patients to a treatment that is offered to increasingly younger children with early signs of SCA-related morbidity. Historical indications for genoidentical HSCT in children <16 years of age [23] were stroke, cerebral vasculopathy with stenoses or silent strokes with cognitive impairment, sickle nephropathy and lung disease, multiple osteonecrosis, erythroid-alloimmunization, and recurrent priapisms. These may now be extended to adults using myelo- [160] or non-myeloablative conditioning regimens [46, 47], depending on organ dysfunction.

Table 4.3 Non-myeloablative and reduced-intensity (RIC) matched/related HSCT for SCA-patients

	Conditioning regimen	GVHD prophylaxis	Number	Age (year)	Death	AGVH	chGVH	DFS
Van Besien et al. (2000) [28]	Melphalan 140 mg/m^2, FLU 120 mg/m^2, ATG	Tacrolimus, MTX	2	40–56	2	2	1 severe	0
Iannone et al. (2003) [69]	TBI 200 cGy, FLU 150 mg/m^2	CSA or tacrolimus, MMF	6	3–20	0	1gr2	0	0
Jacobsohn et al. (2004) [158]	BU 6.4 mg/kg IV, FLU 180 mg/m^2, ATG	CSA, MMF	1	22	1	1	1	0
Horan (2005) [34]	TBI 200 cGy, FLU 125 mg/m^2, ATG	CSA, MMF	3	9–30	0	0	0	1
Shenoy, (2005) [33]	Melphalan 140 mg/m^2, FLU 150 mg/m^2, alemtuzumab	CSA, MTX	1	2	0	0	0	1
Horwitz BBMT (2007) [159]	TBI 200 cGy, FLU 100–120 mg/m^2, CY 2 g/m^2	Alemtuzumab, MMF	2	21–27	0	0	0	2
Krishnamurti et al. (2008) [30]	BU 6.4 mg/kg IV or 8 mg/kg PO, FLU 175 mg/kg, ATG, TLI 500 cGy	CSA, MMF	7	6–16	0	1	1	6
Hsieh et al. (2009) [138] (+2011) [139]	TBI 300 cGy, alemtuzumab 1mg/kg	Sirolimus	23	17–64	0	0	0	20
Matthes et al. Eur J Haematol (2013) [160]	Melphalan 140 mg/m^2, FLU 160 mg/m^2, Thiotepa or TLI, ATG or alemtuzumab	CSA, MMF	8	2–24	0	0	0	8

Table 4.4 Indications for matched/related HSCT for SCA

Indications for matched/related HSCT	
Historical consensus (<16 years) [23]	2013 suggestion with extension to adults
Stroke	Idem
Abnormal TCD despite TP	History of abnormal TCD (even normalized)
Silent strokes with cognitive deficiency	Silent strokes
Frequent VOC despite HU	Frequent VOC before HU initiation
ACS ≥2 despite HU	ACS ≥2 before HU initiation
Sickle nephropathy	ACS ≥2 before HU initiation
Multiple osteonecrosis	ACS ≥2 before HU initiation
Recurrent priapism	ACS ≥2 before HU initiation
Erythroid allo-immunization	ACS ≥2 before HU initiation
	Recurrent splenic sequestration
	TRJV ≥2.9 m/s
	TRJV 2.5–2.8 m/s with NT-proBNP >164.5 pg/mL or 6 min walk-distance <333 m
	Very rare erythroid blood group such as U-, with frequent requirement for transfusions

Several new indications have also emerged. For example, in the case of splenic sequestration recurrence, transfusion programs are recommended until splenectomy (usually after 2 years of age following 23-valence pneumococcal vaccine). However, in the Créteil newborn cohort, children with splenic sequestration history were at risk of frequent crises during the entire childhood follow-up [171]. Considering the fact that spleen function can be at least partially restored following HSCT [20, 21, 25, 172], such procedure should be proposed to those with available donor before considering total splenectomy. Frequent vaso-occlusive crises (VOC) and/or recurrent acute chest syndromes (ACS) are indications for HU. However, long-term safety remains a concern for children requiring several decades of HU treatment, justifying the recommendation for HSCT, even in those who improved with HU or before HU initiation.

Abnormal velocities by TCD require initiating a transfusion program (TP), but its duration has not been clearly defined. Stopping TP exposes the child to a risk of stroke or recurrence of abnormal velocities [139], whereas some patients maintain normalized velocities with or without HU. The ongoing French trial “Drepagreffe” (Clinical Trials.gov number: NCT 01340404) comparing cerebral vasculopathy outcomes between HSCT and long-term TP will hopefully determine if HSCT is the best choice for children with a history of abnormal velocities, even in those with normalized velocities. Silent strokes, which are cerebral ischemic lesions, are a manifestation of severe disease and should be an indication for HSCT, even in absence of cognitive impairment.

TRJV associated with NT-proBNP and the 6 min-walk test are useful to detect patients at risk of pulmonary PH, which is mainly diagnosed as a mean pulmonary artery pressure (mPAP) >25 mmHg by right heart catheterization; however, TRJV >

2.5 m/s was shown to a be a risk factor for early death [134] and prospective trials comparing outcome in the absence of treatment with that following HSCT in patients with elevated TRJV should be performed.

Very rare erythroid blood groups, for example U-, compromise successful management of SCA-related complications and could be another indication for HSCT.

Several studies have attempted to define early predictive criteria of adverse outcomes occurring later in life. It was reported by the CSSCD study [173] that early dactylitis, baseline Hb level<7 g/dL, and leukocytosis correlated significantly with adverse outcomes later in life, but this was not confirmed by the Dallas newborn cohort [174] where none of the events before age 3 predicted death or stroke after age 3. However, in the Créteil newborn cohort, data collected before age 2, such as the presence of Bantou beta haplotype, high reticulocyte count and early age at first SCA-manifestation (<1 year) were independent and significant predictive factors of severity [164]. Moreover, severe basal anemia (Hb < 7 g/dL) has been recognized as a risk factor for the majority of SCA complications, i.e., stroke [137], abnormal TCD [107, 175], silent strokes [107, 144], and early deaths [103]. Thus, severe anemia (Hb < 7 g/dL) in the absence of iron deficiency could be retained as a marker of severity, justifying early recommendation for HSCT.

Considering the high risk of rejection after non-myeloablative procedures while still offering a definitive cure, myeloablative transplantations should only be recommended for children with no organ failure. Non-myeloablative HSCT should be reserved to patients with organ dysfunction. Interesting results for tolerance inducing were observed when using Sirolimus for GVHD prophylaxis; thus, it might also be used in patients undergoing HSCT and prepared with a myeloablative conditioning regimen.

Because the hope of cure is as high as 95% in SCA children with geno-identical HSCT, thereby preventing end-organ failure and preserving cognitive functioning that favors the future socio-professional insertion, this therapeutic approach should be discussed early with families and regarded as standard of care for children who have a suitable sibling-matched donor, not only for those with cerebral vasculopathy but also for those experiencing frequent vaso-occlusive crises and/or acute chest syndrome episodes.

Costs

Estimates of the cost of care may facilitate the assessment of the value of new SCA treatments and treatment strategies and inform the prioritization of research efforts [176]. The total cost of medical care for a population of children and adults with SCA was estimated in the SCA population enrolled in the Florida Medicaid program during 2001–2005. Across the 4294 patient cohort, total health care costs generally rose with age, from $892 to $2562 per patient-month in the 0–9- and 50–64-years age groups, respectively. Average cost per patient-month was $1389 suggesting a lifetime cost of care averaging $460,151 per patient with SCA [176]. Moreover, for the patients requiring a transfusion program with iron chelation for

cerebral vasculopathy the cost has been estimated to be approximately $ 40,000/year [153]· but a stroke requires not only life-long transfusions with chelation, but also additional rehabilitation costs of $ 40,000/year [177]. Genoidentical HSCT is the only procedure offering 95% cure [25, 154] and allowing safe interruption of transfusions in patients with macro-vasculopathy. Its cost (€ 76,237 in France) [178] is about equivalent to 2 years of transfusion program with chelation. These data argue that stem-cell-transplantation is really cost-effective.

Barriers

Despite the increasing acceptance of HSCT as a "standard of care" for severe SCA patients, the contrast remains striking between, on one hand, hundreds of thousands patients bearing the disease and on the other hand, only about 1000 transplants performed worldwide so far [109]. If, as underlined by the title of a recent review [166], "the time is now", for a tremendous development of this curative treatment, we have to better understand the possible reasons of this paradoxical situation .

Of course, the major obstacle lies in the fact that the vast majority of the patients live in Africans low-income countries, where expensive transplant technology is not available, and other health issues such as HIV or Malaria have priority. However, this situation is slowly evolving as illustrated by the Nigerian team who recently performed the first HSCT for SCA in Africa (personal communication) and by "emerging countries" such as Brazil who develop SCA programs including HSCT for selected patients. To help these efforts, it is the responsibility of North-American and European teams to work on "exportable" techniques, both for the selection of patients (TCD, family HLA typing etc) and for the transplant itself. In this context, a highly effective non-myeloablative regimen, which could be used in Africa, would be a major progress.

However, even in Europe and the US, HSCT for SCA is clearly underutilized. In 1996, Walters et al evaluated the barriers [179] observed in a multicenter international trial proposing HSCT to children younger than 16 years of age with eligible criteria and genoidentical donor. They reported that the major barrier to HSCT for SCA was the lack of HLA-identical donors but, they suggested that other significant obstacles remained to be identified as only 6.5% of all SCA-children were considered eligible for transplantation in this series. In fact, wide variations exist between institutions, as the percentage of patients with eligible criteria ranged between 0.9 and 36%. Moreover, HLA typing was only performed in 41% of eligible patients. The reasons for the lack of HLA typing were the absence of siblings (41%), but also the lack of financial support (18%), parental (16%) or physician (7%) refusal, or other reasons (18%). However, among the HLA-typed patients, 34% had a genoidentical donor. Omondi recently conducted focus groups at 3 sites representing the Midwest, South Atlantic, and West South Central U, to better understand participation barriers to HSCT clinical trials for SCA [180]. The main barriers to clinical trial participation included gaps in knowledge about SCA, limited access to SCA/HSCT trial information, and mistrust of the medical professionals. For education about

SCD/HSCT trials, participants highly preferred one-on-one interactions with medical professionals, using electronic media as a supplemental source of information. Hansbury retrospectively analyzed their cohort of 113 SCA-patients on chronic transfusion where 40 (35%) had at least one unaffected full sibling. The families of 23 patients (58%) agreed to HLA-typing of siblings, and 8 (35%) were matched, but only 3 underwent HSCT [181]. Common reasons to decline HLA typing or transplantation included fear of the process, toxicities of the procedure, and comfort with current quality of life on transfusions.

We have a very different experience in our SCD-center at Créteil, France. In the newborn cohort of SCA children (*n* = 280), 156 had severity criteria justifying introduction of intensive therapy (HU or chronic transfusion) [164]. HLA-typing was proposed to those with sibling not affected by the disease (*n* = 100/156: 64%). Only one family refused HLA-typing, and 49 of the 99 HLA-typed (49.5%) had a geno-identical sibling. HSCT has already been performed in 43 of them (15 CBT), 2 are scheduled in the near future whereas HSCT was refused by only 2 families (4%), 1 was not performed because of the departure to another country, and one patient died before HSCT-scheduling.

Thus, several factors may contribute to increasing the chances of having a geno-identical donor in SCA families: (1) early information given to parents concerning the different treatments for SCA; (2) early detection of severity criteria using TCD, cerebral MRI/MRA; (3) early familial HLA-typing as soon as severity criteria are detected or in case of new pregnancy in order to schedule cord blood cryopreservation when there is not yet a geno-identical donor among existent siblings; (4) preimplantation genetic diagnosis with double selection HLA-disease proposition to parents who have a SCA-child with severity criteria and no geno-identical donor and who still desire other child. Concerning this approach, Jae et al. [182] conducted semi-structured interviews with SCA adult patients and parents of SCA children in an urban medical center in the US to examine the perceived barriers of transplant and the use of in vitro fertilization and preimplantation genetic diagnosis (PGD). The financial cost of these technologies was perceived as a significant initial barrier, with the clinical risks of HSCT, and the ethical appropriateness of using PGD also identified as barriers. However, ethical reservations, while present, did not preclude patients' and parents' desire to be informed.

In France also, HSCT for SCA remains underutilized. Considering the fact that, in our Créteil SCA-newborn cohort with severity criteria, the cumulative chance to be transplanted with a geno-identical donor before age 14 was 28% and given the fact that about 350 births with SCA are observed yearly in France, 90 transplants could be proposed per year, whereas only about 25 are currently performed. The (non-exhaustive) list of reasons why patients, families, and more importantly medical professionals, sometimes refuse transplantation could be detailed as follows: (a) the low probability to have a suitable HLA identical donor; (b) the risk of TRM; (c) the fear of morbidity such as chronic GVHD and (d) the long term sequelae concerning fertility. However, as previously detailed in this chapter, none of these reasons are pertinent: (a) In our pediatric experience, 28% of the patients have or will have a geno-identical donor before age 14 and, for the others, new

techniques such as haplo-identical HSCT are currently developed (see relevant chapter) [183, 184]; (b) TRM is clearly very low and in fact lower than the risk of mortality from SCA itself in the absence of transplant; (c) with modern management of transplant, the use of ATG and of cord blood, very few patients (<3%) experience extensive chronic GVHD and limited GVHD is more acceptable than severe SCA; (d) Infertility is not an absolute rule as measures can be taken pretransplant to preserve fertility for both girls and boys, and non-myeloablative regimens have already been shown to preserve fertility. Moreover, transplanted patients experience a huge improvement in quality of life, allowing a better professional and social integration.

Conclusions

An international consensus exists for to perform HSCT with myeloablative conditioning for all thalassemia-major patients with geno-identical donor. For SCA-patients, HSCT with myeloablative conditioning and geno-identical donor during infancy offers 95% chance of cure, but still remains underutilized. HSCT should be proposed as soon as a severity criteria requiring intensive therapy with hydroxyurea or chronic transfusion occur in order to preserve cognitive functioning, quality of life and to offer better social and professional integration.

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Chapter 5
Alternative Donor/Unrelated Donor Transplants for the β-Thalassemia and Sickle Cell Disease

Courtney D. Fitzhugh, Allistair Abraham, and Matthew M. Hsieh

Abstract Considerable progress with respect to donor source has been achieved in allogeneic stem cell transplant for patients with hemoglobin disorders, with matched sibling donors in the 1980s, matched unrelated donors and cord blood sources in the 1990s, and haploidentical donors in the 2000s. Many studies have solidified hematopoietic progenitors from matched sibling marrow, cord blood, or mobilized peripheral blood as the best source—with the lowest graft rejection and graft versus host disease (GvHD), and highest disease-free survival rates. For patients without HLA-matched sibling donors, but who are otherwise eligible for transplant, fully allelic matched unrelated donor (8/8 HLA-A, B, C, DRB1) appears to be the next best option, though an ongoing study in patients with sickle cell disease will provide data that are currently lacking. There are high GvHD rates and low engraftment rates in some of the unrelated cord transplant studies. Haploidentical donors have emerged in the last decade to have less GvHD; however, improvements are needed to increase the engraftment rate. Thus the decision to use unrelated cord blood units or haploidentical donors may depend on the institutional expertise; there is no clear preferred choice over the other. Active research is ongoing in expanding cord blood progenitor cells to overcome the limitation of cell dose, including the options of small molecule inhibitor compounds added to ex vivo culture or co-culture with

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supportive cell lines. There are inconsistent data from using 7/8 or lower matched unrelated donors. Before routine use of these less matched donor sources, work is needed to improve patient selection, conditioning regimen, GvHD prophylaxis, and/or other strategies.

Keywords Alternative donor transplant • Matched unrelated donor • Cord blood transplant • Haploidentical donor

Introduction

The first reported bone marrow transplant for sickle cell disease (SCD) was published in 1984 by the group at St. Jude Children's Research Hospital [1]. An 8 year-old girl with SCD developed acute myeloid leukemia (AML) and underwent sibling donor myeloablative transplantation as part of her AML therapy. She was ultimately cured of both and remains alive almost three decades later. This proof-of-principle report paved the way for a series of small pilot studies also demonstrating that transplantation from HLA-matched siblings could cure SCD [2–5]. Later, the multi-center trial published by Walters and colleagues in 1996 was instrumental in solidifying transplant as a bonafide treatment [6]. In this landmark trial, 22 children with very symptomatic SCD underwent sibling marrow transplantation; overall and event-free survival estimates at 4 years were 91 and 73%, respectively. Since then, the procedure has become even safer and more effective. This success is evidenced by the 95% event-free survival (EFS) in 44 patients undergoing refined transplant procedures after January 2000, as described in the French experience [7]. Such excellent results have prompted physicians to reconsider the methods and timing of treatment.

The concept of transplantation earlier in the disease course was already being considered in the 1980s, as reported by investigators in Belgium [8]. In this study, 50 transplanted patients belonged to one of two groups: permanent residents of a European country who had already developed severe sickle cell phenotype before transplant, or visiting patients who were transplanted much earlier in the disease course due to a desire to return to their country of origin. The combined rate of non-engraftment, mixed chimerism, and death was significantly higher in the more diseased group (25% vs. 7%, $p < 0.001$). While transplanting patients earlier in their disease course was controversial in the 1980s, current understanding of the devastating nature of SCD has led providers to accept early transplant in less severe patients.

Traditionally, myeloablative conditioning was used in SCD transplants to maximize engraftment. This often excluded patients older than 16 years of age who had significant sequelae of SCD, including considerable organ dysfunction. Today, less intense preparative regimens have made curative approaches available. Increased rates of stable mixed donor chimerism are accepted as part of

these less intense regimens because the red cell compartment becomes predominantly donor cells and symptoms of SCD resolve with time. The largest, most successful report to date of such non-myeloablative transplantation was described by Hsieh et al. where 30 adults with severe sickle cell disease safely underwent matched sibling transplant with an 87% success rate using a lower-intensity protocol (manuscript under review).

While the field continues to make matched sibling transplant safer and available to more patients with SCD regardless of disease severity and age, there remains the inherent problem of donor availability. The likelihood of two siblings being HLA identical is only 25%, so the odds of finding such a donor are already limited. Notably, a fraction of siblings themselves will have SCD, further limiting the chance of having a suitable match. Some families of patients who do not have siblings have pursued fertility treatments including in vitro fertilization with pre-implantation genetic testing to select embryos that are HLA-matched and disease-free. There are significant ethical and financial considerations to this approach, and it is unlikely that it will become an option for the majority of patients. Therefore, the field has moved to investigate alternative donor-graft sources. Improved outcomes have already been described with matched unrelated donor (MUD), umbilical cord blood (CB), and haploidentical transplantation techniques for malignancy and immunodeficiency in the last two decades [9–12]. This success is confirmed by the 2012 Center for International Blood and Marrow Transplant Research (CIBMTR) data on allogeneic transplants performed in the United States from 2007 to 2011, which shows that approximately 60% of all transplants are from alternative donor sources [13].

Similarly, the first bone marrow transplant to correct β-thalassemia major was reported by E.D. Thomas and colleagues in 1982 [14]. A 16 month-old boy received minimal red blood cell transfusion prior to transplant and underwent dimethyl busulfan and cyclophosphamide conditioning followed by infusion of matched sibling bone marrow. Two months later, his hepatosplenomegaly had completely resolved; at 6 months post-transplant, the patient had a normal hemoglobin level. The authors concluded that transplant could cure not only thalassemia but also other genetic diseases of the marrow such as SCD. In the 1980s, a series of reports from the group in Pesaro, Italy, described varying preparative regimens used in matched related donor bone marrow transplantation for β-thalassemia [15–17]. In 1990 they reported a large experience of 222 matched related donor transplants for homozygous β-thalassemia in patients under the age of 16 years [18]. EFS was an impressive 94% for the patients with least disease severity (Pesaro Class 1), but this was in stark contrast to the only 53% EFS in the most diseased patients (Pesaro Class 3). This same group of investigators subsequently spent several years working on improving outcomes in the highest-risk patients. Ultimately, they published a regimen, referred to as protocol 26 (hydroxyurea, azathioprine, fludarabine, busulfan, cyclophosphamide), that resulted in an improved EFS of 85% for these high-risk Class 3 patients [19]. A major driver behind pursuing curative transplant in thalassemia is the resulting improvement in quality of life post-transplant, provided there are no long-lasting complications such as severe chronic graft-versus-host disease

(GvHD) [20]. Also, life expectancy in developing countries where chelation is not optimal results in a significantly shorter lifespan, so curative transplant becomes a more attractive treatment modality in these areas. However, relying on a matched sibling donor again leaves the majority of patients without a donor, leading to the search for an alternative graft source.

The transplant field is continuing to make improvements in the approach for hemoglobin disorders with better outcomes on the horizon for unrelated donor and cord transplants, but the major limitation remains donor availability. In one report from 2003 that evaluated searches done by the National Marrow Donor Program (NMDP), the chance of finding a potential 6/6 HLA-matched MUD for a patient with thalassemia or SCD was approximately 60% in each case [21]. In the same report, the chances of finding a 5/6 or 6/6 HLA-matched cord donor were approximately 62 and 30%, respectively. However, this matching was done at the serological level for HLA-A and -B and at the potential allele level for -DRB1. A recent look at this scenario that incorporated more detailed matching demonstrated that the chances of a sickle cell patient finding a potential allele 8/8 HLA-A,-B,-C and -DRB1 MUD was only 19% [22]. Combining 5/6 and 6/6 HLA-matched cords (HLA-A,-B antigen; DRB1 potential allele) with higher cell doses as needed for hemoglobin disorder transplants (total nucleated cell count of at least 5×10^6/kg) could increase the alternative donor option. When this was examined, the probability of a sickle cell patient finding a potential 8/8 MUD, 5/6 or 6/6 unrelated cord improved but remained relatively low at 45% [22]. This leaves a significant number of patients without a potential donor if only using HLA matched siblings, 8/8 MUDs, 5/6 and 6/6 cords with good cell doses. In both of these reports describing donor availability, over 95% of patients would have a donor option if double cord transplants or 7/8 MUD donors were used.

Over the last 2 decades, there has been significant progress in transplantation using umbilical CB units, haploidentical donors, and unrelated donors, to make this curative procedure more accessible to those who are eligible.

Umbilical Cord Blood Transplantation for Patients with Hemoglobin Disorders

Since the first report of a successful umbilical cord blood (CB) transplant in 1989 in a pediatric patient with Fanconi Anemia [23], this option has increasingly been used to treat patients with malignant and nonmalignant hematologic diseases. While CB grafts are smaller in cellularity and volume as compared to bone marrow, the proliferative capacity and numbers of progenitor cells contained within the graft are often greater [24, 25]. Further, due to immunological differences such as lower numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells with a higher CD4/CD8 ratio and a higher percentage of naïve $CD45RA^+$ T-cells [26], CB transplants have been associated with a lower risk of GvHD as compared to bone marrow transplants. Besides the lower risk

of GvHD, other advantages associated with CB grafts include easy availability, reduced time to complete the pre-transplant process, little or no donor morbidity, a decreased risk of transmitting latent viral infections, possibility of directed sibling banking, transplant at earlier disease stages, potential for greater HLA-mismatching, an expandable donor pool, and a greater frequency of rare HLA haplotypes in the donor pool as compared to bone marrow registries [27–32].

For Patients with Sickle Cell Disease (SCD)

Table 5.1 describes the results to date for patients with SCD who have undergone related and unrelated CB transplantation (CBT).

Related Umbilical Cord Blood Transplantation

The first report of CBT in a patient with SCD occurred in 1996 [33]. The patient received a myeloablative conditioning regimen consisting of busulfan, cyclophosphamide, and anti-thymocyte globulin (ATG) with cyclosporine for prophylaxis against GvHD. The patient engrafted without evidence of GvHD and had hemoglobin electrophoresis results consistent with sickle trait donor by 9 months post-transplant. The largest study was recently reported by the Eurocord and European Blood and Marrow Transplantation group [34]. A total of 325 patients with β-thalassemia major (TM) and 160 patients with SCD underwent HLA-identical CBT or bone marrow transplantation (BMT); among patients with SCD, 30 underwent CBT and 130 received BMT. The 6-year disease-free survival (DFS) was 90 ± 5% after CBT and 92 ± 2% after BMT. Of all patients transplanted, those who received a CBT had significantly longer time to neutrophil and platelet engraftment as compared to those who underwent BMT ($p < 0.005$). None who received CBT developed grade IV acute GvHD as compared to 8 (2%) who received BM cells. Twenty-nine percent of the BMT patients experienced chronic GvHD as compared to none of the CBT recipients.

A subgroup analysis that included all of the CBT recipients revealed that MTX affects outcome [34] as noted in their previous report [35]. Patients who did not versus did receive MTX had disease-free survivals of 90 ± 4% versus 60 ± 11%, respectively ($p < 0.001$). The time in which transplant was performed also influenced outcome, with CBT performed after 1999 faring significantly better as compared to those transplanted earlier (hazard ratio 0.033, confidence interval 0.12–0.89, $p = 0.02$). The use of thiotepa and patients belonging to Pesaro Class 1 also did better, though those variables were not significant in multivariate analysis. The total nucleated cell dose infused did not influence outcome in patients that received CBT, though median TNC was sufficient at 3.9×10^7 cells/kg.

To date, 44 children or young adult patients with SCD have received related CBT as reported in the literature (Table 5.1, top). The grafts have primarily been 6/6

Table 5.1 Umbilical cord blood transplantation for patients with sickle cell disease

Refs.	Transplant regimen	TNC dose median (range) × 10^7/kg	HLA match	No. of patients (age)	Alive without SCD	Acute GvHD (Gr 2–4)	Chronic GvHD (extensive)	Death (cause)
Related cord blood donors								
Brichard et al. [33]	Bu 16 mg/kg, Cy 200 mg/kg, ATG, CSA	4.6	6/6	1 (5)	1	0	0	0
Miniero et al. [71]	Bu 16 mg/kg, Cy 200 mg/kg, CSA ± MTX	(3.5–6.0)	6/6	3 (3–11)	2	0	0	0
Gore et al. [72]	Bu 726 mg/m^2, Cy 200 mg/kg, ATG, CSA	2.3	6/6	1 (9)	1	0	0	0
Walterset al. [44]	NR	NR	6/6 42 pts, 4/6 4 pts[a]	8 (NR)	6	NR	NR	1 (intractable seizures)
Matthes-Martin et al. [73]	TLI (2 Gy), Flu 160 mg/m^2, Mel 140 mg/m^2, Alem 1 mg/kg, CSA, MMF	NR	6/6	1 (11.1)	1	0	0	0
Locatelli et al. [34]	Bu ± Flu ± Cy ± ATG/ALG ± TT, CSA ± MTX	3.9 (1.5–14)[b]	6/6	30 (2–20)[b]	27	11% (Gr 2–3)[b]	0	3 (2 hemorrhage, 1 organ failure)[b]
Total	–	–	Mostly 6/6	44	38 (86%)	11%	0	9% (of total)
Unrelated cord blood donors								
Mazur et al. [74]	Rituximab, Alem, TT (600 mg/m^2), 600 cGy TBI, tac + MMF[3]	4.2	4/6	1 (9)	1	0	0	0
Adamkiewicz et al. [75]	Mixed, 4 pts myeloablative, 3 pts reduced-intensity	(1.5–9.3)	5/6 2 pts, 4/6 5 pts	7 (3.4–16.8)	3	4	1	1 (multi-organ failure)
Sauter et al. [76]	Reduced-intensity	3.8 and 2.0[c]	5/6	1 (22)	1	0	0	0

Ruggeri et al. [36]	Mixed, 9 pts myeloablative, 7 pts reduced-intensity	6 (2–12)	6/6 (2)	16 (6)	8	23%[d]	16%[d]	1 acute GvHD
			5/6 (4)					
			4/6 (10)					
Kamani et al. [37]	Alem 48 mg, Flu 150 mg/m², Mel 140 mg/m², CSA or tac + MMF	6.4 (3.1–7.6)	6/6 (1)	8 (7.4–16.2)	3	2 (Gr 2)	1 (extensive)	1 (respiratory failure)
			5/6 (7)					
Radha-krishnan et al. [77]	Bu 12.8–16 mg/kg, Flu 180 mg/m², Alem 54 mg/m², MMF, tac	NR	NR	8 (1–10)	4	4	1 (limited)	3 (infection)
Total	–	–	Mostly mis-matched	41	20 (49%)	20% (of total)	7% (of total)	15% (of total)
						40% (of engrafted)	15% (of engrated)	

Abbreviations: TNC total nucleated cell, SCD sickle cell disease, GvHD graft-versus-host disease, Bu busulfan, Cy cyclophosphamide, ATG anti-thymocyte globulin, CSA cyclosporine A, N/A not applicable, MTX methotrexate, Gr grade, NR not reported, pts patients, TLI total lymphoid irradiation, Gy gray, Flu fludarabine, Mel melphalan, Alem alemtuzumab, MMF mycophenolate mofetil, ALG anti-lymphocyte globulin, TT thiotepa, TBI total body irradiation, tac tacrolimus

[a]Includes 47 patients in the entire cohort: 14 patients with thalassemia, 8 patients with SCD, 25 other. Ten patients received peripheral blood stem cells or bone marrow from the same sibling donor

[b]Includes subjects with SCD and thalassemia. GvHD percentage estimated with the assumption that GvHD incidence was the same in both populations since incidence not differentiated in patients with SCD versus thalassemia. Hemoglobin disorder type was also not differentiated among patients who died

[c]Patient received a double umbilical cord unit transplant

[d]Includes 51 patients: 35 have TM and 16 have SCD

HLA-matched. The conditioning regimens have been mixed, but most contained busulfan and cyclophosphamide with or without ATG. Related CBT have been successful in patients with SCD, with an overall survival of 91% and DFS of 86%. Eleven percent of patients have developed grade 2–4 acute GvHD, but none of the patients has experienced chronic extensive GvHD. Therefore, while a standard conditioning regimen has not been developed, the DFS results of related CBT for patients with SCD are at least as good as HLA-matched sibling BMT and with a lower incidence of chronic GvHD.

Unrelated Umbilical Cord Blood Transplantation

In contrast, unrelated CBT have been much less successful. The largest study was reported in 2011, and includes 35 patients with TM and 16 patients with SCD [36]. Nine of the SCD patients received myeloablative regimens while seven underwent reduced intensity conditioning. While the overall survival was 94%, DFS was only 50%. The authors did not distinguish the incidence of GvHD based on hemoglobinopathy type, but 22% of all patients experienced grade 2–4 acute GvHD and 3% extensive chronic GvHD.

A total of 41 patients with SCD have been reported to undergo unrelated CBT (Table 5.1, bottom). The conditioning regimens varied in each study and ranged from myeloablative to reduced-intensity. Compared to the related setting, the majority of patients received 1 or 2 HLA-mismatched grafts. Overall survival was 85%, DFS about 50%, but was reported as low as 37.5% in one study despite only selecting 0 or 1 HLA-mismatched grafts [37]. About one third of patients has developed grade 2–4 acute GvHD, and 7% extensive chronic GvHD. Therefore, the results of unrelated CBT for patients with SCD are suboptimal. As these data reported the composite results of 6/6, 5/6, and 4/6 matches, there is insufficient information to decipher if 6/6 HLA matches would render better outcomes. More effective conditioning, better GvHD prophylaxis, and/or other graft sources should be sought for patients who do not have an HLA-matched sibling donor.

For Patients with Thalassemia Major (TM)

Table 5.2 shows the results for patients with TM who have undergone related and unrelated CBT.

Related Umbilical Cord Blood Transplantation

There are many more reports of related than unrelated CBT for patients with TM. The first patient with TM who underwent CBT was reported in 1995 [38]. The cord was 6/6 HLA-matched from a sibling, and the patient received busulfan,

Table 5.2 Umbilical cord blood transplantation for patients with thalassemia major

Refs.	Transplant regimen	HLA match	TNC dose median (range) × 10^7/kg	Pesaro Class	No. of patients (age)	Alive without TM	Acute GvHD (Gr 2–4)	Chronic GvHD	Death (cause)
Related cord blood donors									
Issaragrisil et al. [38]	Bu 14 mg/kg, Cy 200 mg/kg, CSA, MTX	6/6	3.9	NR	1 (2.5)	1	0	0	0
Chik et al. [78]	Bu 16 mg/kg, Cy 150 mg/kg, ATG 110 mg/kg	6/6	2.9	NR	1 (10)	0	0	0	0
Lau et al. [79]	Bu 20 mg/kg, Cy 200 mg/kg, ATG 90 mg/kg, CSA, MTX	6/6	8.8 (6.2–11.4)	NR	2 (2.2–3.8)	2	2 (Gr 3)	0	0
Chan & Lin [80]	Bu 22 mg/kg, Cy 200 mg/kg	6/6	NR	NR	1 (2.1)	1	0	0	0
Hongeng et al. [81]	Bu 20 mg/kg, Cy 200 mg/kg, ATG 40 mg/kg, methylpred, CSA	4/6	6.1	NR	1 (3)	0	1 (Gr 2)	0	1 (sepsis)
Zhou et al. [82]	Bu 20 mg/kg, Cy 200 mg/kg, ATG 90 mg/kg, CSA, MTX	Class 1	4.8[a]	5/6	1 (1.7)	1	1	0	0
Fang et al. [49]	Mixed	6/6 (6) 5/6 (1) 3/6 (2)	6.6 (3.4–12.7)	Class 2, 6 Class 3, 3	9 (3.5–10)	4	2	1	1 (GvHD)
Vanichsetakul et al. [83]	Bu 16–20 mg/kg, Cy 200 mg/kg, ATG, CSA ± methylpred	6/6 (3) 4/6 (1)	2.9 (1.49–5.3)	Class 1, 1 Class 2, 1 Class 3, 3	5 (2.1–15)	3 (60%)[b]	1	0	1 (Sepsis)
Walters et al. [44]	NR	NR	NR	6/6 42 pts, 4/6 4 pts[c]	14 (NR)	12	NR	NR	NR

(continued)

Table 5.2 (continued)

Refs.	Transplant regimen	HLA match	TNC dose median (range) × 10^7/kg	Pesaro Class	No. of patients (age)	Alive without TM	Acute GvHD (Gr 2–4)	Chronic GvHD	Death (cause)
Sun et al. [84]	Bu, Cy, ATG[d], CSA ± MMF	6/6 (8)	(3.63–16.0)	Class 1 or 2, 10	12[e] (1.3–8.3)	7	4	0	1 (infection)
		4/6 (4)		Class 3, 2					
Sun et al. [39]	Bu, Cy, ATG[d]	6/6	20.8 (19.5–23.3)	Class 2, 1	3	3[f]	0	NR	0
				Class 3, 2					
Goussetis et al. [45]	Bu 16 mg/kg, Cy 200 mg/kg, ATG 40 mg/kg ± Flu, CSA	6/6 (7)	2.5 (0.3–8)	Class 1, 1	8 (3–15)	7[g]	NR	NR	0
		5/6 (1)		Class 2, 2					
				Class 3, 5					
Locatelli et al. [34]	Bu ± Flu ± Cy ± ATG/ALG ± TT, CSA ± MTX	6/6	3.9 (1.5–14)[h]	Class 1 40	66 (2–20)[h]	53	7[h]	0	3 (2 hemorrhage, 1 organ failure)[h]
				Class 2, 23					
				Class 3, 2					
Total	–	Mostly 6/6	–	–	124	94 (76%)	15% (of total)	1%	6% (of total)
							19% (of engrafted)		
Unrelated cord blood donors									
Fang et al. [85]	Bu 20 mg/kg, Cy 200 mg/kg, Flu 150 mg/m², TT 6 mg/kg, ATG 90 mg/kg, tac, MTX, methylpred	6/6	7.5	Class 3	1 (5)	1	1 (Gr 3)	0	0
Tan et al. [86]	Bu 18 mg/kg, Cy 120 mg/kg, ATG, CSA, MTX	4/6	6	Class 2	1 (5.5)	1	0	0	0
Hall et al. [87]	Bu 320 mg/m², Cy 200 mg/kg, ATG 90 mg/kg, CSA, Methylpred	4/6	19.1	NR	1 (0.17)	1	0	0	0
Bradley et al. [88]	Bu, Flu, ATG, tac, MMF	5/6	9.5	NR	1 (0.5)	0	0	0	0

Ruggeri et al. [36]	Mixed, 30 pts myeloablative, 5 pts reduced-intensity	6/6 (5)	4.9 (1.1–9)	Class 1, 9	35 (4)	8	23%[i]	16%[i]	12 (7 transplant-related complications, 5 primary graft failure)
		5/6 (14)		Class 2, 2					
		4/6 (15)		Class 3, 4					
				NR 20					
Jaing et al. [40]	Bu 14 mg/kg, Cy 200 mg/kg, ATG, CSA, methylpred	6/6 (8)	7.8 (2.8–14.7)	NR	35 (1.2–14)	28[j]	28	1	4 (3 hemorrhage), 1 septicemia)
		5/6 (16)							
		4/6 (27)							
Gumuscu et al.[k] [89]	Bu 12.8 mg/kg, Cy 200 mg/kg, ATG 120 mg/kg, tac, MMF	5/6	4.9	N/A	1 (3.7)	1	1	0	0
Total	–	Mostly mis-matched	–	–	75	40 (53%)	51% (of total)	9% (of total)	21%
							95% (of engrafted)	18% (of engrafted0	

Abbreviations: TNC total nucleated cell, TM thalassemia major, GvHD graft-versus-host disease, Bu busulfan, Cy cyclophosphamide, CSA cyclosporine A, MTX methotrexate, NR not reported, N/A not applicable, ATG anti-thymocyte globulin, Gr grade, methylpred methylprednisolone, MMF mycophenolate mofetil, Flu fludarabine, ALG anti-lymphocyte globulin, TT thiotepa, tac tacrolimus, pts patients

[a]Patient has hemoglobin Bart's disease. TNC is total count, not per kg

[b]One additional patient maintained mixed chimerism but remains anemic

[c]Includes 47 patients in the entire cohort: 14 patients with thalassemia, 8 patients with SCD, 25 other. Ten patients received peripheral blood stem cells or bone marrow from the same sibling donor

[d]HLA-mismatched patients received hypertransfusions, continuous intravenous desferrioxamine, Hydroxyurea, Flu, Bu, Cy, and ATG

[e]One donor was not a sibling

[f]All patients received accompanying bone marrow from the same sibling donor

[g]Two patients were re-transplanted and five patients received accompanying bone marrow from the same sibling donor

[h]Includes subjects with SCD and thalassemia. GvHD percentage estimated with the assumption that GvHD incidence was the same in both populations since incidence not differentiated in patients with SCD versus thalassemia. Hemoglobin disorder type was also not differentiated among patients who died

[i]Includes 51 patients: 35 have TM and 16 have SCD

[j]Includes six patients who required second transplants (five CB, one peripheral blood stem cell)

[k]Patient had homozygous α-thalassemia disease

cyclophosphamide, and ATG. The transplant was successful, and the patient did not experience GvHD.

In the study mentioned previously [34], 66 patients with TM underwent CBT, and 259 received BMT. A greater percentage of patients categorized as Pesaro Class 2 and 3 underwent BMT (44%) as compared to those who received CBT (39%, $p = 0.01$). In contrast to patients with SCD (92 ± 2%), the 6-year DFS in patients with TM was 84 ± 2% ($p = 0.04$). Upon multivariate analysis, a diagnosis of SCD was the only variable which favorably influenced the DFS probability for all patients transplanted in the study (hazard ratio 0.52, 95% confidence interval 0.28–0.97, $p = 0.04$). The 6-year DFS was 86 ± 2% in patients with TM who received BMT as compared to 80 ± 5% in patients who underwent CBT. This largest study to date does not report a significant difference in DFS in patients who receive CBT as compared to BMT. On the other hand, the incidence of grade 2–4 acute GvHD was similar at 15%, and the rate of chronic extensive GvHD was close to non-existent. Therefore, the incidence of GvHD is significantly lower in patients with TM who undergo CBT as compared to BMT.

Currently, 124 patients with TM have undergone related CBT as reported in the literature (Table 5.2, top). Similar to patients with SCD, the majority of conditioning regimens have included busulfan and cyclophosphamide with or without ATG. Patients have had all stages of disease with Pesaro Class ranging from 1 to 3. The majority of the studies have employed 6/6 HLA-matched grafts. The overall survival is excellent at 94%. However, as compared to an 86% DFS found in patients with SCD, DFS in patients with TM appears to be lower at 76%. Notably, while hypertransfusion and cytoreductive therapy pre-transplant has been associated with improved DFS in subjects with Pesaro Class 3 disease [19], these additional therapies were only employed in one study [39]. Patients with TM may require more intensive therapy pre- and post-transplant to suppress the enhanced erythropoietic drive which may out-compete the immature immune and progenitor cells located within the CB graft. The contribution of hypertransfusion and the remaining portion of the 'pre-transplant conditioning regimen' to differences in DFS should be further evaluated.

Unrelated Umbilical Cord Blood Transplantation

Two relatively large studies involving patients with TM who have undergone unrelated CBT have been reported. The first study described above included 35 patients with TM [36]. Thirty patients received myeloablative conditioning, and five reduced-intensity conditioning. Pesaro classification was not available in the majority of the subjects. Most received 1 or 2 HLA-mismatched grafts, and 1 patient received a 3 HLA-mismatched graft. Thirty-four percent of the patients died: 7 patients died from transplant-related complications and 5 died as a result of graft failure. The transplant was successful in only 8 patients (21%). As described above, 22% of the total patients transplanted experienced grade 2–4 acute GvHD while only 3% developed extensive chronic GvHD.

Conversely, another group reported their results for 35 patients with TM who underwent unrelated CBT [40]. They received busulfan, cyclophosphamide, ATG, and cyclosporine and methylprednisolone for prophylaxis against GvHD. Pesaro classification was not reported, and again the majority received 1 or 2 HLA-mismatched cords with one patient receiving a 3 HLA-mismatched cord. Overall survival was 89%. By the end of the study, about 80% of patients were free of their disease. However, 6 of the patients required a second transplant: 5 received second CBT and 1 patient underwent peripheral blood stem cell transplantation. All of the patients experienced acute GvHD: 18% grade 1, 35% grade 2, 44% grade 3, and 3% grade 4. However, only 3% developed extensive chronic GvHD.

A total of 75 patients with TM have been reported to have received unrelated CBT (Table 5.2, bottom). The regimens are varied, but most patients are administered busulfan, cyclophosphamide, ATG, and/or fludarabine. Most of the patients received mismatched grafts. The overall survival is 79% with a DFS of 53%. About half of the patients developed grade 2–4 acute GvHD, and only 2.5% extensive chronic GvHD. Therefore, mortality (21%) associated with unrelated CBT is high in patients with TM, and should not be routinely applied to patients with Pesaro Class 1 who have <10% mortality with transfusions and iron chelation [41–43]. Since many patients died from graft failure, survival may be improved with second transplants, even using a second CB graft. The incidence of grade 2 and 3 acute GvHD is high, though the rates of grade 4 acute and extensive chronic GvHD are low. Again these studies reported composite results—6/6 HLA matches with 5/6 and 4/6 matches—thus there are no sufficient data to convincingly decipher 6/6 matches would lead to better outcomes. Conditioning regimens to induce tolerance, more intensive supportive care post-transplant, and other donor sources are necessary for patients with TM who do not have an HLA-matched sibling donor.

Summary and Future Directions for Umbilical Cord Blood Transplantation

The most encouraging results are from related donor CBT; this option is most favorable when the cord is 6/6 HLA-matched and the TNC is greater than 4×10^7/kg [28]. It is disappointing that the transplant results from using unrelated CB grafts have been suboptimal to date. The major obstacles with unrelated CBT remain graft failure in SCD, and low DFS and excessively high mortality in TM. Thus CB grafts have not emerged as a clear alternative cell source for patients without matched sibling donors. While cord blood banking should be discussed among patients, parents, and providers, the enthusiasm for routine cord blood banking, including directed to siblings with hemoglobin disorders, has been dampened by favorable results obtained only when using fully matched related units. Donation of cord blood units for research purposes, however, should be encouraged.

Methotrexate should not be used as GvHD prophylaxis due to the associated inferior results [30]. The incidence of graft failure may be decreased by infusing

bone marrow cells from the same sibling donor [39, 44, 45]. These results have led us to conclude that improvements to CBT outcome can come from several areas currently in active research.

Optimizing Supportive Care and Conditioning Regimen

One study reported that CBT performed more recently had a higher success rate [34], suggesting that greater experience, better supportive care, and improved antibiotic prophylaxis has led to better results. Indeed, improved success over the past few decades in patients with hematologic malignancies has been at least partially attributed to better selection of CB units, more experienced supportive care, and better selection of eligible transplant recipients [29, 46], and those principles will need to be optimized in the non-malignant hematologic disease setting as well. Additional factors that may contribute to graft failure and should be evaluated are the effects of red cell alloimmunization and donor-specific HLA antibodies.

Cord blood transplants for hemoglobin disorders have been plagued by graft failure as demonstrated in the BMT CTN 0601 SCURT trial (NCT00745420), where the premature closure of the cord donor arm was related to an unacceptably high graft failure rate [37]. This study suggests that even one antigen-mismatched grafts and optimal cell dose may not be sufficient [37]. Thus adding more chemotherapy agents was a reasonable next step to overcome the engraftment barrier. Preliminary data suggest that improvement in engraftment has been achieved by adding hydroxyurea and thiotepa to the preparative regimen of alemtuzumab, fludarabine, and melphalan used in the SCURT study [47]. While 5 out of 8 patients showed graft rejection in the SCURT trial, only 1 of 12 rejected with the new regimen that included thiotepa and hydroxyurea. Further follow-up of these early findings is warranted. The use of thiotepa has also shown some preliminary success in other studies [34, 48]. However, increasingly intensive conditioning was not sufficient to significantly improve the graft failure rate in nine patients with TM who received related CBT [49].

Regimens that instead focus on tolerance induction, such as promoting regulatory T-cells (sirolimus) or eliminating alloreactive T-cells following graft infusion (post-transplant cyclophosphamide), may decrease the risk of graft failure. Mixed donor chimerism has been found to be associated with a decreased incidence of GvHD in patients with SCD or TM who receive BMT or peripheral blood stem cell transplantation (PBSCT) [50, 51]. Further, mixed donor chimerism (10–95%) was not found to be associated with an increased risk of graft failure in 27 patients with TM given CBT from a related donor [48]. All 27 patients were alive, transfusion-independent, off immunosuppression, and did not have GvHD. Therefore, a low intensity regimen which leads to stable mixed chimerism and tolerance induction may be optimal. This approach in the unrelated donor setting would need to be balanced with the higher risks of graft failure and GvHD.

Allelic HLA Matching

There has been an international effort to expand allelic level typing for all matched unrelated donors, and recently this is increasingly performed for cord blood units. In a recent report from CIBMTR, where they performed such allelic level HLA-typing in single unit CBT for hematologic malignancies, neutrophil recovery was not more delayed in one and two antigen mismatched units. However, non-relapse mortality was higher, evidenced by higher hazard ratios, in any mismatched setting (even one antigen mismatched) [52]. Due to these apparently conflicting results and the novelty of the data, further investigation is warranted. A marrow donor recruitment group from Washington, DC, performed DNA sequencing and assigned HLA haplotypes, and showed that there were novel HLA-B, -C,-DRB1, and -DQB1 associations in African-American mothers and their cord blood units, again highlighting HLA diversity in this population [53]. Thus higher resolution HLA-typing could provide detailed information for the possibility of better HLA matching.

Double Cord Blood Transplant

Double cord transplant, while mostly described in the malignant transplant realm, remains the subject of research for hemoglobin disorder transplants. The currently open trial NCT00920972, which evaluates alemtuzumab, fludarabine, and melphalan conditioning in non-malignant transplants, has strata dedicated to single and double cord transplants for hemoglobin disorders [54].

Ex vivo Expansion

Another significant limiting factor has been cord size/cell dose. A study published on behalf of the Eurocord Registry, the CIBMTR, and the New York Blood Center describing outcomes of unrelated cord transplants for hemoglobin disorders recommended that a total nucleated cell count of at least 5×10^6/kg be targeted for single cord transplants to maximize engraftment [36]. Such high cell doses can significantly restrict cord options for patients, especially as adulthood is approached and cord units become too small. In the last few years, the transplant field has seen a bevy of approaches used to expand hematopoietic stem cells in the basic science lab now being translated to clinical practice. The most impressive report to date describes 31 adults with hematologic malignancy who received unrelated double umbilical cord transplants [55]. In this trial, one of the cords was co-cultured ex vivo with mesenchymal stromal cells prior to infusion, resulting in a 12-fold increase in total nucleated cell doses and a 30-fold increase in $CD34^+$ cell content. The rate of neutrophil engraftment by day 42 was significantly improved in patients who

received the expanded cord as part of their transplant (96%) when compared to two separate control cohorts who received unmanipulated double cords (83 and 78%). While the expanded cord transplant contributed to hematopoiesis early on, at 1 year the predominant donor-derived chimerism was from the unmanipulated unit in the vast majority of the patients. The finding that the unmanipulated cord is the one ultimately responsible for long-term engraftment brings into question whether expanded units, despite having increased numerical cell doses, behave functionally similar to unmanipulated grafts of similar size and are capable of life-long engraftment. Promising preliminary data in transplants for malignancy have supported the NiCord® trial for SCD (NCT01590628), which is evaluating the use of double cord transplantation after myeloablative conditioning where one of the cord units is expanded ex vivo in culture with cytokines and nicotinamide [56]. The study is estimated to complete in the spring of 2014.

Haploidentical (and Mismatch Related) Transplantation for Patients with Hemoglobin Disorders

Haploidentical transplantation has increasingly been explored as a viable treatment option for patients with hemoglobin disorders. Many benefits exist including a readily available donor pool, since parents, children, and half-matched siblings can serve as donors, relatively short time to collect the graft as compared to unrelated donors, and the option for repeat collections as opposed to a CB graft. However, due to the higher immunologic barrier, there has traditionally been a high risk for graft rejection, GvHD, and transplant-related mortality.

For Patients with Sickle Cell Disease (Table 5.3)

The first report of a haploidentical BMT in a patient with SCD occurred in 2004 [57]. The 14 year-old patient was conditioned with fludarabine and 200 cGy TBI, and received CSA and MMF for prophylaxis against GvHD. The patient survived free of SCD and did not develop GvHD. In 2012, the Johns Hopkins group reported their experience [58]. Their regimen was based on the success seen in their patients with hematologic malignancies who underwent haploidentical BMT and included post-transplant infusion of cyclophosphamide to decrease the incidence of GvHD [59, 60]. Fourteen patients, predominantly adults, were treated. While the rate of graft rejection was expectedly high due to the non-myeloablative regimen, 7 of the 14 patients are completely free of SCD. One additional patient displays a mixed donor and recipient erythroid phenotype with severe anemia and sickle hemoglobin elevated more than would be expected in a subject with sickle cell trait. Importantly, none of the patients developed GvHD and all of the patients are living.

Further, six of the patients with complete donor chimerism have been weaned off of immunosuppressive therapy.

Recently, Dallas and colleagues described eight pediatric patients who received haploidentical PBSCT [61]. The patients underwent reduced-intensity conditioning consisting of fludarabine or pre-transplant cyclophosphamide, busulfan, thiotepa, and muromonab-CD3 with or without ATG. They were treated with MMF for GvHD prophylaxis. The patients received $CD34^+$-selected PBSC on day 0 and $CD3^+$-depleted PBSC on day +1. These patients were compared to 14 pediatric patients who received myeloablative conditioning: 13 patients underwent matched sibling BMT and 1 patient received matched sibling CBT. The overall survival and DFS were 93% for patients who underwent matched related donor transplantation. Conversely, for patients who received haploidentical transplants, overall survival was 75% and DFS 38%. Two patients (25%) developed grade 2 acute and two patients chronic extensive GvHD; both patients died from GvHD.

Therefore, haploidentical transplantation is associated with a high risk of graft rejection in patients with SCD, with a total DFS rate of 43% (Table 5.3). While the most recent report was associated with a prohibitive rate of transplant-related mortality, both as a result of GvHD, the Hopkins group transplanted 14 patients, including 12 adults, and none of the patients died or developed GvHD. The improved survival and absence of GvHD may be due to the bone marrow stem cell source and/or the use of post-transplant cyclophosphamide. Additional studies to attempt to decrease the rate of graft rejection and GvHD should be performed in the context of a clinical trial.

For Patients with Thalassemia Major (Table 5.4)

In two earlier reports, up to three antigen mismatch family donors were used and the majority of the patients belonged to Pesaro Class 2 or 3. In the report by Gaziev et al. [62], reduced doses of busulfan down to 8 mg/kg were used to reduce the potential liver and other transplant-related toxicity, which was one explanation of the surprisingly high graft rejection rate (55%). There was also an equally high rate of acute and chronic GvHD, 47 and 38% respectively. Separately, Sullivan and colleagues also reported a high rejection rate at 28%, but rates of GvHD were not reported [63]. Both reports contained mortality rates close to 30%, which dampened the enthusiasm of relying on this donor source for transplantation. However, in the most recent report of 16 patients, the results were much improved with only one death and GvHD rates of 15–20% [64].

In 2004, a patient with Pesaro Class three disease was reported to have undergone haploidentical PBSCT after being conditioned with busulfan, fludarabine, anti-lymphocyte globulin (ALG), and 500 cGy TBI [65]. The patient survived free of disease but developed grade 2 acute GvHD and extensive chronic GvHD. The largest study to date was reported in 2011 [66]. Thirty-one patients with TM received myeloablative conditioning with fludarabine or cyclophosphamide, busulfan, thiotepa,

Table 5.3 Haploidentical (and mismatched related) transplantation for patients with sickle cell disease

Refs.	Transplant regimen	Graft Type	No. of patients (age)	Alive without SCD	Acute GvHD (Gr 2–4)	Chronic GvHD (extensive)	Death (cause)
Raj et al. [57]	Flu 90 mg/m^2, 200 cGy TBI, CSA + MMF	BM	1 (14)	0	0	0	0
Bolanos-Meade et al. [58]	Cy 29 mg/kg, Flu 150 mg/m^2, 200 cGy TBI, ATG 4.5 mg/kg, PT-Cy 100 mg/kg, tac or sir + MMF	BM ± GCSF	14 (15–42)	7[a]	0	0	0
Dallas et al. [61]	Flu 150–200 mg/m^2 or Cy 200 mg/kg, TT 10 mg/kg, Bu, muromonab-CD3 ± ATG 30 mg/kg, MMF	PBSC[b]	8 (4.2–17.1)	3	2	2	2 (GvHD)
Total	Mixed	–	23	43%	9% (of total) 20% (of engrafted)	9% (of total) 20% (of engrafted)	9% of total

Abbreviations: SCD sickle cell disease, GvHD graft-versus-host disease, Flu fludarabine, Gy gray, TBI total body irradiation, CSA cyclosporine A, MMF mycophenolate mofetil, BM bone marrow, N/A not applicable, Cy cyclophosphamide, ATG anti-thymocyte globulin, PT post-transplant, tac tacrolimus, sir sirolimus, GCSF granulocyte colony-stimulating factor, TT thiotepa, Bu busulfan, PBSC peripheral blood stem cell, Gr grade

[a] One additional patient with mixed chimerism is transfusion-independent and free from SCD crises, but remains anemic

[b] Patients received a $CD34^+$-selected product on day 0 and a $CD3^+$-depleted product on day +1 post-transplant

ATG, and CSA for prophylaxis against GvHD. Donor grafts consisted of T-cell depleted BM and PBSCs to achieve mega doses of enriched $CD34^+$ cells. Overall survival was 94% with a DFS of 70%. None of the patients developed acute or chronic GvHD.

To date, a total of 159 patients with TM have been reported to undergo haploidentical transplantation (Table 5.3). Conditioning regimens are mixed, but all except one contain fludarabine. Total overall survival is 93% and DFS is 74%. Despite most donor grafts including PBSCs, GvHD rate was low: 13% grade 2–4 acute and 4% extensive chronic GvHD.

Interestingly, unlike in CBT where DFS is higher in patients with SCD as compared to TM, DFS appears to be higher in patients with TM as compared to SCD when undergoing haploidentical transplantation. The mega doses of $CD34^+$-cells in more than half of the patients with TM may have helped to overcome the engraftment barrier. Therefore, future studies should consider mega doses of $CD34^+$ cells ($>10 \times 10^6$ cells/kg) to help decrease the rate of graft rejection along with ex vivo or in vivo T-cell depletion and/or post-transplant cyclophosphamide to help decrease the risk of GvHD and again should be performed as part of a clinical trial.

Matched Unrelated Donor Transplantation for Patients with Hemoglobin Disorders (Table 5.5)

Due to the lack of donor availability, matched unrelated donor (MUD) marrow transplants have not been performed in sufficient number of patients with SCD. There is one report from Germany that included two children who received matched unrelated marrow transplant successfully [67]. The Sickle Cell Unrelated Transplant (SCURT) trial (NCT00745420), which has been open since 2008, has transplanted close to 20 patients from MUD (European Bone Marrow Transplant meeting 2012). When published, the study results will provide useful information about this donor option for patients with SCD.

With respect to MUD marrow transplants in TM, there have been slightly over 130 patients transplanted. La Nasa et al. reported MUD marrow transplants from 10/10 donors, but included more Pesaro Class 3 (more severe) patients [68, 69]. Rates of acute GvHD, chronic GvHD, and mortality were 35, 20, and 24%, respectively. A more recent cohort was described in mostly Pesaro Class 2 patients where they allowed up to one allele HLA-mismatch donors [70]. There was only 8% acute GvHD (grade 2 not reported), no extensive chronic GvHD (limited GvHD not reported), and 8% mortality. Having more Class 2 patients, using reduced doses of cyclophosphamide, and employing more current supportive care certainly contributed to the better results reported in this recent cohort. There is preliminary report from an international collaborative collection of 23 children with thalassemia, 16 of them received transplant from unrelated donors. The DFS was 78%, with acute and chronic GvHD rates of 30 and 9% respectively (Shenoy et al., American Society of Hematology Annual Meeting, 2013).

Table 5.4 Haploidentical (and mismatched related) transplantation for patients with thalassemia major

Refs.	Transplant regimen	Graft type	Pesaro Class	No. of patients (age)	Alive without TM	Acute GvHD (Gr 2–4)	Chronic GvHD (extensive)	Death (cause)
Sullivan et al. [63]	Bu, Cy, MTX, CSA	1–2 alleles mismatch	Class 2 and 3	60 (1–19)	28	NR	NR	15
Gaziev et al. [62]	Bu 8–16 mg/kg, Cy 120–200 mg/kg, ALG or radiation, CSA, MTX	BM, 1–3 antigen mismatch	Class 1, 6	29 (1–33)	6	NR	NR	10
			Class 2, 17					
Hongeng et al. [65]	Bu 4 mg/kg, Flu 175 mg/m^2, ALG 25 mg/kg, TLI 500 cGy, CSA, MTX	Haplo, PBSC	Class 3	1 (18)	1	1	1	0
Jaing et al. [90]	Bu 14 mg/kg, Cy 200 mg/kg, CSA, MTX	Haplo, PBSC	NR	1 (5)[a]	1	0	0	0
Hao et al. [91]	Flu 100 mg/m^2, Bu 16 mg/kg, Cy 200 mg/kg, ATG	Haplo, BM	Class 1, 1	5 (1.6–9.3)	3	2	0	0
			Class 2, 2					
			Class 3, 2					
Wang et al. [92]	Flu 150–240 mg/m^2, Bu 520 mg/m^2, Cy 100 mg/kg, ATG 10 mg/kg ± TBI 3 Gy, CSA, MTX, MMF	Haplo, PBSC	Class 1, 3	16 (3–11)	13	4	1	2 (graft failure, GvHD)
			Class 2, 2					
			Class 3, 11					
Sodani et al. [66]	Flu 150 mg/m^2 or Cy 200 mg/kg, Bu 14 mg/kg ± TT 10 mg/kg, ATG 50 mg/kg, CSA	Haplo, BM and PBSC	NR	31 (NR)	22	0	0	2 (EBV cerebral lymphoma and CMV pneumonia)

Gaziev et al. [64]	Bu 14 mg/kg, Cy 90–200 mg/kg, TT 10 mg/kg, ATG	BM, 1 antigen mismatch	Class 1, 1	16 (1–24)	15	3	2	1
			Class 2, 5					
			Class 3, 10					
Total	Mixed	–	–	159	89 (56%)	6% (of total)	3% (of total)	19% (of total)
						11% (of engrafted)	4% (of engrafted)	

Abbreviations: TM thalassemia major, GvHD graft-versus-host disease, Bu busulfan, Flu fludarabine, ALG anti-lymphocyte globulin, TLI total lymphoid irradiation, Gy gray, CSA cyclosporine A, MTX methotrexate, PBSC peripheral blood stem cell, Gr grade, N/A not applicable, Cy cyclophosphamide, ATG anti-thymocyte globulin, TBI total body irradiation, MMF mycophenolate mofetil, TT thiotepa, BM bone marrow, NR not recorded, EBV Epstein Barr Virus, CMV cytomegalovirus

[a]Patient had received a previous 6/6 matched unrelated donor bone marrow transplant at 2 years of age and an unrelated double cord unit transplant at 5 years of age. Haploidentical transplant was performed 46 days after his UCB transplant

Table 5.5 Mismatch related marrow or matched unrelated donor transplantation for patients with thalassemia major

Refs.	Transplant regimen	Graft type	Pesaro Class	No. of patients (age)	Alive without TM	Acute GvHD (Gr 2–4)	Chronic GvHD (extensive)	Death
Sullivan et al. [63]	Bu, Cy, MTX, CSA	Unrelated	Class 2 and 3	4 (1–16)	2	NR	NR	1
La Nasa et al. [68, 69]	Bu 14 mg/kg, Cy 200 mg/kg, TT 10 mg/kg, ATG, CSA, MTX,	Unrelated BM, 10 of 10 match 85%	Class 1, 14	68 (2–37)	Class 1+2: 24 (80%)	24 (35%, gr 2–4)	5	16 (most were in class 3)
			Class 2, 16		Class 3: 21 (55%)			
			Class 3, 38					
Feng et al. [93]	Bu 16 mg/kg, Cy 200 mg/kg, ALG	Unrelated, BM, includes 1 and 2 alleles mismatch	Class 1, 3	9 (1–9)	7 (78%)	6	1	1
			Class 2, 4					
			Class 3, 2					
Resnick et al. [94]	Bu 16 mg/kg, Flu 180 mg/kg, ATG	Unrelated, 1 BM, 1 PBSC	Both Class 1	2 (2 and 4)	1	NR	NR	NR
Li et al. [70]	Bu 12–13.2 mg/kg, Cy 100–120 mg/kg, TT 5 mg/kg, Flu 160 mg/m^2, ATG, MTX, CSA, MMF	Unrelated, PBSC 8/8: 32, 7/8: 19, 6/8: 1	Class 1, 9	52 (2–15)	50 (96%)	4 (8%, grade 3–4)	0	4, 2 directly from GvHD
			Class 2, 82					
			Class 3, 9					
Total	Backbone of Bu, Cy, ATG			135	105 (78%)	26% (of total)	5% (of total)	17% (of total)
						33% (of engrafted)	6% (of engrafted)	

Abbreviations: SCD sickle cell disease, GvHD graft-versus-host disease, Bu busulfan, Cy cyclophosphamide, ATG anti-thymocyte globulin, CSA cyclosporine A, MTX methotrexate, Flu fludarabine, Mel melphalan, MMF mycophenolate mofetil, ALG TT thiotepa, Gr grade, NR not reported, Gy gray

Summary and Recommendations

With the passing of each decade, substantial progress in allogeneic transplantation from the different donor options has been made for hemoglobin disorders: matched sibling/related donors in the 1980s, matched unrelated and cord blood in the 1990s, and haploidentical donors in the 2000s. These alternative options have greatly expanded transplantation to those who were otherwise eligible for a conventional matched sibling transplant. From our review of the literature, improvements in supportive care and high resolution HLA-typing have further made transplantation safer, and modifications to the conditioning regimen have allowed those with end-organ damage or higher Pesaro classification to receive this curative procedure (Fig. 5.1).

The conditioning regimens have gradually been adapted from the most common backbone of busulfan (14–16 mg/kg), cyclophosphamide (200 mg/kg), anti-thymocyte globulin, and cyclosporine for GvHD prophylaxis. Busulfan has mostly been switched to intravenous for consistent pharmacokinetic measurements and less risk of sinusoidal obstructive syndrome (veno-occlusive disorder). Cyclophosphamide dose can be reduced to as low as 120 mg/kg to prevent significant liver injury peri-transplant. For haploidentical or allelic mismatched donors, thiotepa and/or fludarabine have been added to enhance engraftment, and post-transplant cyclophosphamide and other immunosuppressants (e.g. sirolimus) to reduce GvHD.

The results of these transplant studies that we surveyed can be summarized into the following recommendations, which can be applied with children or adults, and in β-thalassemia or SCD (Fig. 5.2).

1. Marrow, peripheral blood derived, or cord blood progenitor cells from matched sibling/related donors offer the best results of transplantation, thus this donor source should be sought whenever possible.
2. Fully matched unrelated marrow (8/8 or 10/10 allelic match) would be the next best option (Table 5.5), as the evidence for this donor choice in patients with thalassemia has been reported in two reasonably sized studies in different populations [68, 70]. There are insufficient data in SCD thus far, but the results from SCURT trial (BMT CTN 0601, NCT00745420) should be available soon. This donor source may be more difficult to find for patients with SCD, compared to patients with thalassemia.
3. While fully matched unrelated cord blood units (6/6 match) would appear to be the next reasonable option, the reports only included limited number of patients and the results were combined 6/6 match with lower HLA matches. There are favorable results in some reports but high rates of GvHD with low rates of engraftment in others (Tables 5.1 and 5.2). Thus at this time, this source (whether 6/6 match or lower) is not clearly more preferable than haploidentical donors. The institutional expertise would likely dictate which source is better suited. Additionally there are other factors that make this source less desirable than match unrelated marrow.

   a. Since total nucleated cell count can vary, this source is better suited for children.

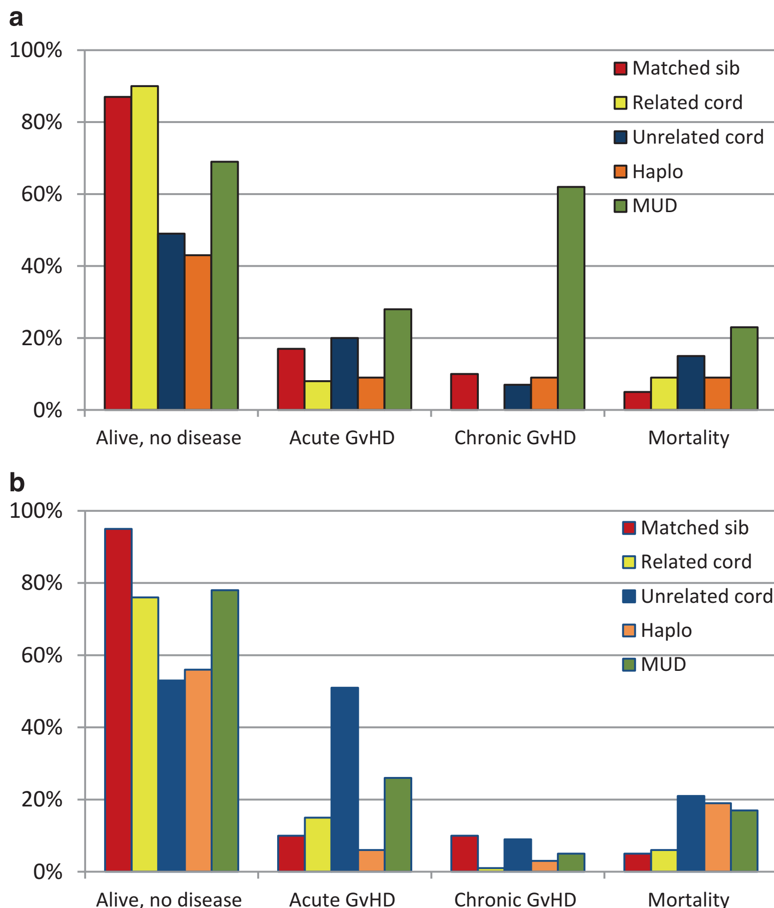

Fig. 5.1 (**a**) Summary of transplants performed in sickle cell disease. The average rates of event-free survival (Alive, no disease), acute GvHD, chronic GvHD, and mortality are plotted with respect to the different donor sources. *There are insufficient matched unrelated donor (MUD) data to date, but the sickle cell unrelated transplant (SCURT) trial results will be available soon. (**b**) Summary of transplants performed in thalassemia. The average rates of event-free survival (Alive, no disease), acute GvHD, chronic GvHD, and mortality are plotted with respect to the different donor sources

b. Fully matched unrelated cord blood units are rare for patients with SCD.
c. Delayed platelet engraftment that is typical with CBT and may lead to excessive morbidity should be considered: hypersplenism in patients with thalassemia with risk of platelet transfusion refractoriness, or CNS vasculopathy in patients with SCD with risk of intracranial hemorrhage.

4. Haploidentical (or mismatch related) donors have emerged as a reasonable and safe alternative from the studies published in the last decade (Tables 5.3 and

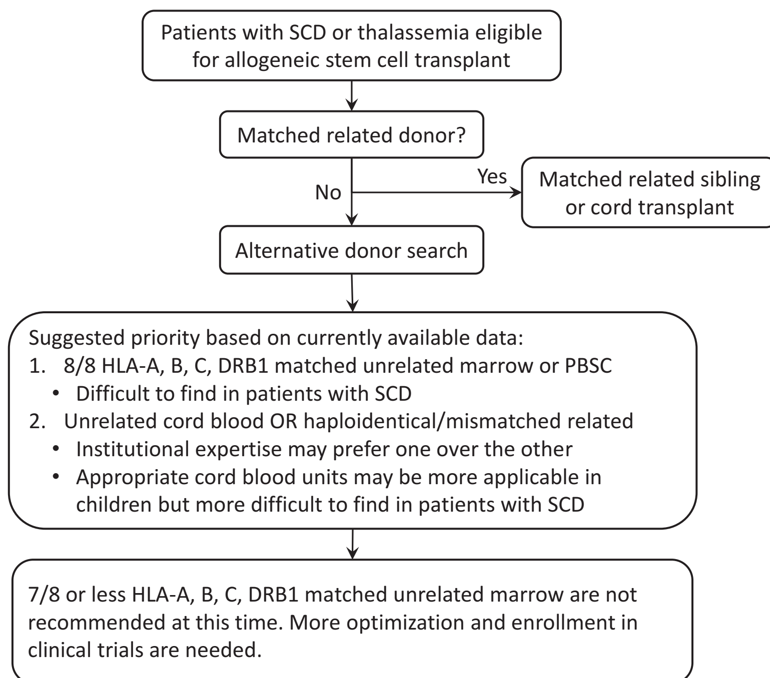

Fig. 5.2 Suggested priority of alternative donors. Hematopoietic progenitors from matched sibling donors remain the best option. From the studies reviewed in this work, fully matched unrelated donor is the next best option for patients with thalassemia. The corresponding data in sickle cell disease will be available soon. Since the results from unrelated cord blood and haploidentical/mismatched related transplant are variable, there is no one preferred source at this time. Enrollment in clinical trials is strongly encouraged. Mismatched unrelated marrow source is not recommended until after further optimization.

5.4), but the cumulative experience is less than that of cord blood transplants. The typical conditioning regimen backbone of busulfan, cyclophosphamide, and ATG has been adapted to include fludarabine and/or low dose radiation in thalassemia. Although at a glance, the outcomes from this source appear to be suboptimal, the data from the most recent series have better engraftment and lower GvHD rates [64]. The conditioning regimens in SCD are significantly different from busulfan, cyclophosphamide, and ATG. The addition of post-transplant cyclophosphamide has been shown to decrease the incidence of GvHD, but more optimization is needed to improve this donor option.

5. Although data from other nonmalignant diseases (immune deficiencies or aplastic anemia) are encouraging, the results from using mismatched unrelated marrow (7/8 or less) in hemoglobin disorders are overall inferior to the other options based on studies to date. Therefore, this donor source should be studied in the context of clinical trials, testing newer conditioning regimens, other immunosuppressive combinations, and/or other strategies.

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Chapter 6
Gene Addition Strategies for β-Thalassemia and Sickle Cell Anemia

Alisa C. Dong and Stefano Rivella

Abstract Beta-thalassemia and sickle cell anemia are two of the most common diseases related to the hemoglobin protein. In these diseases, the beta-globin gene is mutated, causing severe anemia and ineffective erythropoiesis. Patients can additionally present with a number of life-threatening co-morbidities, such as stroke or spontaneous fractures. Current treatment involves transfusion and iron chelation; allogeneic bone marrow transplant is the only curative option, but is limited by the availability of matching donors and graft-versus-host disease. As these two diseases are monogenic diseases, they make an attractive setting for gene therapy. Gene therapy aims to correct the mutated beta-globin gene or add back a functional copy of beta- or gamma-globin. Initial gene therapy work was done with oncoretroviral vectors, but has since shifted to lentiviral vectors. Currently, there are a few clinical trials underway to test the curative potential of some of these lentiviral vectors. This review will highlight the work done thus far, and present the challenges still facing gene therapy, such as genome toxicity concerns and achieving sufficient transgene expression to cure those with the most severe forms of thalassemia.

Keywords Beta-thalassemia • Sickle cell anemia • Hemoglobinopathies • Hemoglobin disorders • Gene therapy • Oncoretrovirus • Lentivirus • Hematopoietic stem cells • Cell-based therapy • Mixed chimerism

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Introduction

Sickle cell anemia (SCA) and beta-thalassemia are the most frequently inherited blood disorders worldwide. Altogether, roughly 100,000 Americans are affected by these disorders. Both disorders are characterized by mutations in the beta-globin gene, a subunit of hemoglobin (Hb). SCA is an inherited disorder characterized by only a single mutation in the beta-globin gene, leading to the formation of hemoglobin S (HbS) [1]. HbS exhibits a marked decrease in solubility, and an increase in viscosity and polymer formation. Ischemic stroke, caused by large vessel arterial obstruction with superimposed thrombosis is one of SCA's most devastating complications. Blood transfusions are administered to prevent thrombosis. Unfortunately, periodic blood transfusions are associated with significant risks of iron overload and other complications, and must be accompanied by iron chelation [2, 3]. Beta-thalassemia on the other hand is characterized by one or more of over 300 various mutations in the beta-globin gene. Based on the combinations of these mutations, patients might be affected by a milder form, indicated as beta-thalassemia intermedia or non-transfusion dependent thalassemia (NTDT), or the most severe form, beta-thalassemia major [4, 5]. Beta-thalassemia major requires regular transfusions to sustain life. However, due to the negative progression of this disease, very often NTDT patients become transfusion dependent as well [6, 7]. Major problems are progressive splenomegaly from extra medullary hematopoiesis and iron build-up in the heart and other organs, often resulting in fatal outcomes for some patients in their teens or early 20s [8, 9]. Current palliative therapeutic options to treat these two disorders are red blood cell transfusion and iron chelation [2, 10].

In addition to life-threatening anemia, patients may present with inherent and treatment-related complications that exacerbate the pathology. For patients with SCA, common complications include painful episodes, acute chest syndrome, and stroke [1]; in patients with thalassemia, hepato-splenomegaly, recurrent infections, and spontaneous fractures [11–13]. In both cases, transfusion-associated infections and organ damage are side effects of long-term treatment and unsatisfactory iron chelation. Iron overload is observed also in NTDT patients because of ineffective erythropoiesis [6, 14]. Ineffective erythropoiesis triggers a cascade of compensatory mechanisms resulting in erythroid marrow expansion, extramedullary hematopoiesis, splenomegaly, and increased gastrointestinal iron absorption [15]. Ineffective erythropoiesis triggers increased iron absorption by reducing the expression of hepcidin, the hormone that controls dietary iron absorption [14, 16–18].

Although both transfusion and iron chelation treatments have remarkably improved over the years and, thus, improving the quality of life, they do not provide a definitive cure, as they do not address the inherent genetic cause. To this end, hematopoietic stem cell (HSC) transplantation is the only presently available cure. Allogeneic bone marrow transplant (BMT) can be curative, but only a small proportion of patients have suitable donors. Furthermore, myeloablative HSC transplantation carries a 5–10% mortality rate. Graft-vs-host disease and adverse immune reactions can limit the success of allogenic BMT as well [9]. Given these limitations,

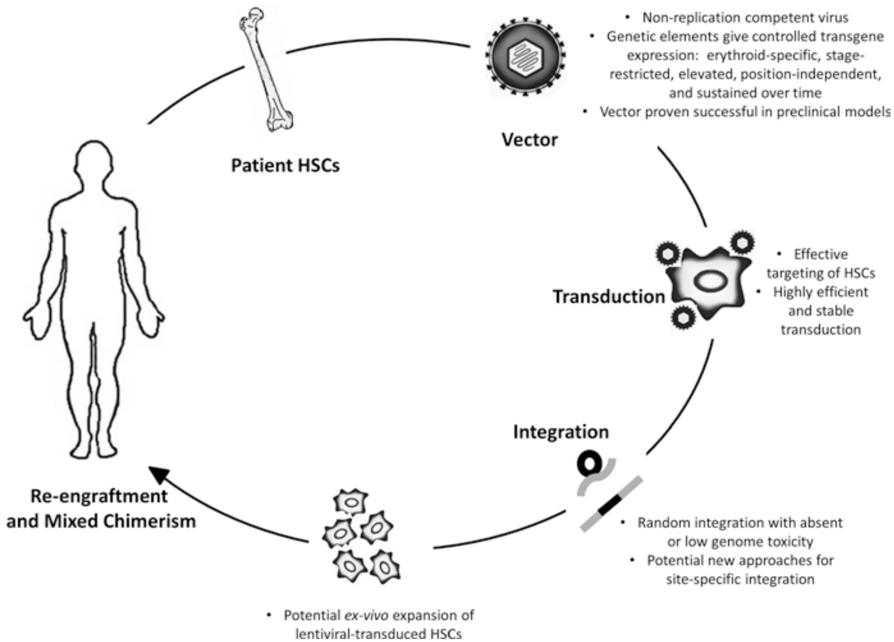


Fig. 6.1 Schematic of gene therapy for beta-thalassemia and sickle cell anemia

gene therapy using a patient's own HSCs represents an alternative and potential cure because it aims at the direct recovery of the hemoglobin protein function via the addition of a functional copy of the beta- or gamma-globin gene. The development of gene therapy tools for SCA and beta-thalassemia has been the object of research of the last few decades and has been proved successful in mouse model studies, in vitro human cell studies, and thus far in one clinical trial. This review will highlight key findings from these gene-addition studies.

The conditions for a clinical-grade gene therapy vector can be summarized as follows: (1) controlled transgene expression: erythroid-specific, stage-restricted, elevated, position-independent, and sustained over time; (2) effective targeting of HSCs; (3) highly efficient and stable transduction; (4) absent or low genomic toxicity; and (5) correction of the phenotype in preclinical models. Figure 6.1 provides a schematic of gene therapy and key issues found in each stage.

Oncoretroviral Vectors

The first studies of gene addition were done with oncoretroviral vectors and helped paved the way for current lentiviral vectors. Oncoretroviruses, like lentiviruses, belong to the Retroviridae family and are RNA-based viruses [19]. Multiple studies showed oncoretroviruses are capable of transferring genetic material without transferring any

viral material, and are able to achieve expression of human beta-globin in murine cells. However, the expression of beta-globin was extremely low and nowhere near therapeutic [20–22].

Studies then moved on to determining what other elements needed to be incorporated to achieve higher beta-globin expression. Discovery of the locus control region, or LCR, brought significant advancements to vector design. The LCR is a regulatory region upstream of the beta-globin locus and is critical for high-level, sustained, erythroid-specific, and position-independent globin expression [23, 24]. The LCR is made up of four DNaseI hypersensitive sites (HS) that contain many motifs for transcription factors and chromatin remodeling factors. It is thought to regulate globin gene expression through a looping mechanism, bringing various transcriptional modifiers to the globin promoter [25]. Incorporation and modification of these HS sites was done in a number of works. Work by Plavec et al. in 1993 [26] showed HS2, HS3, and HS4 elements increased beta-globin expression by 10-fold in mouse erythroleukemia (MEL) cells, but still remained relatively low for mice transplanted with oncoretrovirus-transduced cells: 0.04–3.2% of endogenous mouse beta-globin RNA. Furthermore, there were problems with stability and high viral titer production. Leboulch and coworkers [27] undertook modification of the LCR in order to overcome these problems. They saw instability in all combinations of LCR sequences, with HS2 alone conferring a single common rearrangement and other combinations showing multiple rearrangements. Beta-globin gene mutagenesis and elimination of a 372 base pair intronic sequence and multiple reverse polyadenylation and splice sites resulted in higher titer viruses and more stable proviral transmission. Sadelain saw additional success in 1995 [28] with producing a high-titer retroviral beta-globin vector, but unfortunately the vector did not give high position-independent expression and large clonal variation was seen.

Other methods were also tested for their ability to achieve therapeutic levels of expression: addition of a chromatin insulator [29]; use of the ankyrin promoter driving the gamma-globin gene [30]; use of a mutant gamma-globin enhancer characterized from patients with hereditary persistence of fetal hemoglobin (HPFH) driving gamma globin [31]; addition of the HS40 regulatory region from the human alpha-globin gene locus [32]; and use of an anti-sickling beta-globin [33]. Many important insights were gained by these experiments, however, as with other oncoretroviral studies, their success was limited. Eventually oncoretroviral studies gave way to the lentiviral studies discussed below.

Lentiviral Vectors

In the mid-1990s, lentiviral vectors based on the human immunodeficiency virus (HIV-1) arose as an option for gene transfer. Engineered to be devoid of any pathogenic or replication competency, these viruses are efficiently able to encompass large therapeutic transgene cassettes. Like all retroviruses, lentiviruses exhibit receptor-mediated entry, capsid uncoating, reverse transcription, and integration

into the host genome [19]. However, lentiviruses have a more intricate genome, notably the Rev response element, or RRE. The RRE helps stabilize the proviral RNA by interacting with the viral protein Rev. This allows for stronger unspliced RNA export from the nucleus [19, 34, 35]. Another important element discovered in lentiviruses is the central polypurine tract/central termination sequence element. The cPPT/CTS element is a short noncoding part of the *pol* gene sequence that increases lentiviral transduction efficiency.

Importantly, for hematological gene therapy purposes, lentiviruses are able to infect dividing and non-diving cells [36]. Gene therapy for hematological disorders typically infect HSCs, so that the genetic modification is passed on short term to differentiating cells, and long-term to more stem cells through self-renewal. HSCs, however, are difficult to culture and transduce ex vivo due to a delicate balance between dividing/proliferation and engraftment potential. Normally, proliferation and engraftment potential are negatively correlated: increasing the proliferation is detrimental because the cells subsequently do not engraft [37]. Thus, ability to infect non-dividing cells that retain engraftment potential is an extremely beneficial property of lentiviruses. Lentiviruses containing regulatory elements, promoters, enhancers, and beta-globin or gamma-globin have been successful with correction of mouse models of thalassemia and SCA, and with in vitro correction of human CD34+ peripheral blood (PB) cells. A list of beta-globin vectors can be found in Table 6.1.

In 2000, May [38] and colleagues used the TNS9 vector to correct a mouse model of thalassemia intermedia. Later in 2003, Rivella [39] and colleagues showed that this same vector could be used to rescue lethality in a new model of Cooley's anemia (thalassemia major). TNS9 exhibits position-effect variation though, and in the Cooley's anemia model, was unable to be therapeutic in all mice, with average human beta-globin expression between 3.6 and 9.4 g/dL. Two mice models of SCA were corrected in 2001 by Pawliuk [40] and colleagues: the S-Antilles-D Punjab model (SAD) and the Berkeley (BERK) model. The SAD mouse model expresses human alpha and a human "super S" beta-globin that has two point mutations [41]. The BERK model expresses human alpha and human sickle beta-globin, but additionally does not express any endogenous mouse alpha or mouse beta globin [42]. As a result, the BERK model has a more severe phenotype, in part because of suboptimal expression of the human beta-globin gene as compared to the endogenous mouse gene. Pawliuk used a transgenic "βT87Q" form of beta-globin, an anti-sickling mutant form which has an amino acid substitution at the 87th position. With this vector, Pawliuk saw transgenic Hb could make up to 12% and 52% total Hb for the SAD and BERK models, respectively. In 2013, another anti-sickling mutant form was tested with three point mutations: T87Q for blocking the lateral contact with HbS, E22A to disrupt axial contacts with HbS, and G16D, which confers a competitive advantage over HbS for interaction with alpha-globin [43]. Named "CCL-βAS3-FB", this vector could reduce the relative amount of sickled red blood cells differentiated in vitro; and, using vector copy numbers of 0.5–2, could make up 15–25% of total Hb.

Table 6.1 Beta-globin vectors

Name	Author and year	Main characteristics
TNS9	May 2000 Rivella 2003	- Correction of a mouse model of thalassemia intermedia (2000) and prevention of lethality in a mouse model of thalassemia major (2003) - Variable expression
$\beta^{A(T87Q)}$	Pawliuk 2001	- Correction of two SCA mouse models - Anti-sickling (T87Q) form of beta-globin
BG-1	Puthenveetil 2004	- Full-length cHS4 enhancer used in 3′LTR (1.2 kb) - Amount of beta-globin approached normal levels - Low viral titers
T10	Lisowski 2007	- Addition of HS1 to HS2-4
Globe	Miccio 2008	- Higher titer with removal of HS4
$\beta^{A(T87Q)}$ LentiGlobin	Cavazzana-Calvo 2010	- Used in first European clinical trial to achieve transfusion independence in a β^0/β^E heterozygous patient - Transgenic $\beta^{(T87Q)}$-globin made up only 1/3 of total Hb, rest combination of HbE/HbF - Integration sites near potential oncogenes - Saw expansion of one clone with a *HMGA2* integration site
G-Globe	Miccio 2011	- No HS4, incorporates HS2 enhancer of the GATA1 gene
AnkT9W T9AnkW	Breda 2012	- Incorporates the Ankyrin insulator - Shows improved β-globin expression over TNS9
CCL-βAS3-FB	Romero 2013	- "FB" insulator containing the minimal 77 bp binding site for CTCF - 3 mutations to beta-globin to confer anti-sickling properties

In order to increase the safety of lentiviral vectors and improve expression, insulators were tested by Puthenveetil in 2004 [44]. An insulator is a genetic element which usually has two properties: (1) enhancer-blocking activity, when placed between an enhancing element and a promoter and (2) preventing the spread of heterochromatin into the integrated transgenic cassette from a nearby heterochromanized region [45–47]. By adding an insulator, one can prevent the beta-globin LCR from acting on nearby oncogenes it might have integrated near. It can also help reduce vector silencing, to ensure sustained and high transgene expression. Puthenveetil et al.'s vector, named BG-1, added a 1.2 kb cHS4 insulator, taken from the chicken beta-globin hypersensitive site 4. As reviewed by Nienhuis and Persons [48], the cHS4 insulator has a "core" that contains five footprints. The footprints are involved in: recruiting CTCF, an enhancer blocking protein; binding USF proteins in order to recruit histone-modification enzymes that make transcription-activation marks; and binding VEZF1, which prevents DNA methylation in the transcribed region. Human beta-thalassemic cells treated with BG-1 and differentiated in vitro showed similar amounts of hemoglobin as non-thalassemic controls. Upon transplantation into immunodeficient mice, treated cells underwent effective

erythropoiesis and expressed normal amounts of beta-globin. In 2007, Arumugam [49] and colleagues compared vectors with the cHS4 insulator to those without and consistently saw approximately double the beta-globin expression with the insulator in vitro with MEL cells and in vivo with transplanted and transduced murine HSCs. While beneficial to expression, the 1.2 kb cHS4 insulator causes low viral titers. Thus, in 2009, Arumugam [50] identified a 400 bp extended core region of the cHS4, that still exhibits full insulator activity but does not have a severe an impact on titer. They found the previously identified core only reduced clonal variegation.

Further studies have also been done on the LCR. In 2007, Lisowski et al. [51] showed addition of HS1 from the LCR to HS2-4 significantly increased globin expression. Miccio in 2008 [52] used a "GLOBE" vector containing the HS2 and HS3 regions without the HS4 region. They used it to rescue Cooley's anemia lethality, but high copy numbers were required for correction [34]. Interestingly, upon transplantation, transduced cells expressing a high level of beta-globin were preferentially selected in vivo. Roselli [53] produced preclinical data using the "GLOBE" vector in 2010 on a diverse set of CD34+ patient samples to restore adult Hb (HbA) synthesis. Integration analysis revealed integration preference in transcriptionally active regions but no preference for cancer-related regions. "GLOBE" was later modified into "G-GLOBE" by adding the HS2 enhancer of the GATA-1 gene [54]; it too could achieve high expression of beta-globin. The GATA-1 HS2 bound GATA1 and CBP acetyltransferase, leading to the establishment of an open chromatin region.

Lentiviral transduction of gamma-globin has additionally been shown to be therapeutic. Table 6.2 lists gamma-globin vectors that have been successfully used. In 2003, Persons et al. [32] developed the "d432$\beta^A\gamma$" vector, which expressed gamma-globin under beta-globin LCR elements. Although they saw expression of fetal hemoglobin, they still saw high variation due to position and vector copy number. Hanawa in 2004 [55] used a longer LCR to achieve more consistent expression

Table 6.2 Gamma-globin vectors

Name	Author and year	Main characteristics
d432$\beta^A\gamma$	Persons 2003	- High variation depending on integration region and vector copy number
mLARβΔγV5 (V5)	Hanawa 2004	- Larger LCR reduced position effects and improved expression
G9	Samakoglu 2006	- Includes shRNA against sickle β-globin - Test only in vitro on MEL and HeLa cells stably expression sickle beta globin
V5m3	Pestina 2009	- 3′UTR of beta-globin corrected BERK SCA model
V5m3-400	Wilber 2011	- Added 400 bp core of cHS4 insulator
GGHI	Papanikolaou 2012	- No LCR elements, but instead contains a gamma-globin promoter with a -117 point mutation associated with HPFH, the HS40 enhancer from the alpha-globin locus, the HPFH-2 enhancer, and the cHS4 insulator - Only mild HbF production

across animals. In 2006, Samakoglu [56] and colleagues showed that a combination vector could be made that expressed gamma-globin and concurrently knocked-down sickle beta-globin via small hairpin RNA. They tested this vector on HeLa and MEL cells stably expressing sickle beta globin. The discovered that the placement of the shRNA was critical, as it affected interferon response, siRNA production, and the amount of gamma-globin expression. Pestina [57] tested a gamma-globin vector in vivo on the BERK SCA model. The gamma-globin was modified to contain a 3′UTR from beta-globin, since proteins are believed to bind the beta-globin 3′UTR and increase mRNA stability. This was further modified by Wilber in 2011 [58] with the addition of the 400bp core of the cHS4 insulator, and tested on human CD34+ PB cells from three beta-thalassemia patients. They saw fetal Hb (HbF) production ranged between 45 and 60% of total Hb, and up to a threefold increase in total Hb content. Papanikolaou in 2012 [59] published a report of a gamma-globin virus without the LCR, but instead containing a gamma-globin promoter with a -117 point mutation associated with HPFH, the HS40 enhancer from the alpha-globin locus, the HPFH-2 enhancer, and the cHS4 insulator. They saw mild improvement of HbF synthesis compared to mock-transduced controls.

Most recently, our laboratory has done work to find new insulators. The aforementioned cHS4 insulator is subject to rearrangements and loss (see Clinical Trials, below). To this end, we generated a new lentiviral vector, which we have named AnkT9W. AnkT9W contains the erythroid-specific ankyrin 5′ hyper-sensitive barrier insulator [60, 61]. This insulator does not exhibit enhancer-blocking activity, but does prevent the spread of heterochromatin, and significantly increases expression of beta-globin as compared to vectors without this insulator. This vector was able to maintain high, yet stable levels of Hb synthesis in MEL cells and human CD34+ PBMCs. Analysis indicated that in MEL cells, AnkT9W expressed the transgenic mRNA and hemoglobin at higher levels than the parental T9W (a modified TNS9). Interestingly, AnkT9W was additionally able to correct the phenotype of SCA cells by modifying the *proportion* of sickling vs functional Hb, without changing the overall Hb content. This could be clinically relevant since there is a concern that adding transgenic beta-globin into SCD HSCs increases the total amount of beta-chains, both sick and transgenic. This new total amount might exceed the amount of α-chains, leading to an alpha-thalassemia like phenotype.

Addition of Non-globin Genetic Elements

On top of the numerous studies to add back beta and gamma globin genetic sequences, there are have been additional studies based on adding other genetic elements which can modify beta or gamma-globin gene expression. The gamma-globin repressor *BCL11A* has been identified as target to increase gamma-globin gene expression. Xu and colleagues were able to demonstrate that affecting *BCL11A* alone was able to increase endogenous gamma-globin expression and ameliorate the sickle cell phenotype in mice [62]. Since *Bcl11A* knockout is postnatal lethal,

they used a floxed *Bcl11A* mice crossed with the *EpoR-GFP Cre* mice, which express Cre recombinase under the erythropoietin receptor promoter. As in the full *Bcl11A* knockout, the switch from HbF to HbA did not occur. These mice were then bred with SCD mice. In the combination *SCD/Bcl11A$^{fl/fl}$* mice, sickle cells were absent and blood parameters were markedly improved, thus showing that *Bcl11A* deletion alone was sufficient to ameliorate SCD. Along the same lines, Wilber and colleagues [58] tested a lentiviral construct encoding a *BCL11A* shRNA on CD34+ human PB cells and saw a 3-fold increase in gamma-globin expression. Most recently, Bauer and colleagues [63] have done a genome-wide association study concerning *Bcl11A*. They found a sequence in intron-2 that causes developmentally restricted, erythroid-specific *lacZ* reporter expression in mice. Disruption of this sequence with transcription activator-like effector nucleases (TALENs) in MEL cells lead to reduced expression of *Bcl11A*. As such, this sequence might be a new target to lower *Bcl11A* expression and increase HbF production.

Oct-1 is another gene that negatively regulates gamma-globin gene expression. Oct-1 is a transcription factor that recognizes the octamer ATGCAAAT. The gamma-globin promoter contains three Oct-1 consensus sequences. The -175 consensus sequence has been shown to be associated with HPFH and mutagenesis of the -280 consensus sequence leads to increased gamma-globin expression. Xu and colleagues [64] tested the ability of a "decoy oligonucleotide" to compete for Oct-1 binding, therefore reducing Oct-1 binding at the endogenous gamma-globin locus. In K562 cells, they saw an increase in gamma-globin gene expression after addition of the decoy oligonucleotide.

Genetic elements can also be engineered to affect beta and gamma-globin gene expression. Advances in zinc-finger (ZF) development have allowed the creation of domains able to recognize any 18 base pair DNA sequence. ZF domains can be paired with transcriptional activation domains to create "artificial transcription factors". In 2010, Wilber and colleagues [65] extensively examined one such engineered ZF transcription factor, termed GG1-VP64. GG1-VP64 recognizes the -117 position of the gamma-globin promoter. The -117 position is the site of a naturally occurring mutation which causes HPFH, and is thus a known region important for modulating gamma-globin gene expression [66]. They discovered in wild-type CD34 PB cells that up to 20% HbF could be produced, as compared to 2% in untransduced controls. Later in 2011 [58], they tested beta-thalassemic samples and found a therapeutic 20-fold increase in gamma-globin could be achieved. In 2012, Deng et al. published a paper concerning an artificial ZF linked to the protein Ldb1 [25]. Ldb1 is a critical part of GATA1-mediated chromatin looping of the LCR to the beta-globin promoter. Deng and colleagues created Ldb1-ZFs that recognized the beta-major promoter (P-ZF) or the HS2 site of the LCR (L-ZF). They showed in murine GATA-1 null cells that beta-major expression could be induced by P-ZF alone or P-ZF and L-ZF, but not by L-ZF alone. Furthermore, they showed that the self-association domain of Ldb1 was sufficient for this activity as well [25].

Beta-thalassemia is characterized by over 300 mutations. A subset of these mutations creates new cryptic splice sites and, even though the original splice sites are intact, leads to incorrect splicing. The most common splice mutations involve the

creation of a splice site in intron 1 or intron 2 of the beta-globin gene, and are termed IVS1 or IVS2 for intravenous sequence as such. Specific mutations are followed with a number denoting the site of the mutation, such as IVS1-110 or IVS2-654. Since the correct splice sites are still intact, approaches have been made to create splice switching oligonucleotides, which cover the aberrant splice site and restore splicing to the original sites. A splice switching oligonucleotide has to achieve a number of goals: (a) it must bind to the aberrant splice site and prevent it from being recognized by the splicing machinery and (b) the duplex it creates must not be recognized by RNaseH, as to prevent degradation of the RNA. To this end, Svasti and colleagues [67] have developed a morpholino oligomer conjugated to the cell-penetrating peptide P005. The conjugation leads to efficient uptake of the oligomer into the cell. The oligomer targeted the aberrant splice site in the IVS2-654 mutation, and upon in vivo delivery, improved hemoglobin synthesis in an IVS2-654 mouse. Laccera et al. [68] investigated this in vitro on human CD34+ cells as well. They found dose-dependent and sequence-specific correction for one IVS2–654 and two IVS2–745 thalassemic patient samples. The IVS2–745 pre-mRNA splicing was corrected more efficiently than that of IVS2–654 pre-mRNA. The authors say this coincides with the clinical phenotype of the two diseases, in that IVS2-654 is more severe.

Clinical Trials

The first successful gene therapy trial for beta-thalassemia was done in Paris and reported by Leboulch in 2010 [69]. It was a small trial involving only two patients. The first patient failed to engraft due to technical issues unrelated to the vector. The second patient, however, has been transfusion independent now for several years. The patient is a compound heterozygote (β^E/β^0), in which one allele (β^0) is nonfunctioning and the other (β^E) is an HbE mutant allele whose mRNA may either be spliced correctly (producing a mutated β^E-globin) or incorrectly (producing no beta-globin).

The βT87Q LentiGlobin vector was used for this trial (see Table 6.1). As with the βT87Q vector, this vector expresses a mutated beta-globin distinguishable from transfused beta-globin due to an anti-sickling mutation at the 87th amino acid. It also contains two core copies of the cHS4 insulator. Analysis of the patient's transduced cells revealed an intact coding sequence for the vector, however, with the loss of one copy of the cHS4. Of the twenty-four chromosomal integration sites (IS) found, one of the sites, high mobility group AT-hook 2 (*HMGA2*), caused transcriptional activation of *HMGA2* and became the dominant clone. Cells from the clinical trial patient with a HGMA2 IS showed loss of the 3′UTR of *HGMA2*, preventing the binding of let-7 miRNAs to complementary sequences. Erythroid cells from the *HMGA2* clone exhibited a dominant, myeloid-biased cell clone. *HMGA2* mRNA was undetectable in granulocyte-monocytes, thus the expression was reported to be erythroblast-specific. However, the clonal dominance of *HMGA2* was represented

in all populations in similar proportions (erythroblasts, granulocyte-monocyte and LTC-IC cells). The authors hypothesize that this dominance is due to a transient expression of *HMGA2* in a myeloid-restricted LT-HSC during β-LCR priming, before the β-LCR becomes restricted to the erythroid lineage.

Overexpression of *HMGA2* is found in a number of benign and malignant tumors and can lead to a clonal growth advantage [70]. Overexpression is often associated with mutations affecting the 3′ untranslated region (UTR), which contains binding sites for the regulatory miRNA let-7 [70]. Let-7 miRNA binding to the 3′UTR of *HMGA2* negatively regulates *HMGA2* mRNA and thus the level of protein expression [71]. Transgenic mice carrying a *HMGA2* with a shortened 3′UTR expressed increased levels of *HMGA2* protein in multiple tissues including hematopoietic cells. These mice showed splenomegaly, erythropoietin-independent erythroid colony formation, and an increased number of peripheral blood cells in all lineages. Furthermore, BM cells derived from these animals had a growth advantage over wild-type cells. Thus, overexpression of *HMGA2* is associated with clonal expansion at the stem cell and progenitor levels [70].

At the time of reporting, the patient had been transfusion-independent for 2 years, and showed stable Hb levels from 9 to 10 g/dL^{-1}. The patient has undergone frequent phlebotomies to increase iron clearance. Therapeutic Hb-βT87Q LentiGlobin however only accounted for 1/3 of the total Hb, with endogenous HbE and HbF making up the rest. Without the additive effect of these endogenous Hb's, this first trial might not have been a success. This suggests that we not only need a predictive in vitro model with which to evaluate potential trial patients, but better vectors that can achieve higher therapeutic Hb expression.

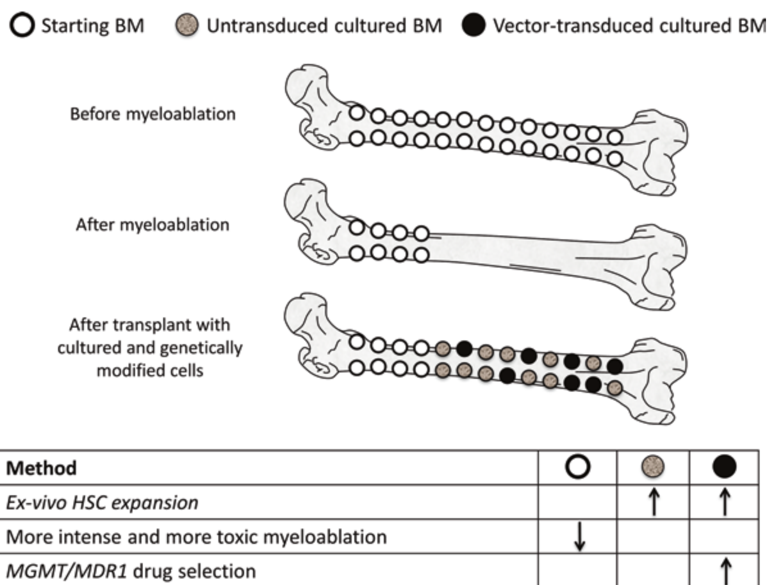
Currently the first United States phase I clinical trial has received FDA approval and is enrolling patients. The strategy was briefly described by Sadelain and colleagues in 2010 with the main goals of assessing insertional oncogenesis and replication-competent lentivirus safety, and determining levels of engraftment and vector expression [72]. The study plans to use $CD34^+$ cells mobilized by granulocyte colony-stimulating factor (G-CSF). Using G-CSF, Sadelain et al. have already achieved successful mobilization of $CD34^+$ cells in three beta-thalassemia patients in amounts sufficient for transduction. In 2002 Li et al. [73] studied G-CSF peripheral blood stem cell mobilization in beta-thalassemia patients and found up to a 21.5 fold increase in $CD34^+$ cells could be collected. In 2012, Yannaki and colleagues studied different mobilization methods in 23 patients with beta-thalassemia [74]. They studied patients with or without splenectomy, and found that non-splenectomized patients tolerated G-CSF, but splenectomized patients could not tolerate it without a 1-month pretreatment with hydroxyurea. They additionally examined Plerixafor, which reversibly inhibits the CXCR4-SDF1 interaction with the BM microenvironment, to mobilize HSCs. Plerixafor proved successful for both splenectomized and non-splenectomized patients.

For the Sadelain trial, the previously described TNS9 vector [38, 39] will be used to induce transgenic expression of beta-globin. Small unpublished modifications have been made to this vector to increase titer, but the gene, promoter, enhancers, and LCR remain intact. Two more trials are also in the works. (1) A trial St. Jude

Children's Research Hospital is planned using gamma-globin coding sequences under control of the beta-globin promoter. (2) The company Bluebird Bio, a company specializing in genetic and orphan diseases, is planning a trial in the San Francisco area using a LentiGlobin BB305 T87Q virus. The identifiers for the TNS9, gamma-globin, and LentiGlobin trials are NCT01639690, NCT00669305, and NCT01745120, respectively; and at the time of writing were all recruiting participants.

Mixed Chimerism and In Vivo Selection of Transduced Cells

When undergoing autologous stem cell transplant, patients first need to undergo a myeloablative conditioning regimen. The success of conditioning regimen intensity depends on a balance between toxicity and the amount of mixed transgenic-chimerism, i.e. the amount of BM made up of transplanted cells that carry the vector (Fig. 6.2). Full myeloablation can, theoretically, result in a complete transgenic-chimerism, where the BM is made entirely of cultured cells, with a greater amount of therapeutic vector-transduced cells than in a partial myeloablation setting. However,



Method	○	◍	●
Ex-vivo HSC expansion		↑	↑
More intense and more toxic myeloablation	↓		
MGMT/MDR1 drug selection			↑

Fig. 6.2 Mixed chimerism. *White:* starting bone marrow of the patient before procedure; *Textured Grey:* Bone marrow that has been cultured but not transduced; *Black:* Bone marrow that has been cultured and successfully transduced with therapeutic vector. Ex-vivo expansion would increase the total amount of bone marrow that been cultured. More toxic myeloablation would decrease the amount of residual bone marrow. Selection with *MGMT* or *MDR1* would increase the amount of vector transduced cells

full myeloablation is more toxic and puts the patient at greater risk, especially in the case of graft failure. A reduced-intensity conditioning regimen can be less toxic; however it can also lead to a lower composition of therapeutic vector-transduced cells due to lower transgenic-chimerism. When a reduced-intensity conditioning regimen is used, beta- globin expression from the vector must be high enough to give transduced cells a survival advantage compared to untransduced cells. In studies done of traditional allogeneic BMT, patients with as low as 20% donor contribution were still able to achieve transfusion independence and normal hemoglobin levels. However, a high initial engraftment (>90% at 60 days post-transplant) is necessary for good chances of stable mixed chimerism [9].

Lucarelli and colleagues identified three Pesaro risk classes for beta-thalassemia patients based on previous iron chelation, hepato- and splenomegaly, and liver fibrosis [75]. Patients with irregular iron chelation and more liver damage fall into class 3 while those patients with less iron overload and liver damage fall into class 1 and 2. A study of 886 beta-thalassemia patients who received transplants from HLA-matched siblings or parents showed a 91% and 84% probability of Thalassemia-free survival with a normal conditioning regimen for class 1 and class 2 patients, respectively [76]. There has been a recent trend to lower-intensity, non-myeloablative conditioning regimens though based on the following data: (1) lower morbidity and mortality is associated with these regimens, (2) patients not eligible for the traditional full myeloablative regimen have been safely transplanted with these regimens, and (3) mixed chimerism can be sustained and still lead to amelioration of disease in patients with allografts [77]. Multiple groups have had success with reduced-intensity regimens with lower doses of busulfan, or by using alternatives such as thiotepa, treosulfan, fludarabine, busulfex, or antithmocyte globulin (as reviewed in [77, 78]). All of these myeloablation reports, however, relate to beta-thalassemia transplants with HLA-matched donors and not to autologous transplants done with vector-transduced cells. The TNS9 trial with vector-transduced cells will use a reduced-intensity regimen based off of data from successful allogeneic transplants [72] and data from autologous transplants with vector-transduced cells in immunodeficiency disorders [79, 80].

If after transplant a patient were to have a non-therapeutic level mixed chimerism, or suboptimal transgene expression, it would be helpful to have an in vivo strategy to increase the chimerism. Conferring a cytoprotective drug resistance to lentiviral-transduced cells is one of these strategies. The human multidrug resistance 1 (*MDR1*) gene encodes a P-glycoprotein drug efflux pump that confers resistance to several chemotherapy drugs, including paclitaxel and doxorubicin, both of which under normal conditions are hematopoietically toxic. Researchers discovered a modest positive selection of peripheral blood progenitor cells that had been transduced with *MDR1* could be achieved by giving paclitaxel [81, 82]. In a second set of studies, researchers used a mutated *MGMT* gene. *MGMT* encodes the enzyme O^6-methylguanine-DNA methyltransferase, and confers resistance to nitrosoureas and O^6-benzylguanine drugs. A dual vector was created encoding both MGMT under a constitutive promoter and gamma globin under erythroid control elements (V5 from Table 6.1). Murine wild-type [83] and murine beta-thalassemic bone

marrow [84] were transduced with this vector. This dual vector was able to increase the number of fetal hemoglobin expressing cells in vivo after treatment with drug. Furthermore, for thalassemic bone marrow, researchers could achieve amelioration of the anemia. Researchers also showed that ex vivo selection of lentiviral-transduced and transplantable cells was possible. They pretreated the cells with drug prior to transplantation and saw that a greater number a mice achieved therapeutic fetal hemoglobin levels as compared to untreated controls [84].

MGMT has been studied in both dogs [85] and non-human primates. One study with non-human primates showed mostly mild and transient enrichment of MGMT-transduced cells [86], while another group showed more stable enrichment [87]. The dog [85] and one non-human primate study [87] showed no significant enrichment for vector integration sites near proto-oncogenes after drug treatment. In the dog study, two dogs had to be euthanized due to health complications, but these complications seemed to be unrelated to the MGMT-transduced cells. The other non-human primate study [86] did not extensively study genome toxicity, but also saw no evidence of clonal dominance or leukemic transformation. *MGMT* and *MDR1* have additionally been combined, with bicistronic vectors encoding both genes. The stoichiometry between the two genes has even been examined. With an *MDR1-IRES-MGMT* vector, Maier and colleagues [88] saw a similar cytoprotective effect for monotherapy with paclitaxel or O^6-BG/temozolomide, and a greater cytoprotective effect with the combination therapy. Later studies showed that a F2A provided the best stoichiometry between the two drug resistant genes for the best cytoprotective effect [89].

Concerns and Genome Toxicity

Although lentiviral vectors offer a number of benefits, there are still many unmet concerns. Sustained, high expression is still difficult to achieve, as transgene silencing by chromatin modifications is still a problem. Insulators have helped this situation, but it has not been solved entirely. Additionally, there is a mild concern with replication-competent lentivirus; although as generations of lentiviruses progress, they resemble the original HIV-1 genome less [90]. The SIN, or self-inactivating design for a lentivirus removes a 400bp region from the 3′ long terminal repeat. This deletion abolishes the enhancer/promoter activity of the virus, therefore reducing transcriptional interference. It is less likely to recombine with cells that have been infected with HIV-1 or make replication-competent lentivirus as it has less similarity [91].

Several studies have been done on non-viral methods to achieve gene transfer [92]. However, these methods have not been as efficient and still have difficulty achieving sustained and stable expression. The Sleeping Beauty transposase (SB) system is a non-viral method; it is a synthetic transposon system, reverse engineered from defective copies in fish [93]. Sjeklocha and coworkers used SB to transduce human CD34+ cord blood cells. They saw integration and expression of

the beta-globin gene, and in studies with K562 cells, saw sustained transgene expression [94].

One of the greatest concerns with lentiviral gene therapy is random integration. Random transgene integration can potentially disrupt a tumor suppressor or cause activation of an oncogene. In trials for X-linked severe combined immunodeficiency, leukemia developed as a result of aberrant gene activation from random integration [95]. One method of preventing malignancy with lentiviruses is to analyze the insertion sites before transplantation and select those "safe harbor" sites which are least likely to cause endogenous gene perturbation. A safe harbor is an integration site that is more that 50–100 kb away from known coding, miRNA, and ultraconserved regions. In 2011, Papapetrou published a paper concerning genomic "safe harbors" and induced pluripotent stem cells (iPSCs). They found that about 10% of integrations occurred in safe harbors and permitted beta-globin gene expression [96, 97].

Another method is to have a failsafe way of getting rid of vector-transduced cells should they become malignant. In addition to the therapeutic gene, a suicide gene can also be transduced at the same time. If malignancy occurs, this suicide gene can be induced with drugs to cause apoptosis and ablate vector-transduced cells in the body. Two such suicide genes studied in the context of gene therapy are the herpes simplex virus type 1 thymidine kinase (HSVtk) and inducible caspase 9 (iCasp9) [98]. HSVtk-transduced cells can be eliminated with the phosphorylation of acyclovir or ganciclovir by HSVtk. iCasp9 is expressed as a monomer, but upon addition of AP1903, dimerizes and causes apoptosis. In a study with T-cells, iCasp9 effected immediate death, but HSVtk needed 3 days of treatment [99].

Last, site-specific integration—which does not disrupt other genes—is a new area being explored. Site-specific correction of the beta-globin gene has been done with iPSCs. Making iPSCs from thalassemic cells usually requires the addition of four factors: Oct4, Sox2, Klf4, and c-Myc, although a number of other gene combinations have been successfully tried [100, 101]. This field alone is a large area of research, and reprogramming can be done in a variety of ways: lentiviruses, episomes, nonintegrating viruses, synthetic RNA, or proteins. In 2009, Ye and colleagues showed iPSCs could be successfully generated from thalassemic patients and upon differentiation, could be stained for HbF [102]. Zou [103] and Sebastiano [104] showed in two separate papers that thalassemic iPSCs could undergo site-specific correction of beta-globin using zinc finger nucleases and homologous recombination. This provided the scientific basis for potentially non-integrating in situ correction. While the iPSCs generated in the above papers were done with random integration, it is possible to combine a non-integrating method of iPSC generation and site-specific correction, thus avoiding integration-associated genome toxicity. Most recently, Ma in 2013 used non-integrating episomal technology to create iPSCs and non-integrating TALEN to perform in situ correction [105]. All of these studies have been met with very limited success though, as iPSCs express extremely low and nowhere near therapeutic levels of beta-globin upon differentiation. Site-specific insertion into the adeno-associated virus preferred integration site (AAVS1) has also been done with limited success. In 2008, Howden and colleagues

used bacterial artificial chromosomes and components from adeno-associated virus to preferentially insert beta-globin into the AAVS1 in K562 cells [106]. Of the 36 insertion sites analyzed, only 6 of them (17%) occurred in AAVS1, and 5 out of those 6 were intact and functional.

Conclusion

Beta-globin gene addition strategies have come a long way in the past 25 years. Many different vectors with a wide-range of genetic elements have proven successful in preclinical tests and some will be tested in clinical trials. However, work still needs to be done to improve the safety and efficacy. Genomic toxicity and malignancy are some of the largest hurdles to overcome in order to move gene therapy to widespread clinical application. Consistently therapeutic transgene expression for those with thalassemia major is an additional problem. For those vectors that do prove safe and effective, research into increasing the number of engraftable lentiviral transduced cells would help with cases of insufficient mixed chimerism.

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Chapter 7
Reactivation of Fetal Hemoglobin for Treating β-Thalassemia and Sickle Cell Disease

Shuaiying Cui and James Douglas Engel

Abstract Reactivation of fetal hemoglobin (HbF) in adult hematopoietic cells has the potential for great clinical benefit in patients bearing deleterious mutations in the β-globin gene, such as β-thalassemia and sickle cell disease (SCD), since increasing the production of HbF can compensate for underproduction of β-globin chains (in β-thalassemia) and it can also disrupt sickle hemoglobin polymerization (in SCD). Thus for the past few decades, concerted efforts have been made to identify an effective way to induce the synthesis of HbF in adult erythroid cells for potential therapeutic relief from the effects of these β-globinopathies. Chemical inducers of HbF as well as a number of transcription factors that are able to reactivate HbF synthesis in vitro and in vivo in adult erythroid cells have been identified. However, there has been only limited success in attempts to manipulate either the drugs or regulatory proteins, and in only a fraction of patients, and there is wide variation in individual response to these drugs or transcription factors. These studies highlight the importance for understanding the molecular mechanisms underlying hemoglobin switching so that future studies can be designed to treat these disorders.

Keywords Gamma-globin • Repression • Activation • Therapy • Repressors • Mechanisms

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Introduction

Reactivation of fetal hemoglobin (HbF) in adult hematopoietic cells has the potential for great clinical benefit in patients bearing deleterious mutations in the β-globin gene, such as β-thalassemia and sickle cell disease (SCD), since increasing the production of HbF can compensate for underproduction of β-globin chains (in β-thalassemia) and it can also disrupt sickle hemoglobin polymerization (in SCD). Thus for the past few decades, concerted efforts have been made to identify an effective way to induce the synthesis of HbF in adult erythroid cells for potential therapeutic relief from the effects of these β-globinopathies. Chemical inducers of HbF as well as a number of transcription factors that are able to reactivate HbF synthesis in vitro and in vivo in adult erythroid cells have been identified. However, there has been only limited success in attempts to manipulate either the drugs or regulatory proteins, and in only a fraction of patients, and there is wide variation in individual response to these drugs or transcription factors. These studies highlight the importance for understanding the molecular mechanisms underlying hemoglobin switching so that future studies can be designed to treat these disorders.

Globin Gene Clusters

Vertebrates express multiple globin genes (of both α- and β-type) that are arranged in groups along the chromosomes and have recognizably similar sequences. In humans, there are two clusters of globin genes: the α-globin genes on chromosome 16 and the β-globin genes on chromosome 11 (Fig. 7.1). The human α-globin genes span about 30 kbp and include one embryonic ζ- and two identical α-globin genes [1, 2]. The human β-globin cluster is composed of five functional genes: one embryonic ε-globin, two genes, $^{G}\gamma$ and $^{A}\gamma$, that are expressed in mid- and late gestation which differ by a single amino acid, and two adult (δ- and β-) globin genes. These genes all differ in their 5′ untranslated regions and introns, but they differ most in their respective 3′ untranslated regions [3]. There are also several globin pseudogenes of unknown function. However, recent studies indicate that the hemoglobin beta pseudogene 1 (HBBP1) is actually functional during early embryogenesis and that a single base mutation in HBBP1 may be associated with β-thalassemia [4, 5].

In other species, the globin gene organization in these clusters and the distribution of the clusters on chromosomes differs from humans. For example, the mouse α-globin genes bear one embryonic ζ- and two identical α-globin genes near a telomere on chromosome 11 and the mouse β-globins, located on chromosome 7, express four developmentally regulated genes: the embryonic εy and βh1, and adult β_{major} and β_{minor} transcripts [6].

Expression of all of these genes is regulated in every vertebrate case by delocalized *cis*-regulatory DNA elements, called locus control regions (LCRs), which are invariably characterized by a group of developmentally stable DNaseI hypersensitive

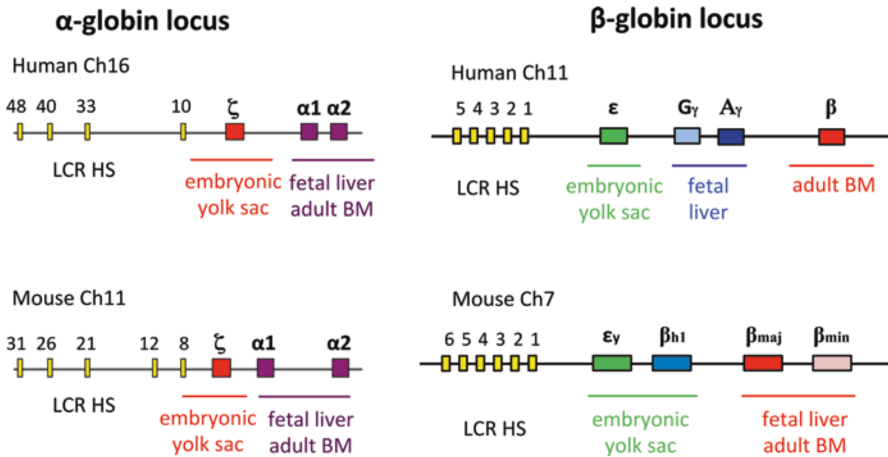

Fig. 7.1 Diagram of the α and β-Globin Loci. A physical map showing the relative positions of the human (*top*) and murine (*bottom*) α- and β-globin structural genes (*colored boxes*) as well as the location of the individual locus control region (LCR) DNaseI hypersensitive sites (DHS; *yellow boxes*) on their resident chromosomes are depicted. *BM* bone marrow

sites [7, 8]. Furthermore, the spatial organization of the genes directly reflects the temporal differentiation of their expression in different tissues during development, with the embryonic stage gene located closest to the LCR. For example, human ε-globin is the most 5′ gene and is expressed in the embryonic yolk sac, followed (in a 5′ to 3′ sense) by the $^{G}\gamma$- and $^{A}\gamma$-globin genes, which are expressed in the fetal liver. The δ- and β-globin genes are at the 3′ end of the whole group and are expressed in adult bone marrow [9]. Addition of the LCR results in high-level, copy number-dependent expression of linked globin or heterologous genes in the erythroid cells of transgenic mice [7]. If the globin genes are rearranged relative to the LCR, the globin gene transcripts are expressed at improper stages of development [10].

Hemoglobin Switching (Embryonic-Fetal-Adult)

Different types of globins are synthesized to form the tetrameric hemoglobin molecule, consisting of two α-like and two β-type globin chains, at different stages of development. Hemoglobin is the primary respiratory protein present in the red blood cells, and transports oxygen from the lungs to all the body tissues and also carries CO_2 from the tissue back to the lungs for respiratory exchange. More than 98% of the oxygen in blood is carried through hemoglobin [11, 12].

Each subunit of the hemoglobin molecule contains a globin polypeptide bound to a heme group. In human embryos, four different types of hemoglobin have been identified. Hb Portland-1 (ζ2γ2), Hb Gower-1 (ζ2ε2), and Hb Gower-2 (α2ε2), are the three most common embryonic hemoglobins synthesized by erythroid cells in

the embryonic yolk sac during the first few months of life [13–16]. However, Hb Portland-2 (ζ2β2), another embryonic Hb, is found infrequently and most usually in an extreme type of α-thalassemia ("hydrops fetalis") where ζ-chains substitute for α-chains when synthesis of the latter is severely impaired [17, 18].

During the second month of human gestation, the first switch in β-globin transcription results in the silencing of ε-globin and concomitant activation of fetal γ-globin when definitive erythroid cells first develop in the fetal liver. The association of two γ-globin chains with two α-globin chains produces fetal hemoglobin (HbF α2γ2), and thus the embryonic hemoglobins are gradually replaced by HbF [19–22]. The maternal blood that is supplied to the developing fetus is lower in oxygen than the blood present in normal arterioles. Hence, HbF has a higher affinity for oxygen as compared to adult hemoglobin. The presence of γ-globin chains instead of the β-globins of adult hemoglobin is one of the ways to achieve such higher affinity [23].

From the sixth month of human gestation, the synthesis of HbF declines, and that of adult hemoglobin increases. Gradually, around the time of birth, this second switch from γ- to β-globin transcription occurs as the site of hematopoiesis shifts again to the adult bone marrow [24]. At birth, about 50–80% of the hemoglobin is comprised of HbF, and its level continues to gradually decline after birth. By 6 months postnatally, HbF is reduced to about 8% of the total hemoglobin content.

The two major types of hemoglobin found in adult red blood cells are: HbA (α2β2) and HbA2 (α2δ2). From the age of 6 months, HbA is the predominant type of hemoglobin in humans and accounts for 95–98% of the total hemoglobin molecules. In contrast, HbA2 exists in only small amounts and makes up 2–3% of the total hemoglobin in adults. HbF is also synthesized in adults, and comprises between 0.8% and 2% of the total hemoglobin molecules [25–29].

From genetic analyses of transgenic mice harboring mutated human β-globin loci, two nonexclusive mechanisms for globin "switching" have been postulated: one is autonomous silencing of the embryonic ε- and fetal γ-globin genes by sequences located in their promoters, while the other is competition among the globin genes for activation by the LCR, the *cis* element located 5′ to the globin clusters that is required for abundant expression of all the β-type globin genes [24]. The competition model proposes that the LCR preferentially interacts with, and hence activates, the closest gene *unless* that gene is autonomously silenced, providing a mechanistic basis for silencing of the adult β-globin gene during the embryonic and fetal stages [30]. Thus, these mechanisms envision that opposing activating and repressing forces collectively determine globin gene transcriptional activity at every developmental stage. However, promoter sequences and transcription factors involved in the autonomous silencing of the embryonic ε- and fetal γ-globin genes were not identified until the most recent turn of the century. Identification and characterization of other regulatory factors and their interactions with transcription factors, the DNA region in the α- and β-LCRs, and further characterization of epigenetic modifications in chromatin should provide further details of γ-globin gene regulation and human hemoglobin switching [31].

Hemoglobin Variants and Hemoglobinopathies

Hemoglobinopathies are blood disorders caused by changes in the genetically determined molecular structure of hemoglobin; these have characteristic clinical and laboratory abnormalities that can result in conditions such as hemolytic anemia, sickle cell anemia and thalassemia.

Several hundred hemoglobin variants have been documented, but only a few are common and clinically significant. A few variants are the result of a single mutation in one of the globin genes. The resultant abnormal hemoglobin molecules give rise to a range of disorders, which are broadly grouped as α- or β-thalassemias.

Thalassemias are inherited blood disorders in which there is diminished hemoglobin production that results in anemia. Only in its most severe form do the RBCs lyse and have a shortened life span. The "β_{major}" form (also called Cooley's Anemia) may result in growth problems, jaundice, and severe anemia. In milder forms, where there is no hemolysis, this "β_{minor}" form (sometimes called β_{thal} trait) causes a mild anemia and is asymptomatic. β-thalassemia intermedia is yet another form of β_{thal} which exhibits moderate anemia and requires intermittent blood transfusions.

Some of the abnormal variants are described below, along with the globin chain that is affected and the resulting disease.

Hemoglobin H (β4) is formed by a tetramer of β chains, and hemoglobin Barts (γ4) is formed by a tetramer of γ chains, which are both most commonly present in variants of α-thalassemia.

Some of the most common hemoglobin variants in the β-thalassemias include hemoglobin C ($\alpha2\beta^{C}2$), which can cause mild hemolytic anemia, and hemoglobin E ($\alpha2\beta^{E}2$), which may either be asymptomatic or present with generally mild disease. In addition, when combined with β-thalassemia, hemoglobin E/β-thalassemia results in a Cooley's Anemia-like phenotype.

Hemoglobin S ($\alpha2\beta^{S}2$) is a variant form of hemoglobin found in individuals with sickle cell disease (SCD), which is generated as a consequence of a specific point mutation in the β-globin gene. This mutation causes a change in the "stickiness" of the hemoglobin tetramers for one another, which then leads to rapid and extensive polymerization inside the cell, which ultimately distorts the RBC membranes making them susceptible to lysis and vastly shorter lifetimes. Sickle cell "trait" (when an individual has one mutant and one wild type gene) can cause minor difficulties; sickle cell "disease" (when an individual bears two mutant genes) creates often severe clinical problems: these misshapen blood cells are physically unstable, leading to blocked blood vessels causing pain, anemia, and often severe organ-specific complications.

Fetal γ-Globin Induction as a Therapeutic Agent for SCD and β-Thalassemia

At present, only bone marrow transplantation (BMT) can cure SCD and β-thalassemia. However, given the limited availability of donors and the constraint of performing the procedure only in countries with well-developed health care delivery systems, BMT is not a widely available option.

Hereditary persistence of fetal hemoglobin (HPFH) is a genetic condition in which high-level synthesis of HbF aberrantly persists into adulthood [32]. When an HPFH allele is co-inherited with SCD, the elevated HbF production greatly mitigates the symptoms of the disease [33–36]. Clinical data indicate that induction of HbF to 10–20% of total hemoglobin, or a three to fivefold increase over the normal baseline, significantly alleviates the clinical complications of SCD [37, 38]. Such evidence led to the early hypothesis that fetal γ-globin has an ameliorating effect on SCD, and thus for the past three decades, concerted efforts have attempted to identify an effective way to induce HbF synthesis as a potential therapy for SCD.

To date, the agents that are best characterized that induce HbF include hydroxyurea (HU), an anticancer drug that inhibits ribonucleotide reductase (and thereby DNA synthesis) [39] and DNA methyltransferase inhibitors such as 5-azacytidine (another anticancer drug) [40, 41]. However, all of these are demonstrably inadequate to prevent the major complications of SCD, since only half or fewer of the patients treated with these drugs exhibit sustained long-term increments in γ-globin synthesis and amelioration of disease symptoms [42–44]. Furthermore, since the effects of currently available γ-globin inducers cause long-term incremental global alteration through epigenetic chromatin modifications, those agents are predicted to include various unfavorable cellular and systemic effects. These may include non-specific cytocidal effects on proliferating cells, and non-selective global gene de-repression, which may lead to adverse effects such as teratogenicity as well as bone marrow suppression and skin ulceration, all of which are often seen in patients treated with HU. Such adverse effects limit the therapeutic dosages, application, and clinical efficacy of those drugs. In summary, of numerous preclinical and clinical studies, none of the currently available drugs exhibit an optimal combination of efficacy, safety and broad applicability for SCD treatment. Therefore, it becomes necessary to search for more effective and less toxic SCD therapeutics.

β-thalassemia is characterized by diminished or absent β-globin protein. Correlative clinical studies support the contention that when HPFH is co-inherited with β-thalassemia, it can dramatically ameliorate the pathological effects of β-thalassemia [33–36]. The presence of elevated HbF in adulthood is capable of replacing reduced adult β-globin protein and can lessen the degree of globin chain imbalance that leads to free intracellular excess α-globin [45], which is thought to cause oxidative damage to the red cell membrane and apoptosis of erythroid precursors [46]. Therefore, a considerable number of studies have focused on reactivation of γ-globin gene expression in patients with β-thalassemia. Although many studies have shown clinical efficacy of HbF inducers such as HU, 5-azacytidine and

butyrates in patients with β-globin disorders, and the impact of these therapeutic agents on SCD patients was clear, but had only limited impact in β-thalassemia patients [47]. The discrepancy in efficacy of the γ-globin inducers between the two diseases may be due to the higher levels of γ-globin that are presumably required in β-thalassemia to achieve clinically beneficial effects [48, 49].

Chemical Inducers of Fetal Hemoglobin

It is firmly established that increasing the production of γ-globin leads to a beneficial decrease in adult β-chains and a consequent reduction of hemolysis. Accordingly, many studies have concentrated on potential inducers of HbF, which can be grouped into several classes based on their chemical structures and mechanisms of action, including cell-cycle specific agents (such as HU), histone deacetylase (HDAC) inhibitors, DNA methyltransferase (DNMT) inhibitors, monoamine oxidases (MAO) inhibitors, DNA-binding drugs, and inhibitors of the mTOR pathway.

Clinical studies with some of these HbF inducers have shown that they were effective, in a fraction of patients, in ameliorating clinical pathophysiological conditions. The increase in HbF in response to these drugs varies among patients with β-thalassemia and SCD due to individual genetic determinants [48, 50–55].

Hydroxyurea (HU)

HU is the most studied drug for the treatment of β-globinopathies and it is currently the only U.S. Food and Drug Administration (FDA) approved HbF-inducing drug for individuals with SCD. HU can be administered with minimal known side effects, has a relatively wide therapeutic window, and has mechanisms of action that counteract the pathophysiologic pathways of sickling, vaso-occlusion, hemolysis, and organ damage [56–65]. Patients with β-thalassemia could also benefit from HU that increases fetal and total hemoglobin levels and thereby decreases the need for transfusions [66–73]. HU stimulates HbF synthesis in sickle cell patients, which mechanism of action has been hypothesized to act through the cGMP [74–76], and cAMP [77, 78] signaling pathways. Recent data suggest that HU increases the production of nitric oxide (NO), a potent vasodilator, in sickle cell patients and HU induces fetal hemoglobin by the NO-dependent activation of soluble guanylyl cyclase [79–81]. However, the effect of HU on increasing the number of HbF-containing reticulocytes (F reticulocytes) is extremely variable and not all sickle cell patients treated with HU showed increases in HbF production [82, 83]. There are limited data regarding the ability of HU to prevent or diminish organ morbidity, and the long-term risks of HU therapy remain incompletely defined, suggesting that patients with SCD who have been treated for long periods with HU should be carefully monitored to reduce the risk of carcinogenicity. Recent findings on the safety and efficacy of HU in

infants (mean age 13.6 months) with symptomatic SCD showed that it significantly decreased pain and dactylitis, with some evidence for decreased acute chest syndrome, hospitalization rates, and need for transfusion, although the primary organ protective study end points (preservation of spleen and renal function) were not attained. HU increased hemoglobin and fetal hemoglobin, and decreased white blood-cell count, and toxicity was limited to mild-to-moderate neutropenia [84].

HDAC Inhibitors

Transcriptional regulation of gene expression is controlled by alterations in chromatin structure, such as methylation, phosphorylation and acetylation. HDAC enzymes remove acetyl groups from ε-*N*-acetyl lysine amino acid residues in the histones, leading to chromatin condensation and repression of transcription [85, 86]. It has been reported that inhibition of HDACs by one class of chemical compounds results in hyperacetylation of histones H3 and H4 that determine an open chromatin configuration, the binding of transcription factors and consequent induction of gene transcription [87–89].

Histone acetylation within the globin gene loci correlates with the state of transcription, with the active γ- and β-globin gene promoters being more highly acetylated than the inactive ε-globin promoter [90]. Numerous experimental data have indicated that inhibition of the activity of HDACs causes increased HbF synthesis. Several HDAC inhibitors, such as sodium butyrate, apicidin, scriptaid, trichostatin A (TSA) and thalidomide, have been shown to induce HbF synthesis in vitro [55, 85, 91–94].

Butyrate is a prototype of histone deacetylase inhibitors that is believed to reactivate silent genes by inducing epigenetic modifications. Orally administered butyrate compounds (sodium phenylbutyrate and isobutyramide) exhibited significant stimulation of HbF synthesis in a small number of β-thalassemia patients [95–97] as well as in pediatric and adult patients with SCD [98–100], although the mechanism of this induction has not been fully elucidated [101–115]. Since butyrate is an inhibitor of histone deacetylases, it was proposed that butyrate increases HbF levels by enhancing the transcription rate of the γ-globin gene via changes in histone acetylation at the level of critical promoter regions [48]. In addition to inhibiting the histone deacetylase itself, butyrate has also been postulated to modulate transcription factor binding to specific DNA sequences defined as butyrate response elements (BREs) [116], and butyrate has also been proposed to induce HbF by increasing the translation efficiency of γ-globin mRNA [117].

Apicidin, an antiprotozoan and antiproliferative agent [118, 119], has been identified as a potent inducer of HbF synthesis in the human K562 erythroleukemia cell line, leading to a tenfold stimulation of HbF expression at nanomolar to micromolar concentrations [94]. A comparative analysis of the histone acetylation patterns over approximately 70 kb within the β-globin loci in K562 cells showed that the level of histone H3 acetylation globally increased from the LCR to the promoter of the γ-globin gene upon apicidin treatment [120]. Additionally, p38 mitogen-activated

protein (MAP) kinase signaling was activated following apicidin treatment of cells, and inhibition of this pathway was shown to block the effects of apicidin-induced γ-globin expression and histone H3 acetylation [94, 120]. In addition, the binding of GATA-1, Sp1 and RNA polymerase II were shown to increase in several regulatory regions of the β-globin locus in apicidin treated cells [120].

Scriptaid is a histone deacetylase inhibitor that works in a wide variety of biological systems. It induces cell cycle arrest in colon cancer cells in culture and inhibits tumor growth in vitro and in vivo [121–123]. Scriptaid also facilitates the cloning efficiency and epigenetic reprogramming of the donor nucleus after somatic cell nuclear transfer [124–129]. Scriptaid induces γ-globin expression via p38 MAPK signaling in K562 cells and human erythroid progenitors, and the p38-selective inhibitor SB203580 reversed the ability of scriptaid to induce HbF. In vivo studies in human β-YAC transgenic mice also showed that scriptaid induced reticulocytosis and human γ-globin mRNA synthesis [93].

Trichostatin A reactivates developmentally silenced γ-globin expression in somatic cell hybrids and induces γ-gene expression and concomitantly downregulated β-globin expression in adult primitive erythroid progenitor cells (BFU-e, burst forming unit-erythroid) cultures, as determined by both mRNA quantification and immunofluorescent quantification in γ-globin expressing cells. In addition, trichostatin A induced γ gene expression in adult BFU-e cultures in a maturation-independent fashion [130]. Valproic acid and trichostatin in combination with hemin (all three FDA-approved drugs) preferentially increase γ-globin chain synthesis up to tenfold [57]. It was also reported that a sodium butyrate and trichostatin A combination induces both embryonic and adult-type globin mRNAs in two murine interleukin-3-dependent bone marrow-derived cell lines [102].

Thalidomide, an inexpensive agent with relatively low cytotoxicity, is regarded as a promising therapeutic candidate, especially for malignant diseases [131]. Thalidomide induces γ-globin gene expression through increased reactive oxygen species-mediated p38 MAPK signaling and histone H4 acetylation in adult erythropoiesis [55]. Pomalidomide is a derivative of thalidomide, which was approved in February 2013 by FDA as a treatment for relapsed and refractory multiple myeloma. Recent studies demonstrated that pomalidomide reduced or eliminated transfusion requirements in certain hematologic malignancies and induced HbF ex vivo in $CD34^+$ progenitor cells from healthy and SCD donors [132]. In addition, pomalidomide augmented fetal hemoglobin production without the myelosuppressive effects of HU in transgenic sickle cell mice [133].

DNMT Inhibitors

DNA methylation is another epigenetic modification that occurs on cytosines in CpG islands, usually situated within promoter regions of genes. Methylation of DNA regions prevents the binding of transcription factors to thereby repress transcription. Dnmt1 (DNA methyltransferase 1) is the principal enzyme responsible

for maintenance of cytosine methylation at CpG dinucleotides in the mammalian genome, which is a promising epigenetic target for the development of novel anti-cancer drugs and perhaps for other diseases.

5-azacytidine (5-Aza) was the first DNMT inhibitor approved by the FDA for therapy of β-thalassemia and SCD [41, 134–137]. 5-Aza is a cytidine analogue that is capable of activating repressed genes in tissue-culture cells and has been shown to increase HbF production in anemic baboons [40, 138]. 5-Aza results in hypo-methylation of bone marrow DNA near both the γ-globin and ε-globin promoters and selectively increases γ-globin synthesis approximately sevenfold in a patient with severe β-thalassemia [41]. 5-Aza acts directly on both erythroid precursors and progenitors to increase production of HbF [139]. Increased production of HbF was observed in a patient with sickle cell anemia treated with 5-aza [140]. Increased HbF production was associated with demethylation of two restriction enzyme sites 5′ to the two γ-globin genes. Methylation at five other restriction sites around the two genes was unchanged [134–136, 141–143].

Decitabine (5-aza-2′-deoxycytidine) is a safer derivative of 5-Aza and functions in a similar manner by hypomethylating DNA as a consequence of inhibiting DNA methyltransferase. Decitabine could be effective in increasing HbF in patients with sickle cell anemia who failed to increase HbF with HU [144–146]. Sustained HbF levels without cumulative toxicities was observed after dose interval treatment of patients with sickle cell anemia [147], and weekly subcutaneous decitabine produces cumulative increases in HbF and total hemoglobin through a non-cytotoxic mechanism of action [148]. Decitabine also produces equivalent augmentation of HbF levels in baboons [149–152] and in adult and child patients with advanced solid tumors [153–159].

MAO Inhibitors

Monoamine oxidases (MAO) are family of enzymes that catalyze the oxidation of monoamines, and MAO inhibitors (MAOI) have therapeutic value for several conditions including depression, stroke, neurodegenerative diseases and aging [160]. One of the classical non-selective MAOIs tranylcypromine (TCP) has recently been reported as a new γ-globin inducer [161]. HbF synthesis was enhanced by TCP from 4.6% of total hemoglobin to 31% upon in vitro differentiation of human primary CD34+ cells. In addition, TCP treatment resulted in statistically significant enhancement of H3K4me2 accumulation at the γ-globin promoter, indicating that one possible mechanism for γ-globin induction by TCP is H3K4me2 accumulation at its promoter through LSD1 inhibition [161]. However, since a growing number of transcription factors have now been shown to associate with LSD1, these effects could be mediated through any of these transcription factor proteins [162].

DNA-Binding Drugs

Small molecules that bind to DNA have great clinical significance as anticancer drugs. The DNA-binding drug mithramycin is a potent inducer of γ-globin mRNA accumulation and HbF production in erythroid cells from healthy human subjects and β-thalassemia patients [163]. Angelicin and its analogs are able to induce erythroid differentiation in K562 cells as well as leading to elevated expression of γ-globin genes in human erythroid precursor cells, with only small effects on apoptosis [164, 165] when compared with other known HbF inducers, such as cytosine arabinoside [166] or cis-platin [167–170].

mTOR Inhibitors

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription [171, 172]. Rapamycin is an mTOR inhibitor that possesses immunosuppressive, antifungal and anti-tumour properties. When normal human erythroid precursors were cultured in the presence of rapamycin, γ-globin mRNA accumulation and HbF production increased to levels that were higher than those obtained with HU, and these effects were not associated with inhibition of cell growth. Furthermore, rapamycin was found to increase HbF content in erythroid precursor cells derived from β-thalassaemia patients [173, 174].

Transcriptional Regulation of the γ-Globin Genes

Differential β-globin gene expression during erythropoiesis represents a complex differentiation process employing differential transcription factor utilization during mammalian hematopoiesis, with the imposition of co-activators and co-repressors functioning as requisite activities to elicit gene-specific transcriptional responses adding to both the complexity and specificity of gene regulation. Elucidation of the molecular basis for γ-globin gene silencing during the adult stage of erythropoiesis in particular has been the focus of intense investigation, since it has been observed that coinheritance of genetic conditions that confer elevated γ-globin synthesis can significantly alleviate the symptoms and pathology of inherited β-globin disorders [33, 34, 175].

Recently, a growing number transcription factors, such as GATA1, FOG, EKLF, NF-E2, NF-E4, Ikaros, SOX6, BCL11A, FOP [176] and Myb [177], as well as nuclear receptors COUP-TFII [178] and TR2/TR4 have been implicated as adult-stage γ-globin gene transcriptional repressors.

GATA1 is an erythroid cell-specific transcription factor required for the activation of globin and other erythroid genes as erythroid cell maturation progresses. GATA1 binds to DNA elements in the β-LCR and to the promoters of many erythroid cell-specific genes, and is required for the normal differentiation of erythroid cells. Other GATA-binding proteins cannot fully compensate for the loss of GATA1 in erythropoiesis [179–184]. GATA1 and its cofactor, FOG-1, are required for the physical interaction between the β-globin LCR and the β_{major}-globin promoter [185]. Recent reports suggested a role of GATA1, FOG-1, and the associated chromatin remodeling complex NuRD (or Mi2β) in the developmental silencing of γ-globin gene expression [186–188].

EKLF plays a critical role in β-globin gene activation via its interactions with CBP/p300 and SWI/SNF proteins [189–192]. It also interacts with the mSin3A and HDAC1 corepressors via its zinc finger domain [193, 194].

NF-E2 binds to AP-1-like recognition sites in the upstream regulatory regions of the α- and β-globin loci, and is also essential for activation of the adult β-globin gene although mice deficient in NF-E2 exhibit only mild erythroid defects [195–198]. NF-E2-related factor 2 (NRF2) is a NF-E2-like basic leucine zipper transcriptional activator that binds to an NF-E2/AP1-related motif in the beta-globin LCR [199] and can induce human HbF via the NRF2 antioxidant response signaling pathway [200].

NF-E4 has been shown to play critical roles in chicken globin gene switching and in human fetal globin gene activation [201–204]. Enforced expression of NF-E4 in the human K562 cell line and human cord blood progenitors leads to increased fetal globin gene expression [205]. Site-specific acetylation of NF-E4 prevents its ubiquitination and regulates its interaction with HDAC1 [206].

Ikaros appears to play a major role in the formation of the β-globin active chromatin hub (ACH), and in hemoglobin switching [207]. Ikaros null mutant mice have multiple hematopoietic cell defects including anemia and megakaryocytic abnormalities, and exhibit a delay in murine embryonic to adult β-globin switching and a delay in human γ- to β-globin switching [208]. Point mutation in the DNA-binding domain of Ikaros leads to marked down-regulation of β-globin gene, and up-regulation of γ-globin gene expression [207]. Ikaros reportedly interacts with GATA-1 and enhances the binding of the latter to different regulatory regions across the locus, which may be required for silencing of the human γ-globin genes [209].

Sox6 is a subfamily member of sex determining region y-related transcription factors that are characterized by a conserved high mobility group (HMG) DNA-binding domain and by their ability to bind in the minor groove of DNA [210]. Sox6 is essential for silencing of εy-globin gene expression in adult definitive erythropoiesis and is required for normal erythrocyte maturation [211, 212]. BCL11A and SOX6 interact physically and functionally during erythroid maturation, possibly through co-occupation of sites within the human β-globin locus along with GATA1 [213], and cooperate in silencing γ-globin transcription in adult human erythroid progenitors [214].

BCL11A has been identified as a potential regulator of HbF expression through genome-wide association studies looking for genetic association with variable HbF levels. The high-HbF genotype is associated with reduced BCL11A expression

[215–217]. Abundant expression of the full-length form of BCL11A is developmentally restricted to adult erythroid cells. Down-regulation of BCL11A expression in primary adult erythroid cells leads to robust HbF induction [218]. Developmental silencing of the mouse embryonic globin and human γ-globin genes fails to occur in β-YAC mice in the absence of BCL11A [219, 220]. Recent reports showed that BCL11A expression is activated by EKLF, leading to γ-globin repression [221]. Knockdown of EKLF in human and mouse adult erythroid progenitors and in an in vivo mouse model markedly reduces BCL11A levels and increases human γ-/β-globin expression ratios [222, 223]. Moreover, an erythroid enhancer of BCL11A subject to genetic variation that determines HbF level was recently identified. Disruption of this enhancer reduced transcription factor binding, modestly diminished BCL11A expression, and led to elevated HbF in erythroid precursors [224]. BCL11A was also found in a proteomic screen within multiprotein complexes consisting of erythroid transcription factors, transcriptional corepressors, and chromatin-modifying enzymes [225].

TR2/TR4 were previously shown to bind in vitro to direct repeat (DR) elements in the mouse and human embryonic and fetal β-type globin gene promoters and to play critical roles in the silencing of these genes [226–228]. TR2/TR4 bind to the embryonic β-type globin gene promoters but not to the adult β-globin promoter in adult erythroid cells. TR2/TR4 recruit an array of transcriptional corepressors (DNMT1, NuRD, and LSD1/CoREST repressor complexes, as well as HDAC3 and TIF1β, all known to confer epigenetic gene silencing) to elicit adult stage-specific silencing of the embryonic β-type globin genes through coordinated epigenetic chromatin modifications. Coimmunoprecipitation assays of endogenous proteins indicated that TR2/TR4 complexes consist of at least four distinct molecular species, and TR2/TR4 associated with DNMT1 and LSD1 form a tetrameric core complex to which the NuRD or CoREST complex, HDAC3, or TIF1β binds in a mutually exclusive manner [162].

Interestingly, forced erythroid-specific transgenic expression of TR2 and TR4 unexpectedly led to *induction* of the fetal γ-globin genes in adult erythroid cells of YAC transgenic mice [228] suggesting either that TR2 and TR4 could elicit unanticipated transcriptional effects as activating transcription factors or that the addition of excess TR2 and/or TR4 could squelch the effects of limiting co-repressors by dilution. Consistent with that unexpected observation, forced TR2/TR4 expression in a humanized SCD model mouse lead to elevated γ-globin gene expression coincident with abundant HbF synthesis and alleviation of hematological and pathological indications of SCD without any apparent adverse effects [229].

Summary

In addition to conventional blood transfusion therapy, current treatment of β-thalassemia and SCD includes inducers of HbF synthesis (HU, azacytidine and butyrate). However, because of concerns about dose-limiting myelotoxicity, potential carcinogenicity and high cost of the above agents, an intensive search for

less toxic and more effective drugs is ongoing. As in other current pharmaceutical therapies, the combined use of HbF inducers might also be useful to maximize HbF production while at the same time reducing the side effects of higher concentrations of either drug alone. There is wide variability in the response to these non-specific and less (mechanistically) understood approaches to the augmentation of HbF synthesis and the reasons for this are largely unknown. Fully understanding the genetic and epigenetic factors underlying the variability in therapeutic benefits of these regulators for β-thalassemia and SCD relief is critical for prospectively predicting good responders to specific therapeutics and for designing newer and more effective therapies.

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Chapter 8
Genome Editing for the β-Hemoglobinopathies

Matthew H. Porteus

Abstract The β-hemoglobinopathies are diverse set of disorders caused by mutations in the β-globin (*HBB*) gene. Because HBB protein is a critical component (along with α-globin, heme, and iron) of hemoglobin, the molecule essential for oxygen delivery to tissues, mutations in *HBB* can result in lethal diseases or diseases with multi-organ dysfunction. *HBB* mutations can be roughly divided into two categories: those that cause a dysfunctional protein (such as sickle cell disease but also including varied diseases caused by high-affinity hemoglobins, low-affinity hemoglobins, and methemoglobinemia) and those that cause the insufficient production of HBB protein (β-thalassemia). Sickle cell disease and β-thalassemia are both the most prevalent and the most devastating of the β-hemoglobinopathies.

Keywords β-hemoglobinopathies • β-thalassemia • *HBB* gene • Nucleases • Sickle cell disease • Genome editing

The β-hemoglobinopathies are diverse set of disorders caused by mutations in the β-globin (*HBB*) gene. Because HBB protein is a critical component (along with α-globin, heme, and iron) of hemoglobin, the molecule essential for oxygen delivery to tissues, mutations in *HBB* can result in lethal diseases or diseases with multi-organ dysfunction. *HBB* mutations can be roughly divided into two categories: those that cause a dysfunctional protein (such as sickle cell disease but also including varied diseases caused by high-affinity hemoglobins, low-affinity hemoglobins, and methemoglobinemia) and those that cause the insufficient production of HBB protein (β-thalassemia). Sickle cell disease and β-thalassemia are both the most prevalent and the most devastating of the β-hemoglobinopathies.

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Sickle cell disease is an autosomal homozygous recessive disease caused by a single point mutation in codon 6 of the *HBB* gene in which an adenine (A) is mutated to a thymidine (T) [1]. The essentially non-pathologic heterozygous form of sickle cell disease is called sickle trait. This results in a glutamic acid (charged amino acid) being changed to a valine (a polar amino acid) at the protein level [2].

While the codon 6 glutamic acid to valine change does not affect the affinity of hemoglobin for oxygen or results in the impaired production of HBB protein, it does result in a pathologic dysfunction of the protein leading to the formation of stiff rods of hemoglobin through the polymerization of hemoglobin in the deoxygenated state through hydrophobic interactions [3, 4]. These stiff polymers disrupt the normal, flexible bi-concave shape of the red blood cell often giving it a shape that looks like a sickle [5]. These stiff, sickle RBCs are impaired in their ability to squeeze through the small capillaries and venules of the vascular system, thereby forming occlusions leading to impaired oxygen delivery to tissues. Moreover, the abnormally shaped RBCs are cleared by the reticulo-endothelial system causing a significant anemia (sickle cell disease is also called sickle cell anemia because of the anemia) that can exacerbate the impaired delivery of oxygen to tissues. The impaired oxygen delivery can affect essentially any organ in the body including the brain (both overt and silent strokes and neuro-cognitive deficits), devastatingly painful veno-occlusive crisis of the bone, acute chest syndrome, and chronic kidney damage. Even with contemporary care, patients with sickle cell disease are subject to repetitive veno-occlusive crises leading to extensive morbidity and pre-mature mortality with an average lifespan for both men and women in the mid-40's [6–8]. Sickle cell disease affects >100,000 people in the United States and probably tens of millions of people worldwide, particularly in Africa and India.

In contrast to the pathologic dysfunction of HBB protein in sickle cell disease, β-thalassemia is caused by mutations in the *HBB* gene that cause the insufficient production of HBB protein. Like sickle cell disease, it is also an autosomal homozygous recessive disease. The non-pathologic heterozygous form of β-thalassemia is called β-thalassemia trait. Normally, HBB pairs with α-globin (HBA) in a one to one ratio to form the tetrameric hemoglobin molecule. But with the insufficient production of HBB, unpaired α-globin chains precipitate thereby causing toxic death to the developing erythrocyte or erythrocyte precursor (ineffective erythropoiesis) and leading to the insufficient formation of mature RBCs. The ineffective erythropoiesis leads to anemia. If the anemia is severe such that transfusions are needed to maintain sufficient oxygen carrying capacity in the blood for life, then the patient is classified as having β-thalassemia major (or Cooley's Anemia). β-thalassemia affects >1000 people in the United States but millions of people worldwide including in countries surrounding the Mediterranean sea (hence its name "around the sea"), southeast Asia and China.

The only curative treatment for sickle cell disease and β-thalassemia is allogeneic hematopoietic stem cell transplantation (allo-HSCT) [9–11]. In allo-HSCT, the patient's hematopoietic system is eliminated by high doses of myeloablative chemotherapy (conditioning regimen) and replaced with an immunologically matched hematopoietic system from a donor without the disease. Even using donors with

sickle and β-thalassemia trait can cure these diseases. When a matched sibling donor is used, the cure rate can be >95% [11]. There are several major limitations to allo-HSCT, however, which has precluded its use to just a small fraction of patients that could benefit. These include the lack of availability of immunologically matched donors, the toxicity of the conditioning regimen, the complications from graft vs host disease (GvHD), and the transient but prolonged immunodeficient state following allo-HSCT leading to high risk of lethal infections. An important point is that the replacement of the hematopoietic system, particularly the erythroid system, with cells that are corrected for the disease causing mutation, even in only one allele, can cure the disease. For this reason allo-HSCT has been called "allogeneic gene therapy [9]." An alternative, therefore, to curing the β-hemoglobinopathies using allogeneic cells in which one allele is corrected, is to use genome editing to correct one allele of autologous cells and use the genetically corrected autologous hematopoietic stem cells (HSCs) to cure the patient.

Genome Editing

Genome editing is the precision modification of the nucleotide sequence of the genome. The major approaches to genome editing are to use AAV vectors and to use engineered nucleases. For reasons that remain unclear, AAV vectors will integrate in a targeted fashion into the genome at much higher frequencies (up to 1%) than other DNA vectors [12]. For the focus of this review, however, I will focus on genome editing using engineered nucleases.

Engineered nucleases are designed to create a DNA double-strand break (DSB) at a specific genomic site. This DSB activates the cell's own endogenous DSB repair mechanisms. If the cell repairs the DSB by non-homologous end-joining (NHEJ), a mechanism that conceptually stitches the two broken ends together, small insertions/deletions (in/dels) can be created at the site of the break. The NHEJ process is usually quite accurate in its repair, and it is only after several cycles of cutting and repair do in/dels occur. The sequence of the in/dels is random but the precise location is determined by the specificity of the engineered nuclease. By creating small mutations at a precise location, one can use genome editing to mutate genetic elements, usually coding regions of genes. The use of genome editing to knockout genes has already advanced to clinical trials as investigators have used zinc finger nucleases to mutate the CCR5 gene as a method to create HIV resistant T-cells in HIV infected patients [13–15].

On the other hand, the cell can repair the induced DSB by homologous recombination (HR) (the mechanism by which cells choose to repair a DSB by NHEJ vs HR are increasingly being worked out but still not completely understood [16]). In HR, the cell uses a "copy and paste" mechanism in which it uses an undamaged DNA molecule that has regions of near identity to the damaged site as a template to synthesize new DNA and then recombine the newly synthesized into the damaged site. By providing an appropriately designed "donor" DNA molecule for the cell's HR

machinery to use, precise nucleotide change to the genome, including single basepair changes up to tens of kilobases of new DNA, can be introduced at the site of the break. In this way, HR-mediated genome editing gives both spatial precision, like NHEJ mediated editing, and nucleotide precision to the editing process. The reason the cell chooses to use a provided donor DNA molecule as a template for the HR process is not well understood but it is known that by introducing more donor DNA into the cell, which increases the frequency of HR mediated editing [17–19].

In sum, a fundamental feature of genome editing using engineered nucleases is that the process depends on the use of cellular DSB repair machinery to fix a DSB that has been created by an engineered nuclease at a specific location in the genome. There are now multiple different engineered nuclease platforms available for this process.

Engineered Nucleases

The ability to engineer a nuclease to recognize a specific site in the genome was once the major limitation in using genome editing for both research and therapeutic purposes but is no longer. There are now four fundamental and several hybrid engineered nuclease platforms, which are briefly described here with references to more complete descriptions included.

Meganucleases (Homing Endonucleases)

Meganucleases (named because they have long (>18 basepairs) recognition sites derive from a large family of natural genetic parasites that use DSB mediated homologous recombination to catalyze their transfer among organisms [20]. One of the founding members of the homing endonuclease family, I-SceI, was a critical reagent in the discovery of many of the basic principles of genome editing. While there are extremely large number of natural homing endonucleases that recognize a wide variety of long target sites, it has been a significant challenge to re-engineer this class of enzyme to recognize unique target sites within the human genome. To date, no engineered meganuclease has been reported that recognizes a target within the *HBB* gene.

Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases are artificial hybrid proteins in which a zinc finger DNA binding domain is fused to the non-specific nuclease domain derived from the FokI Type II S restriction endonuclease (Fn) [21, 22]. The zinc finger domain, which consists

of an array of 3–6 individual zinc fingers, determines the sequence specificity of the ZFN. Since each individual zinc finger mediates binding to a 3–4 nucleotide sequence, the target site length can range from 9–18 bps. The Fn domain requires dimerization in order to create a DSB and thus in order to get a break in the genome, two ZFNs must be engineered to bind to specific target sites in order to orient the Fn domain properly to mediated cutting of the genomic target. While this dimerization requirement creates additional challenges in engineering two high quality ZFNs (high quality meaning both high on-target binding activity and low off-target binding activity), the dimerization requirement also provides an important degree of specificity as to get efficient cutting of the DNA you need two ZFNs to bind to the target sequence (creating an "AND" gate). There are a number of different methods to engineer the zinc finger DNA binding domain including modular assembly based approaches [23, 24], selection based approaches [25, 26] and hybrid approaches [27]. All of these approaches have had examples of success but currently the method that has had the most consistent track record of success is a proprietary approach developed first by Gendaq Ltd that was then purchased by Sangamo Biosciences. The proprietary approach involves a combination of a selection strategy developed by Isalan and Choo [28], followed by modular-assembly and then refined by rational design. The first genome editing clinical trials have used ZFNs designed this way [15]. There have been ZFNs that have been reported to target the *HBB* gene using other approaches but these have generally shown relatively poor activity [29, 30]. ZFNs that target the *HBB* gene using the full proprietary and expensive Sangamo strategy have been reported at meetings but have not been published.

TAL Effector Nucleases (TALENs)

TALENs, like ZFNs, are artificial, engineered, synthetic nucleases that fuse a DNA binding domain to the Fn domain [31, 32]. Like ZFNs, because they share the Fn nuclease domain, TALENs also require dimerization in order to create DSBs. The key difference between ZFNs and TALENs is that TALENs use a different DNA binding domain. TALENs use TAL effector repeats to mediate DNA binding. Each TAL effector repeat consists of 33–34 amino acids in which only two amino acids differ (the "repeat variable diresidue" or "RVD"). Each repeat mediates binding to a single basepair and the specificity is determined by the RVD TAL effector code. Table 8.1 summarizes the RVD code [33, 34]. To create a TALEN the desired target site is identified and then the appropriate array of RVD is assembled using either gene synthesis or an assembly protocol. In contrast to ZFNs, the success rate for active TALEN pairs is quite high with >30% of assembled pairs having reasonable activity at their intended target site in cell lines. This high success rate using a platform that was accessible to a wide range of researchers was an important aspect that prepared the genome editing landscape for the explosion of interest that came with the development of RNA-guided endonucleases (see below). Highly active TALEN

Table 8.1 TAL effector code

RVD	Base recognized
NN, NH, or NK	Guanosine
HD	Cytosine
NI	Adenine
NG	Thymidine

pairs have been engineered to recognize the human *HBB* locus and the results of these studies are described in more detail below [35].

RNA Guided Endonucleases (CRISPR/Cas9)

In contrast to meganucleases, ZFNs, and TALENs, RNA guided endonucleases of the CRISPR/Cas9 family (hereafter called "CRISPR") mediated target specificity through Watson-Crick nucleic acid hybridization rather than through protein-DNA recognition [36, 37]. The CRISPR system used in genome editing has been adopted from the system that bacteria use as their "adaptive" immune system to resist invasion by foreign nucleic acids including phage and plasmids. In genome editing, the system consists of two components: (1) An RNA guide molecule that has a twenty nucleotide sequence that is designed to hybridize with the desired genomic target and a tail that mediates interaction with the Cas9 protein; and (2) A multi-functional Cas9 protein that complexes with the guide strand RNA, unwinds the target duplex DNA, and if the guide RNA sequence hybridizes to the genomic DNA, then cuts the target DNA on both strands. Amazingly this guide RNA/Cas9 complex works autonomously in every cell type tested (from bacteria to yeast to cell lines to primary cells) to mediate guide strand directed cutting of the genomic target. In contrast to the other nuclease platforms that create DSBs with different types of overhangs (3′ overhang for meganucleases, 5′ overhang for ZFNs, and variable overhangs with TALENs) the CRISPR system creates blunt breaks. CRISPR's using guide RNAs designed to target the human *HBB* gene have been designed and have shown both remarkable activity in editing the *HBB* locus with remarkable specificity where several of the guide RNAs stimulated editing of the *HBB* gene without having any activity at the nearly identical *HBD* gene [38].

Hybrid Nuclease Platforms

In addition, to the four platforms described above, hybrid platforms have also been developed. "MegaTALs" consist of fusing a short TAL effector DNA binding domain to an engineered meganuclease [39]. "Dimeric CRISPR Nucleases" consist of fusing the Fn domain to a nuclease inactive Cas9 protein such that the nuclease

activity of the protein derives entirely from the Fn domain [40, 41]. Similar to ZFNs and TALENs, because the Fn domain requires dimerization to cut DNA, the dimeric CRISPR nuclease platform requires two guide RNAs to bring the Cas9-Fn fusion into the appropriate location to allow dimerization and cutting by the Fn domain. There have been no published reports of either of these hybrid nuclease platforms targeting the *HBB* gene.

Genetically De-Repressing γ-Globin

For both sickle cell disease and b-thalassemia, upregulating γ-globin to create higher levels of HgbF would be therapeutically relevant. Epidemiologic studies have shown that sickle cell disease patients with higher HgbF levels have lower disease severity, though still suffer from some of the manifestations of the disease. Pathophysiologically the mechanism is that HgbF inhibits the HgbS polymerization in the deoxygenated state. HgbF inhibits polymerization because it does not contain the valine at position six and it has a charged residue (Glutamine 87) that prevents the lateral valine hydrophobic interaction that generates the polymerization. The upregulation of γ-globin would also be therapeutically important for β-thalassemia as the extra γ-globin chains would pair with α-globin chains creating HgbF and thereby decreasing the amount of unpaired α-globin chains to precipitate and thereby decreasing the ineffective erythropoiesis and improving the anemia.

Knocking Out BCL11A As Genome Editing Approach to Increase HgbF

As noted above, patients with sickle cell disease have variable levels of HgbF expression. It was known that part of the genetic variability in HgbF expression was the result of genetic variants within the β-globin gene cluster itself. Genome wide association studies (GWAS), however, identified two additional quantitative trait loci for HgbF expression: BCL11A and MYB [42, 43]. Subsequent studies in mouse have shown that the bi-allelic disruption of BCL11A in either all of hematopoietic lineages or specifically in the erythropoietic lineage results in significant derepression of human γ-globin genes (engineered into mice through a β-globin gene cluster YAC) [44, 45]. γ-globin was derepressed in these studies to a level that would be therapeutically relevant but only when both alleles of *BCL11A* were mutated. Given this mixture of human and mouse human genetics, targeted disruption of *BCL11A* using engineered nucleases is now being pursued. There are several important potential caveats to mutating *BCL11A* in HSCs, however. First, no healthy human has been reported with bi-allelic knockout in *BCL11A* and mouse genetic studies suggest that bi-allelic knockout will lead to significant hematopoietic stem cell and lymphoid defects. Second, the strategy required bi-allelic *BCL11A* knockout to

upregulate γ-globin and thus raises the technical bar to achieve clinical success. Finally, *BCL11A* derives its name because multiple B-lymphoid leukemias have translocations involving *BCL11A*, thus demonstrating that *BCL11A* is a proto-oncogene. Since engineered nucleases can generate translocations, albeit at a low frequency, the strategy of targeting a gene associated with leukemia translocations might not be safe [46].

Knocking Out the Erythroid Specific Enhancer of BCL11A by Genome Editing to Increase HgbF

A potential solution to the stem cell and lymphoid defects seen by mutating *BCL11A* would be to eliminate BCL11A expression specifically in the erythroid lineage. Bauer et al. through a series of human genetics and genomic studies identified a putative intronic erythroid specific enhancer for BCL11A expression in the erythroid lineage [47]. Lee et al. originally had shown that specific chromosomal deletions could be created using a pair of engineered nucleases [48]. Thus, Bauer et al. utilized the idea of creating a defined chromosomal deletion using engineered nucleases to specifically delete the ~11 kb region containing the putative erythroid specific enhancer. They found that bi-allelically deleting this region in erythroid cells but not in lymphoid cells, dramatically decreased expression of BCL11A [47]. As expected, the elimination of BCL11A from the erythroid cells resulted in the de-repression of fetal hemoglobin genes. Thus, engineered nuclease mediated genome editing directly validated the inferred activity of the genetic element. Ongoing studies using engineered nucleases are being used to reduce this element to its minimal essential regions. Moreover, the approach also identified the possibility that genome editing could be used to derepress γ-globin by specifically eliminating expression of BCL11A in the erythroid lineage by deleting this genetic element. The specificity of eliminating BCL11A expression only in erythroid cells would solve the potential problem of HSC and lymphoid defects created by mutating *BCL11A* itself. This strategy, however, would not solve the potential technical problems of achieving bi-allelic deletions and safety problems of potentially creating *BCL11A* oncogenic translocations.

Using Genome Editing to Generate HPFH Genotypes

Hereditary persistence of fetal hemoglobin (HPFH) is a spontaneous human genotype in which alterations in the β-globin gene cluster lead to persistent expression of γ-globin into adulthood. That is, instead of undergoing the normal hemoglobin switch from γ-globin to β-globin within the first year of life, these people maintain high levels of fetal hemoglobin throughout life. People with HFPH are normal and seem to have no pathologic consequences of sustained high levels of HgbF

expression. Importantly, there are rare examples in which a HPFH haplotype has occurred in the presence of homozygous HgbS and these patients have mild disease with almost normal hematologic parameters [49]. While there have been no reports of HPFH combined with homozygous β-thalassemia major, there have been reports of trans-heterozygotes and these healthy people have extremely high levels of HgbF [50]. Thus, these rare examples validate HPFH as a genetic target for genome editing for the β-hemoglobinopathies.

The genetic basis of HPFH is variable and can be classified into deletional and non-deletional forms. Both forms are characterized by the generation of high HgbF in the heterozygous state, that is, only one chromosome 11 needs to have the HPFH genotype in order to generate high levels of γ-globin. There are a myriad of different *deletional* forms of HPFH in which several kilobases to hundreds of kilobases of the β-globin cluster are deleted. Thus, a genome editing approach would be to use engineered nucleases to recreate these deletions in patient derived HSCs. Alternatively, genome editing could be used experimentally to narrow the human deletions down to the smallest functional region and then use engineered nucleases to create this smaller deletion in patient derived HSCs. There are also a number of *non-deletional* forms of HPFH. These are point mutations that lie in the promoter region of the γ-globin genes. Whether these point mutations create binding sites for new transcriptional activators or eliminate binding sites for transcriptional repressors is not clear although genome editing will be the research approach to distinguish between the two. Nonetheless, if one can achieve high frequencies of genome editing by homologous recombination in HSCs, one can imagine knocking-in these non-deletional forms of HPFH in patient derived HSCs. Whether using homologous recombination to create non-deletional HPFH is more effective than using homologous recombination to directly correct the *HBB* gene defect, however, remains to be discovered.

Gene Correction of Induced Pluripotent Cells

One of the most exciting discoveries of the last decade, and recognized with the awarding of the Nobel Prize in Physiology or Medicine to Shinya Yamanaka in 2012, was that somatic cells could be re-programmed into embryonic stem cell-like cells using a small defined cocktail of transcription factors [51]. These reprogrammed cells are called induced pluripotent cells or iPSCs. Not only does this method of deriving pluripotent cells eliminate the ethical issues surrounding generating such cells from pre-implantation embryos, it creates the opportunity of easily creating pluripotent cells from any individual without having to use nuclear transfer into ES cells. In addition, iPSCs can be induced to differentiate into blood cells and HSC-like cells. Thus, several investigators are developing a gene correction approach for the β-hemoglobinopathies in which iPSCs are made from a patient, genome editing is used to correct the disease causing mutations in the iPSCs, the corrected iPSCs expanded and then differentiated into a sufficient number of HSCs

which would then be transplanted as an autologous product back into the patient similar to an allo-HSCT but without the immunologic complications. Hanna et al. gave the first proof-of-concept for this overall strategy using a mouse model of sickle cell disease and subsequently several groups have shown that genome editing using engineered nucleases can be used to correct hemoglobinopathy mutations in patient derived iPSCs [29, 30, 52]. In all of these gene correction studies of human iPSCs, the absolute frequency of gene correction was sufficiently low that selectable markers had to be used in order to enrich for and identify corrected cells.

Overall, this strategy is quite exciting but has two related but important problems to be solved. First, unlike somatic HSCs, HSCs derived from iPSCs have never been transplanted into a patient. Thus, it is unknown whether these cells would either be safe or efficacious. Second, the protocols for differentiating iPSCs into HSCs are still rudimentary. While small numbers of HSC-like cells can be generated, they are not definitive HSCs that can give rise to long-term hematopoiesis and instead only give rise to primitive HSCs that generate transient primitive hematopoiesis. Moreover, a protocol has not been established to generate such iPSC derived definitive HSCs in sufficient quantities to transplant into a patient (probably on the order of ten million CD34+ cells/kilogram or for a young adult about >500 million CD34+ HSPCs). Solutions to these general problems would not only open up this therapeutic approach for the hemoglobinopathies, but for a host of other diseases as well.

Gene Correction of Somatic Hematopoietic Stem Cells

In contrast to the transplantation of HSCs derived from iPSCs, the transplantation of somatic HSCs both in the allogeneic and autologous setting is routine and done at hundreds of centers around the world, including in less developed countries. Even the transplantation of genetically modified HSCs, in this case by the use of retroviral or lentiviral vectors, is becoming increasingly common and has been performed at >10 centers around the world. Thus, the routine use of HSC transplantation means that the developmental and regulatory burdens in bringing genome edited HSCs to the clinic is substantially lower. The challenge is to increase the frequency of gene correction to frequencies that would be therapeutically relevant.

The frequency of gene correction that would be therapeutically relevant has not been determined. For sickle cell disease, the theoretic considerations that non-sickle RBC and RBC progenitors might have a >30-fold selective advantage over uncorrected cells suggests that a correction frequency of 2–5% would result in >70% of circulating RBCs to be non-sickling. Experimental data in mixed chimerism models of sickle cell disease provide experimental support that 2–5% HSC correction would result in >70% non-sickling RBCs in the circulation [53, 54]. Chronic transfusion programs for sickle cell disease show that keeping the percent of non-sickling RBCs greater than 70% prevents ongoing organ damage and can even reverse organ damage as well. Thus, these data support a target of gene correction of 2–5% in

HSCs for sickle cell disease. Genovese et al. have been able to achieve correction of HSCs in this range at a different locus using ZFNs suggesting that when high quality genome editing reagents are developed for the *HBB* locus, therapeutic correction efficiencies can be achieved [55].

For β-thalassemia, the therapeutic correction frequency might be even lower as the selective advantage for corrected erythroid progenitors and mature RBCs is even greater than for sickle cell disease. The single published β-thalassemia patient who received lentiviral gene therapy is deriving greater than 3 g/deciliter of hemoglobin from a single clone that suggests that only a small number of corrected clones will be necessary to make a patient transfusion independent [56]. The long-term safety and efficacy of deriving erythropoiesis from a single or small number of clones remains to be determined, however.

Highly active TALENs and CRISPR/Cas9 nucleases have been published that recognize exon 1 of the human *HBB* [35, 38]. These are significantly more active than the *HBB* exon 1 ZFNs that were used to correct the sickle cell mutation in patient derived iPSCs. While the *HBB* TALENS retain some activity at the nearly identical upstream *HBD* gene, several of the CRISPR/Cas9 nucleases had high on-target *HBB* activity with no editing at activity *HBD*. In comparison to the *CCR5* and IL2RG ZFNs that have been shown to have activity in CD34+ HSPCs, both the TALENs and CRISPR/Cas9 nucleases have equivalent or higher activity in cell lines than the ZFNs. Thus, they are poised for utilization in the primary CD34+ HSPCs.

While sickle cell disease is caused by a single mutation which lends itself to correction using a single nuclease and correction system, β-thalassemia is caused by mutations throughout the *HBB* gene which does not lend itself to one genome editing system for all patients. Voit et al., however, describe a gene correction system that could be applied to all of the β-hemoglobinopathies [35]. In this system a single engineered nuclease is used to target a donor cassette that contains a wild-type *HBB* cDNA and selection cassette to exon 1 of *HBB*. After homologous recombination the wild-type *HBB* cDNA utilizes the endogenous *HBB* initiation start codon for expression, and thus is regulated by all of the natural powerful regulatory elements that evolution has put into play for the precise and high levels for HBB expression, including any potential intronic elements that are not eliminated. Thus, while the exact mutation causing the β-hemoglobinopathy is not corrected, the function of the endogenous gene is corrected thus creating a "functional gene correction." Furthermore, the simultaneous insertion of an expression cassette allows for simple enrichment of functionally corrected cells. Using this system, Voit et al. were able to enrich the number of corrected alleles from 8% to >60% and the number of cells with at least one corrected allele from ~20% to ~100% in K562 cells [35]. Thus, this system has the dual properties of potentially universal for all b-hemoglobinopathies and for the ability to enrich for corrected cells if the initial percentage of cells is not therapeutically sufficient. For α-thalassemia, instead of using a safe harbor approach as described by Change and Bouhassira [57], one could simply target the wild-type α-globin gene to the *HBB* gene and thereby drive α-globin expression from the *HBB* locus.

Summary and Future Directions

In contrast to just a few years ago, there are now a number of different genome editing strategies there are now possible, all of which have the potential to cure or dramatically alter the disease course for patients with β-hemoglobinopathies. And while optimism is high for the potential for genome editing, the history of any new technology shows that there will likely be unanticipated barriers that need to be overcome. Through the thoughtful and careful collaboration among basic scientists, translational researchers, clinicians, patients, patient advocacy groups, and funding agencies, however, there is every reason to believe that these challenges will be reformulated as opportunities and that soon a generation of patients will have a curative treatment available based on the precise modification by genome editing of their own stem cells.

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Chapter 9
Gene and Cell Therapy for β-Thalassemia and Sickle Cell Disease with Induced Pluripotent Stem Cells (iPSCs): The Next Frontier

Eirini P. Papapetrou

Abstract In recent years, breakthroughs in human pluripotent stem cell (hPSC) research, namely cellular reprogramming and the emergence of sophisticated genetic engineering technologies, have opened new frontiers for cell and gene therapy. The prospect of using hPSCs, either autologous or histocompatible, as targets of genetic modification and their differentiated progeny as cell products for transplantation, presents a new paradigm of regenerative medicine of potential tremendous value for the treatment of blood disorders, including beta-thalassemia (BT) and sickle cell disease (SCD). Despite advances at a remarkable pace and great promise, many roadblocks remain before clinical translation can be realistically considered. Here we discuss the theoretical advantages of cell therapies utilizing hPSC derivatives, recent proof-of-principle studies and the main challenges towards realizing the potential of hPSC therapies in the clinic.

Keywords Induced pluripotent stem cells • Reprogramming • Cell therapy • Gene therapy • Beta-thalassemia • Sickle cell disease

Why Use iPSCs?

Gene therapy for beta-thalassemia (BT) using hematopoietic stem/progenitor cells (HSPCs) is already in the clinic [1, 2] and, although its efficacy and safety is still being evaluated, it has shown promising results, including clinical benefit in one patient [1]. One may therefore ask: why consider alternative strategies? We will first

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discuss the challenges and limitations of current HSPC-based gene therapy for BT and sickle cell disease (SCD) and the potential benefits that iPSCs offer over HSPCs.

Limitations of HSPC-Based Gene Therapy

Current cell and gene therapy approaches for BT and SCD, similarly to all cell and gene therapy approaches for genetic disorders of the hematopoietic system more broadly, use HSPCs as the target cell type for harvesting, genetic manipulation and transplantation to the patient (Fig. 9.1). The overwhelming advantage of this strategy is that it exploits the remarkable ability of hematopoietic stem cells (HSCs) to reconstitute the entire hematopoietic system and capitalizes on extensive expertise gained by the field of HSC transplantation, pioneered by E. Donnall Thomas over half a century ago in Seattle. HSPC transplantation is the most widely utilized form of cell therapy today. A total of one million transplantations have been performed worldwide with more than 50,000 transplantations performed annually, based on World Health Organization (WHO) estimates, for malignant (primarily) and non-malignant disorders of the hematopoietic system.

The accumulated experience of the field has produced optimized protocols and standardized practices for the mobilization, harvesting, ex vivo manipulation, cryopreservation and administration of HSPCs to the patient. Adopting these practices,

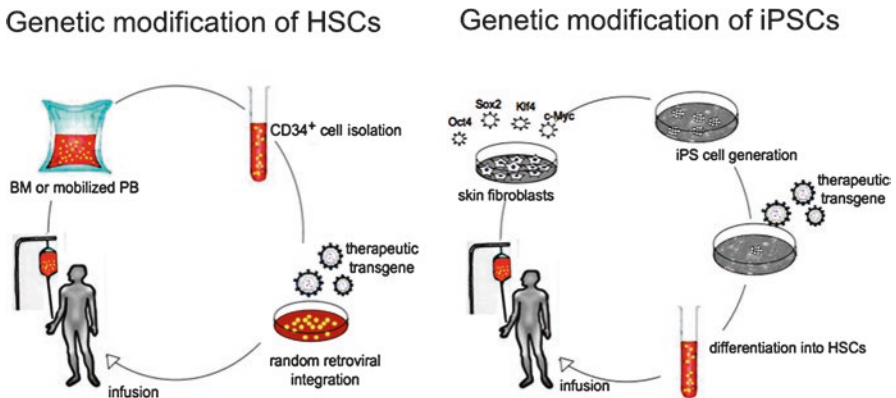

Fig. 9.1 Scheme of autologous cell and gene therapy for blood diseases using HSCs (*left*) or iPSCs (*right*). Left panel: The current paradigm of cell and gene therapy of the hematopoietic system entails harvesting of bone marrow or mobilized peripheral blood from the patient, purification of CD34⁺ cells, transfer of the therapeutic transgene (through retroviral or lentiviral transduction), followed by infusion to the patient. Right panel: The future prospect of using iPSCs instead of HSCs would entail: isolation of somatic cells (skin fibroblasts are depicted in this scheme, but any other somatic cell type, such as blood cells, adipocytes, keratinocytes etc. could theoretically be used); reprogramming to derive one or more iPSC lines; genetic modification to correct the disease (here viral transduction is depicted, but any other strategy of genetic correction can be envisioned); differentiation into HSCs; and infusion to the patient

gene therapists can harvest hematopoietic cells, enrich them for HSPCs and, after a short period of culture, transfer the therapeutic gene and return the cells (some of which are now genetically modified) to the patient (Fig. 9.1).

Despite remarkable progress, some success and future promise, this practice suffers from an important limitation inherent to the use of HSPCs as the target cells. This is the inability to maintain HSPCs ex vivo for more than a short period of time (typically up to 72 h), since, despite extensive efforts, culture conditions permissive for the expansion or maintenance of HSPCs ex vivo at an undifferentiated state and without loss of their long-term engraftment ability, have yet to be found. The need to keep any ex vivo manipulation short-term imposes severe constraints to the genetic modification strategies: it restricts the choice of delivery methods, limits the possibility for "quality control" of the genetically modified cells and precludes sophistication, such as selection or exclusion steps. As a consequence, with these constraints, systems that allow highly efficient recovery of genetically modified cells in the bulk population and, specifically in the cell compartment with repopulating ability, are the only realistic option for a gene therapy regimen with therapeutic efficacy. Viral vectors derived initially from gamma-retroviruses (mainly murine leukemia viruses, MLVs) and subsequently lentiviruses (human immunodeficiency virus 1, HIV-1), have thus to date presented the only gene transfer technologies efficient enough to permit therapeutic levels of expression of the transgene in the reconstituted hematopoietic system. Efficient gene delivery by retroviral and lentiviral vectors hinges on random (or semi-random) integration in the genome, which is well-documented to mediate insertional mutagenesis. The latter entails the serious risk of promoting malignant transformation, most commonly by activation of expression by a vector-encoded promoter or enhancer and secondarily by disruption (leading to fusion or aberrantly spliced gene products) of cancer-promoting genes residing in the vicinity of the integration site. Insertional leukemogenesis constitutes the most severe and alerting shortcoming of HSPC gene therapy to date. Although this risk will likely be substantially reduced with more recent vector platforms (mainly taking advantage of self-inactivating, SIN, vector designs), possibly to levels acceptable for these therapies to become part of standard clinical practice, it will arguably never become negligible. Avoidance of random integration in HSPCs will perhaps become feasible with gene correction approaches using site-specific endonucleases, such as zinc finger nucleases, ZFNs, which have more recently been shown to be potentially efficient enough, at least for some gene therapy applications, but data on a human clinical setting are still pending. Even more importantly, the risk of transforming events in HSPCs mediated by off-target double stand breaks (DSBs) of the genome has not yet been adequately assessed and poses a real concern.

As will be discussed in the subsequent paragraph, the chief advantage of iPSCs as targets for combined gene and cell therapy is that genetic modification is performed in cells that can be maintained ex vivo and characterized extensively for the fidelity and precision of the genetic change, as well as for additional safety parameters, as needed (Table 9.1). The cells that pass this "quality control" and are deemed both adequate to mediate a therapeutic effect and safe can then be expanded to clinical scale and subsequently differentiated into the desired transplantable cell type.

Table 9.1 Comparison of characteristics of human HSCs and PSCs that impact on their use as targets for gene transfer and gene therapy

	HSCs	PSCs
Maintenance/expansion in culture	Very limited	Unlimited (but genetic alterations may arise)
Subcloning	Not feasible	Feasible
Reconstitution of hematopoietic system	Robust	Currently not feasible

iPSCs: Features and Origins

In a breakthrough study in 2006, Shinya Yamanaka at Kyoto University reported the derivation of cells with pluripotency characteristics directly from mouse somatic cells through the transient expression of only four genes, Oct4, Klf4, Sox2 and c-Myc [3]. A little over a year later, the same investigators, as well as the group of Jamie Thomson at the University of Wisconsin, independently, reproduced this in human cells [4, 5]. This amazing discovery—building on: previously discovered principles of reprogramming cell fate by Hal Weintraub (at the Fred Hutchinson Cancer Center in Seattle) [6], inducing pluripotency in somatic cells by John Gurdon (at the University of Oxford) in amphibia in the 50s, and Ian Wilmut (at the University of Edinburgh) in sheep in the 90s [7, 8], and technical advances in the isolation of human embryonic stem cells (hESCs) by Jamie Thomson, also in the 1990s [9]—opened for the first time the possibility of deriving patient-specific human pluripotent stem cells (hPSCs). Two unique characteristics of hPSCs make them extraordinary tools for both research and potential therapeutic applications: (a) the ability of unlimited self-renewal in vitro, providing the opportunity to maintain these cells indefinitely as cell lines, and (b) the potential for directed differentiation into all cell types that are found in the human body, at least theoretically. (Practically, the latter is contingent upon the availability of appropriate in vitro differentiation methods for a given cell type).

Due to these features, hPSCs (including iPSCs) offer opportunities for genetic manipulation that no other primary human cell type presents, critically dependent on the unlimited possibility for subcloning and expansion. This opens two unique possibilities: (1) inefficient but precise methods of genetic modification can realistically be applied, and/or (2) selection and "quality control" of rare single cells carrying a desirable (and no other) genetic modification is feasible.

Initial studies of iPSC generation used permanently integrated gamma-retroviral or lentiviral vectors to express the reprogramming factors (typically OCT4, SOX2, KLF4 and c-MYC, or other factor combinations), as these can mediate very efficient delivery and support factor expression at sufficient levels and duration required for successful reprogramming [10]. Despite the fact that the transgenic factors are typically profoundly silenced in established iPSC lines, their permanent integration in the genome of iPSCs raises a number of concerns: (a) even low levels of residual

factor expression may alter the molecular and possibly functional characteristics of iPSC lines [11]; (b) reactivation of the factors may inhibit differentiation or promote oncogenesis [12]; (c) it is well-known that random integration of retroviral vectors causes insertional mutagenesis.

The observation that factor expression is not required beyond the end of the reprogramming process—as established mouse and human iPSCs were shown to have silenced the transgenic factors and to have activated endogenous pluripotency genes [4, 10, 12, 13]—and the need to move the field towards more clinically relevant methodologies of reprogramming drove several investigators to independently explore techniques for iPSC generation devoid of permanent factor integration. The first generation of vectors circumventing permanent factor integration were excisable vectors, which are effectively vectors—either plasmid or lentiviral—which initially integrate in the genome, but can be subsequently removed, after completion of reprogramming in established iPSC lines when expression of the factors is no longer needed (and in fact is most often already profoundly silenced). Excisable systems that were developed include plasmids [14] or lentiviral vectors [11, 15–19] excisable through the Cre/loxP or the piggyBac transposon/transposase system [14, 20, 21]. The main advantage of excisable systems, especially lentiviral, is that they maintain the very high reprogramming efficiency of integrating vectors. One disadvantage is the requirement of an additional step of transiently exogenously expressing the recombinase (or transposase) and selecting clones with documented complete excision—which extends the passaging time in culture. Another notable disadvantage is that Cre-excisable vectors leave behind a loxP site (~30 nucleotides) as a "footprint" and care should be taken to ensure this does not impact the function of the cell's genome in any way. We proposed that a residual loxP site can be considered practically "harmless" if it is outside coding sequences [22]. By this rule, the vast majority of residual loxP sites in iPSCs should be inconsequential. Alternatively, transposase systems can mediate "seamless" excision, but the possibility of transposition to another genomic site should be excluded.

Subsequently, methods to derive transgene-free mouse or human iPSCs that are completely devoid of integration were developed [23]. These include (a) non-integrating DNA vectors, (b) DNA-free methods. The first category includes adenoviral vectors [24], conventional plasmids [25], oriP/EBNA1 episomes [26] and minicircles [27]. Although all these methods have the potential to generate genetically unmodified iPSC lines, their main disadvantage is the generally low efficiency of reprogramming, which is often several orders of magnitude below this of integrating vectors and therefore often insufficient for derivation of patient-specific iPSC lines from primary biopsies of adult patients. A second concern is that iPSCs derived with these methods still need to be tested for lack of integrated DNA and may harbor randomly integrated vector fragments that may escape detection by standard PCR-based techniques. DNA-free methods are based on RNA-mediated delivery - by Sendai virus-based vectors [28, 29] or repeated transfection of modified mRNAs [30] - or protein delivery using recombinant proteins or cell extracts [31–33]. The main advantage of these methods is the derivation of iPSC lines totally free

of DNA integration. While the extremely low efficiency and reproducibility of reprogramming by protein delivery renders the latter an unrealistic option at this time, RNA delivery offers adequate reprogramming efficiency.

Production and repeated transfection of modified RNAs is rather cumbersome and not suitable for many cell types other than fibroblasts, particularly non-adherent ones, such as cells of hematopoietic origin. Commercialization of Sendai viruses expressing the four "Yamanaka" reprogramming factors has made this technology broadly accessible and increasingly popular. Finally, ongoing efforts are directed towards developing reprogramming methods not requiring any genetic manipulation of cells through the use of small molecules [34] .

Proof-of-Principle Studies Using iPSCs for Gene and Cell Therapy of BT and SCD

The hemoglobinopathies are the most extensively studied inherited monogenic disorders in the human population and their correction by gene therapy has inspired generations of investigators over several decades. It was therefore no surprise that SCD was selected for the first ever proof-of-principle study of gene and cell therapy with autologous iPSCs. Jacob Hanna, at the time a post-doc in Rudolf Jaenisch's lab, performed a tour-de-force study in the mouse, published in a seminal Science paper only 16 months after the original Yamanaka study [36]. Using a humanized mouse model of SCD developed in Tim Townes' laboratory [37], the Hanna et al. study provided first proof of principle of a general scheme of autologous iPSC-based cell and gene therapy, which includes the following steps: (1) reprogramming of somatic cells (specifically, tail-tip fibroblasts) into iPSCs, (2) genetic correction (by "classic" homologous recombination), (3) in vitro differentiation into hematopoietic progenitors and (4) transplantation. Although this study provided clear proof-of-concept early on in the reprogramming field, many aspects of it render it not directly translatable: permanently integrated retroviruses expressing three of the reprogramming factors were used for reprogramming, the iPSC clones were only minimally tested for genetic aberrations and, perhaps most importantly, long-term multi-lineage reconstitution of the hematopoietic system was not shown.

Since the first generation of human iPSCs, a few groups have generated iPSCs from patients with SCD [38–42] and BT major of various β^0/β^0 or β^0/β^+ genotypes [22, 43–46]. Different starting cell types, including skin fibroblasts [22, 38, 41–44], bone marrow fibroblasts (or mesenchymal stem cells, MSCs) [22, 39], amniotic fluid cells [45, 46] peripheral blood mononuclear cells [40] and a variety of reprogramming methods, including retroviral vectors [22, 42–44], a single excisable lentiviral vector [22, 41, 45], EBNA1/oriP-based episomes [40, 42, 46] and a piggyBac transposon vector [39] have been used, reflecting the advances of reprogramming methods in the iPSC field along this period.

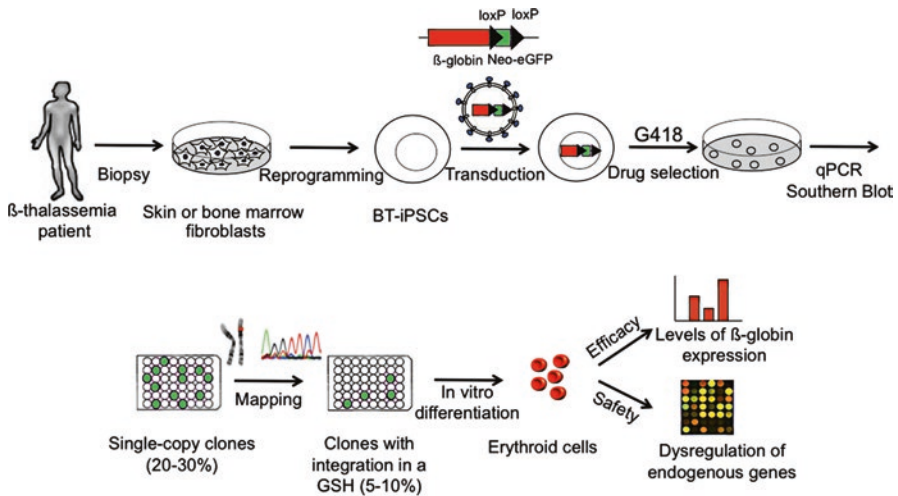

Fig. 9.2 Genetic correction of BT-iPSCs by insertion of a β-globin gene into genomic safe harbors. iPSCs generated from BT patients are transduced with a lentiviral vector driving erythroid cell-specific expression of the β-globin gene. Clones with single integrations can be identified (initially by qPCR and subsequently by Southern Blot) and comprise approximately 20–30% of all G418-resistant clones when transduction is performed at an optimal MOI (0.1–0.3). 1 out of 10 to 20 of these contains integrations at sites that meet the five safe harbor criteria by Papapetrou et al. To further test efficacy and safety in vitro, erythroid cells can be derived and gene expression assays performed to assess β-globin expression and expression of genes in the vicinity of the vector integration (see also text for details)

The first genetic correction study in a human setting was provided by Papapetrou et al. from the Sadelain lab [22] (Fig. 9.2). This study further proposed a new strategy for genetic correction, using gene addition as opposed to in situ correction, followed by selection of corrected clones harboring single copies of a normal beta-globin gene allele inserted into "safe harbor" sites in the human genome. Specifically, we derived multiple BT-iPSC lines from BM fibroblasts from four patients of various genotypes (β^{+}/β^{0} and β^{0}/β^{0}). We developed a lentiviral vector derived from the TNS9 vector originally developed at the Sadelain laboratory [47] and similar to the various lentiviral vectors currently tested in preclinical studies and clinical trials. This vector encodes the human β-globin gene driven by its autologous promoter under control of the locus control region (LCR) elements DNAse I hypersensitive sites HS2, HS3 and HS4 and is modified to also express a floxed PGK-Neo cassette for selection. The strategy developed was as follows: The vector was transduced into BT-iPSC lines at low multiplicity of infection (MOI), so that clones with single vector integrations could be isolated after single-cell subcloning and G418 selection. Clones that were found to harbor a single vector copy and thoroughly confirmed to be clonal were selected for mapping of the vector integration. Integration sites were tested against a set of criteria to select for sites that are less likely to perturb endogenous gene function. The proposed "safe harbor criteria" were

intended to avoid the two types of insertional events that predominantly result in gene dysregulation: transactivation of adjacent genes by a promoter/enhancer present in the vector (which is the most frequent mechanism of insertional mutagenesis/oncogenesis) and gene disruption. Five criteria were thus proposed to exclude regions of the genome in close proximity to coding and non-coding genes—requiring extra distance from genes known to play a role in cancer (in humans or model organisms) and conserved genetic elements. They also excluded integrations inside transcription units. Differentiation along the erythroid lineage showed that vector-encoded β-globin expression can reach therapeutically relevant levels in the majority of clones, including clones with safe harbor integrations. Finally, gene expression analysis was used as an additional safeguard step to ensure that no perturbation of endogenous genes is caused by the therapeutic vector. This is a potentially clinically translatable and universal approach to autologous cell and gene therapy for BT. Aspects warranting further investigation are discussed in the next section.

An alternative and more precise strategy for genetic correction is exploiting homologous recombination to repair the mutation in the endogenous gene locus, a strategy also referred to as homology-mediated gene repair. This strategy takes advantage of the cell's endogenous DNA repair machinery, activated in response to DNA double strand breaks (DSBs), and uses gene targeting tools and principles that have been developed for transgenesis in the mouse over the past two decades [48, 49]. Although conceptually simple, "in situ" genetic correction in human cells, including hPSCs has proven much more challenging than in mESCs. Although the reasons for this are not well understood, different properties of human and mouse PSCs, both biological and culture-related, namely the different growth properties and ability for single-cell subcloning, have been implicated. More recent improvements have made gene targeting in hPSCs feasible and relatively efficient. These include: (a) advances in cell culture conditions, enabling feeder-free single-cell survival of hPSCs (i.e. use of matrices and the Rock inhibitor Y-27632 [50], respectively), (b) improvements of gene delivery methods (i.e. optimized transfection/nucleofection conditions and viral systems for hPSCs) and (c) engineering of designer site-specific endonucleases, which can substantially boost the efficiency of HR [51]. The latter include zinc-finger nucleases [52], meganucleases [53], transcription activator-like effector (TALE) nucleases [54] and more recently the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas9 system [55].

Five studies in the past 3 years reported homologous recombination-based strategies for the genetic correction of mutations in iPSCs from patients with SCD [41, 42, 56] and BT [44, 46] (Table 9.2). One study used "classic" HR [44], one helper-dependent adenoviral vector (HDAdV)-mediated HR [42] and the other three HR facilitated by engineered endonucleases, either ZNF [41, 56] or TALENs [46]. Various donor constructs were used, delivered by electroporation (except in Li et al who used HDAdV) with or without plasmids encoding the nuclease (Fig. 9.3). The selection cassette was inserted in these studies either in the first intron, the second

Table 9.2 Genetic correction strategies in human BT- and SCD- iPSCs

Disease	Genotype	Correction method	Correction efficiency[a]	Beta-globin expression	Refs.
BT	β39/IVS1-110 (β^0/β^+) β39/IVS1-1 (β^0/β^+) β39/β39 (β^0/β^0)	Lentivirally-mediated gene addition in safe harbor sites	5–10% (clones with insertions in safe harbor sites)	Therapeutic levels in vitro (85% of normal allele)	Papapetrou et al.
SCD	βE6V/βE6V	ZFN-mediated HR	1 out of 300 resistant clones	25–40% of normal allele	Zou et al.
SCD	βE6V/IVS-1 βE6V/βE6V	ZFN-mediated HR	Between 5% and 38% depending on ZFN pair (multiple ZFN pairs tested)	Not assessed	Sebastiano et al.
SCD	βE6V/βE6V	HDAdV-mediated HR	81% (39 out of 41 clones)	Not assessed	Li et al.
BT	β41/42/β41/42 (β^0/β^0)	"Classic" HR	0.81% (2 out of 248 clones)	Detected in vivo (in SCID mice) short-term, not quantified	Wang et al.
BT	IVS2-654/IVS2-654 (β^0/β^0) β41/42/β41/42 (β^0/β^0)	TALEN-mediated HR	68% for IVS2-654 (25 out of 37 clones), 40% for β41/42 (4 out of 10 clones)	Detected in vitro, not quantified	Ma et al.

HR homologous recombination; *ZFN* zinc-finger nuclease; *HDAdV* helper-dependent adenoviral vector; *TALEN* Transcription activator-like effector nuclease

[a]Correction efficiency is given as ratio of corrected clones over drug-resistant clones

intron or downstream of the gene. The latter positioning seems preferable to avoid disrupting gene function. Indeed in the Zou et al. study by Linzhao Cheng's group, expression of the corrected β-globin allele after insertion of the selection cassette in the first intron was lower than normal and increased after Cre excision to only reach levels of about 25–40% of these of a normal allele. Even though other reasons (a mutation in the GATA binding site of the 3′ enhancer introduced during or after HR) could not be excluded, this result would warrant caution against targeting a selection cassette in the first intron. The donor DNA design used in Li et al. and Ma et al. containing the entire β-globin gene in the 5′ homology arm is suitable for correction of the majority of BT mutations and deletions (albeit conceivably with varying efficiencies). Expression from the corrected β-globin gene allele was unfortunately

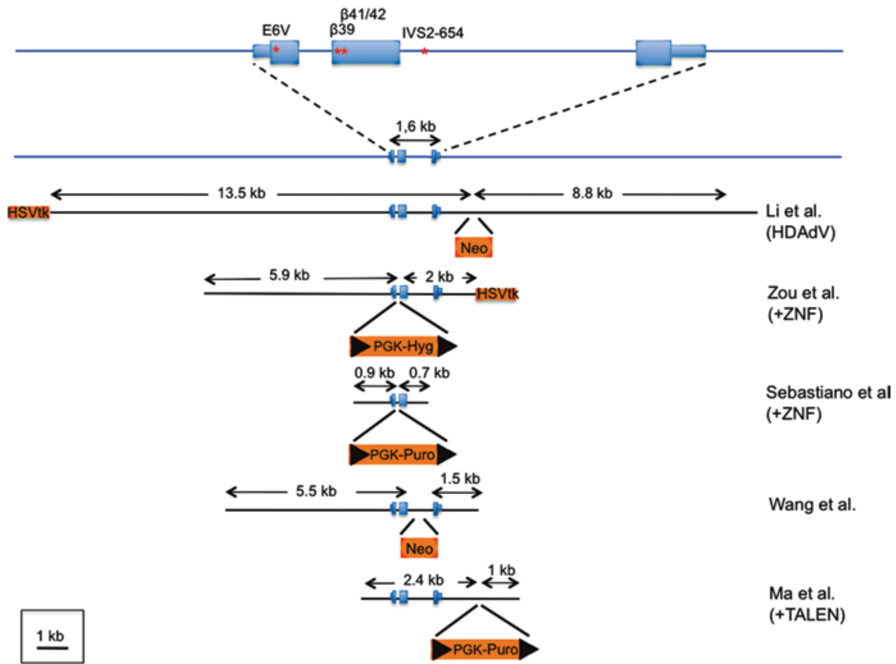

Fig. 9.3 Genetic correction of mutations in BT- and SCD- iPSCs by homologous recombination-based strategies. *Upper panel*: The β-globin gene locus with the BT- and SCD- mutations corrected in patient-derived iPSCs shown with asterisks (see also Table 9.2). The entire human β-globin gene spans approximately 1.6 kb on chromosome 11 and consists of 3 exons. Wide boxes depict coding regions, narrow boxes depict the 5′ and 3′ untranslated regions (UTRs). SCD is caused by a point mutation in codon 6 (E6V). The β39 is a C→T (CAG-TAG) nonsense mutation in codon 39 (at the beginning of exon 2) and is the most common β^0 gene mutation in Italy, mainly Sardenia. β41/42 (-TCTT) is a 4-bp deletion at codons 41/42 resulting in frameshift that generates a downstream stop codon, also leading to absence of β-globin gene expression (β^0). It is the most common BT mutation in China and Thailand. IVS1-1, IVS1-110 and IVS2-654 are single-base substitutions resulting in aberrant splicing of the first or second intron, common in Italy (IVS1-1, IVS1-110) and China (IVS2-654). *Lower panel*: scheme of targeting vectors used in five studies, as shown, for HR-mediated correction of BT- or SCD- iPSCs. Notable differences in the length of the homology arms and in the position of insertion of the selection cassette are depicted (see text for details)

not quantified in the other studies, although it was detectable in Ma et al. and Wang et al., in vitro and in vivo in SCID mice [44].

Although restoration of expression of a targeted allele to normal levels has yet to be demonstrated and the optimal design of the targeting vector has yet to be defined, these recent studies provide proof-of-principle that the β-globin gene locus can be targeted in hPSCs. Remaining roadblocks to clinical translation are further discussed below.

Promises and Challenges of iPSCs for the Gene and Cell Therapy of BT and SCD

Despite the promise of iPSCs in delivering cell and gene therapies for BT and SCD, several roadblocks on the road to clinical translation remain to be addressed.

Issues Common to All Applications of iPSC-Based Cell Therapy

Generation and Quality Control of iPSC Lines

First off, iPSC lines intended for clinical use will need to be generated using methods and procedures that meet both scientific and regulatory standards.

The main issues that need to be determined are: what is the preferred reprogramming method; what is the best starting cell type; what quality control is necessary to deem an iPSC line acceptable for clinical use. Regarding reprogramming methods, DNA-free RNA-based genetic methods seem currently the most appealing, as they combine safety with reasonable efficiency. As additional new methods—substituting some or all reprogramming genes with chemicals, or other treatments—will likely emerge, it is also important to keep in mind that adequate efficiency is required, not only to ensure time- and cost-efficient derivation of sufficiently numbers of iPSC lines to cover all needs, including derivation of lines from rare donors, but also—perhaps more importantly—to avoid selection of rare cells with extreme reprogramming fitness present in the starting cell population. Several connections between reprogramming fitness and transformation potential exist, raising the concern that such cells may be more likely to give rise to a malignant cell clone by acquiring additional genetic lesions before or after transplantation. Indeed, it is being increasingly appreciated that the degree of genetic mosaicism in somatic cells is substantial and that somatic genetic variants can have strong positive or negative effects on the cell's reprogramming ability [57–64].

This consideration also has implications for the choice of the most appropriate starting cell type. The ideal somatic cell to derive iPSCs from should be easily accessible (for example skin or blood), easily reprogrammable and less likely to harbor pre-existing genetic alterations. Studies combining reprogramming with high-throughput genomics will be needed to determine the degree of somatic mosaicism in different human tissues and to catalogue variants with an impact on reprogramming efficiency which may drive or predispose to cancer. Even less clear are standards needed to deem an iPSC line genetically "normal". Several studies have shown that iPSC lines often harbor genetic aberrations, in the form of chromosomal abnormalities, as well as more subtle copy number variants (CNVs) and single-nucleotide variants (SNVs) [65–72], most if not all of which pre-exist in the starting cell [73, 74]. Accumulating genomic data from large projects, such as the 1000 Genomes Project, The Cancer Genome Atlas project and others, will increasingly

over the next years lead to a better classification of genetic variants that confer risk of cancer or of non-malignant diseases. iPSC lines intended for cell therapies will likely need to be tested with high-resolution techniques, like whole-genome sequencing. Additionally, iPSC lines intended for use in cell therapies will need to be compatible with good manufacturing practice (GMP) standards and eligible for FDA approval (and therefore need to meet the FDA's tissue donor standards regarding donor selection, consent and screening) [75]. Finally, advances in the manufacturing procedures, including automated processing and large-scale culture in bioreactors will be required.

Autologous vs Histocompatible iPSCs

The generation of new iPSC lines is very time-consuming and laborious, rendering the prospect of autologous iPSC cell therapy, at least by today's standards, prohibitively expensive and hence unlikely to become routine medical practice. An alternative and more realistic solution would be the generation of cell banks of few allogeneic clinical GMP lines that are compatible for the majority of the population [76, 77]. At least partial human leukocyte antigen (HLA) matching will be required. This requirement may be more stringent in the case of HSC transplantation compared to transplantation of other tissues, according to the paradigm of solid organ transplantation. An attractive possibility is the generation of cell lines from donors homozygous for common HLA haplotypes.

It is estimated that 78% of Northern Europeans, 63% of Asians, 52% of Hispanics and 45% of African Americans would have a matched line if 100 HLA homozygous cell lines from each of these populations were generated, following extensive screening of hundreds of thousands of individuals [78].

Another possibility is the genetic engineering of "universal donor" iPSC lines to generate either HLA-homozygous or HLA class I- and/or class II-negative cell lines, for example by biallelic disruption of the beta-2 microglobulin gene, an obligatory component of HLA class I antigens [79].

All these different options will need to be informed by future studies addressing the immunogenicity of autologous or HLA-matched iPSCs and of their products and their susceptibility to immune rejection, issues that remain at present incompletely understood [80–83]. The antigenicity conferred by the expression of neoantigens by in vitro generated cells is also a matter warranting further investigation. Several open questions remain that include the degree of HLA matching required (which will likely depend on the derivative cell type), what is the best strategy to derive donor cell banks and whether concomitant immunosuppression will be required. Cell banks seem a more attractive option, especially in regions with relatively low ethnic and racial diversity (e.g. Japan). An "off-the-shelf" therapy combined with some level of immunosuppression seems at present the most likely scenario for broad applications of this technology.

Teratoma Formation and Tumorigenicity

A major concern of the transplantation of hPSC-derived cell products is the potential for tumor formation. At least three scenarios can be imagined that pose risks. First, residual pluripotent cells that resist differentiation may persist in a cell graft and give rise to teratomas, non-malignant tumors consisting of tissues of more than one embryonic germ layer, upon transplantation [84–87]. Second, partially differentiated progenitor cells may be present in the graft and result in aberrant proliferation. Even if these cell growths are not malignant, they can cause problems, especially if localized in regions of the body such as the central nervous system or the myocardium. Third, terminally differentiated cells may undergo de-differentiation and malignant transformation. There is some limited evidence that iPSC-derived cells may possess increased ability for transformation. This property may be due to accumulated genetic lesions that predispose to cancer or to unstable epigenetic marks that enhance de-differentiation and/or transformation. The tumorigenic tendency may vary depending on the cell type of origin of the iPSC line [88]. Although it is conceivable that improved differentiation protocols can minimize the chance of the first two scenarios, some method of additional purging of residual undifferentiated cells [89] and/or positive selection of differentiated progeny [90–92] may be beneficial at least in some applications. Furthermore, genetic safety switches can be engineered in the cell lines, for example exploiting suicide genes [93, 94].

Issues Common to iPSC-Based Cell Therapy of the Hematopoietic System

Generation of HSCs with Long-term Engraftment Potential

Two general culture systems are currently employed to induce hematopoietic differentiation of hPSCs: co-culture on stromal cells, typically the OP9 murine bone marrow stroma line, and formation of so-called embryoid bodies (EBs), i.e. aggregates of cells that are forced to stay in suspension, a process that triggers spontaneous differentiation. Although hematopoietic progenitors and more differentiated cells of all hematopoietic lineages can be derived from hPSCs, robust derivation of HSCs—defined by the ability for long-term engraftment and differentiation into all hematopoietic lineages—has not been possible so far.

The current inability to differentiate hPSCs (iPSCs and ESCs) into engraftable HSCs constitutes perhaps the predominant roadblock to cell therapy for blood disorders [95, 96]. Indeed the generation of HSCs from hPSCs has proved much more challenging than anticipated and is still elusive despite substantial efforts from a number of laboratories. Studies from different groups have invariably achieved very low (typically less than 2%) or no engraftment in mice and mostly restricted to the myeloid lineage [97–101]. Intrafemoral injection and transplantation into newborn NSG mice did not result in substantial improvement. Although reproducible and

efficient in vitro derivation of HSCs from hPSCs remains elusive despite intense research efforts, it is clear that this constitutes a technical limitation and that hPSCs inherently possess the biologic potential to give rise to HSCs [102, 103]. A number of reasons may impede success. Current xenograft models may impose "artificial" requirements for the long-term engraftment of hPSC-derived HSCs. Additional signals provided by components of the niche, which may be needed for specification or engraftment, may be critically missing. hPSC-derived hematopoietic cells, similarly to other cell types derived from them, seem to have a more embryonic-like or primitive-like developmental phenotype. Yolk-sac derived hematopoietic progenitors inherently lack the ability to engraft adult recipients and several studies have shown that at least a proportion of hematopoietic cells derived from hPSCs cultures (typically an early "wave") resembles this type of progenitors, although these cultures have been convincingly shown to also give rise to definitive-type hematopoietic cells. Finally, insufficient knowledge of the appropriate culture conditions limits the ability to "capture", maintain and expand HSCs, which may only transiently emerge in these cultures. Insights into the ontogeny of the mammalian hematopoietic system from the developmental hematopoiesis field [104] and/or "trial-and-error" testing of culture conditions, growth factors and cell-to-cell signals by the stem cell field, will likely eventually make the long sought-for goal of HSC generation from hPSCs a reality.

Strategies for Genetic Correction

As discussed above, iPSCs open many more possibilities for sophisticated genetic manipulation than HSCs. Two main approaches for genetic correction discussed above include gene addition in safe harbor sites and HR-mediated gene repair. Gene addition in safe harbors provides a potentially universal approach to disease correction for many diseases caused by reduced or absent expression of a gene and allows the simultaneous expression of supernumerary genes, such as suicide or drug selection genes. A scheme like the one proposed in Papapetrou et al. (Fig. 9.2), where clones with random integrations are screened prospectively or, alternatively, gene targeting of a pre-selected safe harbor site, can be envisioned. However, although the safe harbor criteria provide a starting point, additional data will be needed to establish universal safe harbor sites. Ongoing annotation of the human genome and better understanding of its function can help refine current criteria, for example by incorporating information on long-range chromatin interactions, non-coding RNAs and regulatory DNA.

Editing of the endogenous β-globin locus is also an attractive approach. Although the β-globin gene is silent in hPSCs, and thus its targeting challenging [105], several studies—discussed above—have now demonstrated feasibility. BT is caused by a very large number of different mutations spanning the entire gene, but the β-globin gene comprises only 3 small exons and 2 small introns spanning approximately 1.6 kb. Therefore, the development of a targeting correction vector appropriate for most mutations is conceivable, even though the efficiency would vary between patients

with different genotypes and with potential polymorphisms in the homology regions. Monoallelic correction would be sufficient for phenotypic correction, provided that the corrected locus is expressed at the levels of a normal allele. A considerable body of work involving systematic comparisons of different donor vector designs with or without DSB induction by endonucleases and different strategies for delivery of the donor DNA will be needed, as well as additional data on the efficacy and safety of different nucleases from different families and with different site specificity. ZFNs are ahead of other nuclease technologies in establishing a clinical record in cell therapies [106], but targeting stem cells rather than more differentiated cell types imposes a much higher burden of proof of safety. In parallel, assays of endonuclease-induced genotoxicity, primarily due to off-target cleavage, will need to be established and standardized and acceptable thresholds will need to be defined and amended, as more data become available.

Since iPSCs are clonable and expandable cells, lower efficiency of gene targeting can be afforded and traded for enhanced safety, as obtaining only one or a few corrected clones is sufficient. Regardless of the strategy of genetic correction, it is important that extended culture be avoided, as this will increase the frequency of acquisition of genetic abnormalities [66]. Genetic manipulation will still almost unavoidably necessitate prolonged passaging of the iPSC lines. Thus, standards for verifying genomic integrity, as discussed in the previous section, will be essential.

Issues Specific to iPSC-Based Cell Therapy for BT and SCD

Transfusion Products from iPSCs

Many patients with BT and SCD would also benefit from the development of transfusion products from iPSCs. Although this would not provide long-term treatment, autologous or compatible red blood cells (RBCs) could be valuable for patients with antibody sensitization, often occurring in these patients during multiple transfusions. Either erythroblasts or mature RBCs could be used to this end. The latter would provide a shorter-term treatment and require more prolonged in vitro manufacturing, likely raising the associated cost. On the other hand, the possibility to irradiate the RBC product before transfusion could eliminate the major safety concerns associated with stem cell therapies. Indeed, infusions of RBCs derived from hPSCs represent one of the most appealing near-term strategies for iPSC-based therapies. Although RBCs do not express HLA, they do express a number of surface antigens. Antibody formation against almost 400 antigens belonging to thirty families (blood group systems) has been described [107]. This makes it hard to envision iPSC banks matching all combinations, but the possibility of a master bank of only a few lines generated by donors negative for Rhesus and for as many as possible of the minor antigens is conceivable.

Several studies have demonstrated the feasibility of generating erythroid cells from human ESCs and iPSCs in vitro using different protocols [22, 108–110]. It is clear from these studies that hPSC-derived erythroid cells express predominantly embryonic (ε) and fetal (γ) globins and practically no β-globin. Although protocols

to induce the globin switch in vitro may be developed in the future, an intriguing idea is to harness this developmental immaturity of hPSC-derived erythroid cells to derive phenotypically "corrected" cells from SCD and BT patients without genetic engineering.

Studies assessing the functionality and immunogenicity of in vitro generated RBCs, as well as advances in cell manufacturing, will be needed before protocols for cost-efficient, routine, large-scale production of RBCs can be implemented [111]. An intriguing possibility is the derivation of self-propagating erythroid progenitor lines capable of induced terminal differentiation from iPSCs that can be expanded and banked at the progenitor stage, thus reducing variability, time and cost [107].

Concluding Remarks

The iPSC technology has received a lot of attention from the scientific community and the media alike and holds great promise for the development of new treatment options for BT, SCD and dozens of other inherited and acquired hematopoietic disorders. Some of its applications in regenerative medicine, like the generation of blood transfusion products, seem imminent. Others, on the other hand, like the derivation of transplantable HSCs, seem further away. Advances in several fronts, such as the derivation of high-quality iPSC lines, the development of better differentiation protocols, standards for quality assurance, regulatory frameworks and cost-efficient manufacturing procedures, will be essential for moving this exciting new technology from proof of concept to the clinic.

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